Analgesics encompass those drugs whose outstanding characteristic is their ability to reduce physical pain. Some may have other effects like antipyretic and anti-inflammatory effects as well. The analgesics can be grouped under 2 main heads: narcotic and non-narcotic. The former includes natural opium, its components and derivatives, and a large number of synthetic opiates. As the present work includes two analgesic drugs we would like to make a short review here on the works on cytotoxic and genotoxic effects of various analgesics, which will be of great interest.

The recent spurt in the use of narcotic analgesics for medical and non-medical purposes is a great human concern throughout the world. The teratogenic (Iuliucci and Gautieri, 1971; Smith and Joffe, 1975) as well as mitodepressive (Kabarity et al., 1974) actions of morphine, the major alkaloid of opium and the most widely used narcotic, are on record. It was shown to inhibit the growth of *E. coli* (Simon, 1964). Positive clastogenic effects of both morphine and pethidine in bone marrow cells of mice have been reported from this laboratory using metaphase chromosome analysis (Swain et al., 1980) as well as micronucleus test (Das and Swain, 1982). The chromosome breaking activity of morphine was recorded earlier also in plants (Oehlkers, 1952, 1956) and human addicts (Amarose and Schuster, 1971; Kushnick et al., 1972). But morphine hydrochloride failed to induce sex-linked recessive lethal mutation, II-III.
translocation or dominant lethal mutation in Drosophila melanogaster (Knaap and Kramers, 1976). In a series of studies Falek and his associates (Falek et al., 1972; Falek and McFadden, 1973; Falek and Hollingsworth, 1980) have shown highly significant increase of chromosome damages in opiate abusers; however, prolonged methadone maintenance or drug-free detoxification has been shown to reduce these chromosome damages to almost control level. They have also demonstrated higher SCE levels in addicts compared to controls (Falek and Hollingsworth, 1980). Opiate is also found to increase concomitantly SCE frequencies over controls, both at base level and after UV induction following in vivo and in vitro exposure (Shafer et al., 1983). Higher incidence of SCEs inducted by natural opiates is supposed to be due to repair inhibition (Shafer et al., 1983; Perry et al., 1983). Perry et al. (1983) demonstrated significant increase of SCEs by pyrolysis products of morphine and opium (it contains 10-15% morphine) in Chinese hamster ovary (CHO) cells as well as in human blood lymphocytes; and morphine was shown to be more potent than opium. In bacterial studies though crude opium samples showed a little or no mutagenic activity, the pyrolysed samples of opium and its derivatives gave dose dependent increases in reverse mutation in the presence of S9 mixture (Hewer et al., 1978; Bartsch et al., 1980). According to Malaveille et al. (1982) the pyrolysis products of opium and morphine are supposed to be the mutagenic factors and responsible for the cause of cancer of oesophagus in addicts. An increased frequency of chromosome aberrations, compared to controls, was noticed in lymphocytes of heroin (a derivative of morphine) users (Gilmour et al., 1971) and in lymphocytes of infants exposed to heroin in utero.
(Abrams and Liao, 1974). Similarly Shafer et al. (1979) noticed elevated frequencies of chromosome aberrations and SCEs, and depressed unscheduled DNA synthesis in peripheral blood lymphocytes of 'street heroin' (it contains 20% heroin) users. 'Street heroin' was also shown to increase SCE frequencies in bone marrow cells of monkey in vivo (Roizin et al., 1980). However, Gendel et al. (1974) failed to find an increased frequency of chromosome breaks in heroin addicted mothers as well as in their new born babies. Earlier Matsuyama and Jarvik (1975) made a nice review on the clastogenic potentials of opiates and other psychoactive drugs.

The other category of analgesics (i.e. non-narcotic analgesics) includes salicylates, aniline derivatives, pyrazolone derivatives and others. In addition to the analgesic effect they have an antipyretic effect, and some have an anti-inflammatory or antirheumatic action. They are available in the market under different brand names and, compared to opiates, weak analgesics. Some of them have been tested for genotoxic potentials by using various test systems.

Aspirin (acetyl salicylic acid), a salicylate, happens to be the most widely used drug in the world (Collier, 1963). The therapeutic use of this wonderful drug as analgesic, antipyretic, antirheumatic and uricosuric is in practice for the last hundred years or so. Tests for carcinogenicity of a mixture of aspirin, phenacetin and caffeine led to contradicting results (IARC publication, 1980). Variable claims have been made on the chromosome breaking activity of this wonder drug.
Jarvik and Kato (1968) and Meisuer et al. (1970) reported increased incidences of chromosome aberrations in human lymphocytes exposed to the drug in vitro. Loughman (1971) could also obtain 4 fold increase in the occurrence of simple chromatid gaps. Higher incidence of chromosome aberrations was also noted with aspirin in a Chinese hamster lung fibroblast cell line (Ishidate, 1981). Aspirin induced mitotic abnormalities involving particularly spindle functioning in Allium cepa is on record (Shanthamurthy and Rangaswamy, 1979). On the other hand, Mauer et al. (1970) and Sankar and Geisler (1971) found no significant change in the frequency of chromosome aberrations in human and mouse lymphocytes exposed in vitro. Interestingly, the mouse in vivo system responded positively at early hours (8-24 h) with a dose of 150 mg/kg (Das and Paul, 1978). Bruce and Heddle (1979) evaluated the effect of aspirin from a comparative study based on MNT, sperm abnormality assay and the Ames Salmonella assay, and found a negative response in all the cases. Similar negative result was also obtained very recently by Jasiewicz and Richardson (1987) from MNT as well as Salmonella/microsome assay system.

Paracetamol (N-acetyl p-aminophenol) and phenacetin (N-acetyl-p-phenetidine) are two widely used aniline compounds with non-narcotic analgesic and antipyretic effect. Paracetamol (PC) is a metabolite of phenacetin and was in fact introduced in the market as a substitute of phenacetin which is classified as a carcinogen (IARC, 1982). Flaks et al. (1985) detected bladder and liver tumors in rats following chronic exposure to 1% PC also. It was shown to have very weak clastogenic and extremely poor
C-mitotic influence in mouse bone marrow system following single as well as repeated treatments (Anantha Reddy, 1984). Dybing et al. (1984) could not also find mutagenic potential of paracetamol and its metabolite in Salmonella assay system; its derivative was, however, cytotoxic to the bacterium. Absence of mutagenic capacity of paracetamol was recorded very recently in rodent micronucleus test as well as in the Ames test with and without S9 mixture (Jasiewicz and Richardson, 1987). On the other hand, Kocisova et al. (1988) reported a significant increase in the incidence of chromosome aberration in peripheral blood lymphocytes of some volunteers following intake of 3 X 1000 mg of paracetamol in the course of 8 h. An increase in chromosome aberration was also found in bone marrow cells of mice and Chinese hamsters after single or chronic exposure to PC (Yoshida et al., 1980). In V 79 Chinese hamster cells PC increased the frequency of SCEs without S9 mixture, reduced the rate of replicative DNA synthesis, and induced dose-dependent single strand breaks of DNA (Holme et al., 1988; Hongslo et al., 1988). Human lymphocytes exposed to PC in vitro also exhibited clastogenic activity (Watanabe, 1982). In root tips of Allium cepa the drug exhibited strong mitodepressive and chromatoclastic properties (Anantha Reddy and Subramanyam, 1978, 1981a).

With respect to the mutagenic activity phenacetin was found to be negative in the standard Ames test with rat S9, but positive with hamster S9 as well as with a mixture of rat-hamster S9 (Shudo et al., 1978; Weinstein et al., 1981; Camus et al., 1982). Camus et al. (1982) further documented using S. typhimurium
TA 100 in plate assays that of the metabolic products of phenacetin N-hydroxyphenacetin was mutagenic and higher yield of it in the liver of hamsters as compared to rats was supposed to be the reason for differential response. However, in rat in vivo system (Granberg-Ohman et al., 1980) and in Chinese hamster lung fibroblast cell line (Ishidate, 1981) it was reported to increase the incidence of chromosome aberrations remarkably; but the SCE frequency in rat bone marrow cells remained unaltered (Granberg-Ohman et al., 1980).

Flupрогuаzone, an analgesic anti-inflammatory agent, was judged negative with respect to its mutagenicity from micronucleus and dominant lethal tests using mice and the Ames test using Salmonella typhimurium (Ruttimann et al., 1981).

A number of pyrazolone compounds are now in use as non-narcotic analgesics. Some of them are known to have side effects like general toxicity which includes rashes, giddiness, sweating and agranulocytosis. However, they are widely used because of their potent analgesic and anti-inflammatory effect. Pyrazolones like aminopyrine, sulpyrine, antipyrine, isopropylantipyrine, etc. themselves exhibit very little or no mutagenic effect, but if they are administered in combination with nitrites they are converted into nitrosamines which are highly mutagenic and/or carcinogenic (Kramer, 1980; Imui et al., 1980; Sakai et al., 1981; Marshall et al., 1982). Aminopyrine when given alone (10^-5 - 10^-4 M) to DON cell line failed to elevate the base line frequency of SCE (Abe and Sasaki, 1977). Aminopyrine, sulpyrine and antipyrine in combination with nitrite were found to cause mutation in one or the other of the bacterial strains
TA 98 and TA 100 (Arisawa et al., 1978). Similarly Sakai et al. (1981) showed highly mutagenic effect of N-nitroso compounds produced by the reaction of sulpyrine with nitrite in Salmonella/microsome system. 8-AG resistant mutations also increased greatly in hamster embryos exposed in utero to aminopyrine and sodium nitrite simultaneously (Inui et al., 1980). They (Inui et al., 1980) also showed 5-6 fold increase in the number of transformed colonies of the cells from mothers receiving both the compounds. The drug Cibalgin (trade name) which contains aminopyrine and allobarbitone was shown to have strong mitodepressive, but mild clastogenic and non-disjunction capacity in mouse in vivo system (Anantha Reddy, 1982; Anantha Reddy and Subramanyam, 1981b). The two drugs: metamizole and phenylbutazone, which the present chapter deals with are also pyrazolone compounds, and the works on them will be reviewed in subsequent sections.
3.1 Effect of Metamizole (Analgin)

3.1.1 Introduction

Metamizole is, as mentioned before, a pyrazolone compound and synthesized by heating 4-methyl aminophenazone in the presence of formaldehyde and sodium hydrogen sulfite (Farben, 1922).

\[
\text{CH}_3-\text{N}-\text{CO} \xrightarrow{\text{NaHSO}_3/\text{HCHO}} \text{CH}_3-\text{N}-\text{CO} \text{CH}_2-\text{SO}_3\text{Na}
\]

4-methyl aminophenazone Sodium-1-phenyl-2,3-dimethyl-3-pyrazoline-5-one-4-methylamino methane sulfonate (Analgin)

It is a very efficient non-narcotic analgesic and very widely used against all types of pain and by all routes. Its analgesic effect is so powerful that even those conditions of pain could be treated which would otherwise require administration of preparations containing opiates. It is an established and proven antipyretic analgesic having spasmylytic and antirheumatic effect. Being readily soluble in water this drug can be administered parenterally by intramuscular or intravenous route, which is an advantage over other non-narcotic analgesics. It is used as a symptomatic remedy in various clinical conditions, so much so that now it is being misused by laity being freely available over the counter. This drug is available in the market under different trade names like Novalgin, Analgin, etc., and also in
various combinations and formulations, e.g. Algesin-O, Anadex, Baralgan, Oxalgin, Pamazin, Promalgin, Orphalgin, Kilpane etc. Consumption of this drug is very high in India. The total production for the year 1986-87 was estimated to be 150 metric tons (OPPI, 1987); from this figure one can have some idea about the wide spread use of the drug.

Unfortunately it is not included in the British or the Indian Pharmacopoea though has found a place in the U.S.S.R. Pharmacopoea. It is listed in Extra Pharmacopoea as sodium noramidopyrine methane-sulfonate.

From the foregoing paragraphs one can have an idea how important this drug is and how widely it is used. However, our knowledge on its potential genotoxic effect is extremely limited. Most of the works so far on record in this line were done by Anantha Reddy and his co-workers (Anantha Reddy 1983a, b; Anantha Reddy and Subramanyam, 1983). Discernible clastogenic effect was recorded neither in bone marrow cells (Anantha Reddy, 1983a) nor in spermatocytes (Anantha Reddy and Subramanyam, 1983) of mice following single as well as cumulative doses. However, strong mitodepressive effect was evidenced in root tip cells of onion (Anantha Reddy, 1983b) and at higher dose level (15.6 mg/animal) in mouse bone marrow cells (Anantha Reddy, 1983a). However, study based on host-mediated assay using Salmonella typhimurium G46 strain in mouse host revealed a weakly mutagenic capacity of analgin at a dose 2 mM/kg together with equimolar dose of nitrate (Braun et al., 1980).
All this prompted us to evaluate further the possible toxic effects of metamizole at the cytological and genetic level in mouse in vivo system.

The details of the working parameters followed for this drug are given below:

I. Bone marrow metaphase analysis
   A. Single treatment
      1. Time-response study
      2. Dose-response study
   B. Repeated treatment
      1. Time-response study

II. Spermatocyte chromosome analysis
   A. Repeated treatment
      1. Time-response study

III. Sperm test
   A. Sperm morphology assay
      1. Repeated treatment
         a. Time-response study
   B. Sperm count assay
      1. Repeated treatment
         a. Time-response study

3.1.2 Materials and Methods

Healthy adult mice of the age group 10-14 weeks and weighing 20-25 g constituted the experimental animals. Base free sample of the drug metamizole (Batch number not supplied,
99.23% pure) was obtained from its manufacturer, Hoechst India Limited, Bombay as gift. The drug was administered via oral route without any vehicle for all the protocols of metamizole. With the help of two pairs of blunt forceps the drug was forcefully pushed into the throat of the animal. Sex and age matched untreated animals were used as controls.

3.1.2.1 Bone marrow metaphase analysis:

For single treatment time-response study the mice were fed with a dose of 50 mg/kg of the drug and bone marrow cells were collected at 8, 16, 24, 48 or 96 h post-treatment. For single treatment dose-response study the drug was fed with a dose of 25, 50 or 100 mg/kg and animals were killed at 24 h after the treatment. For each sampling under single treatment regimen 3-5 animals of either sex were employed. The data of the treated series were compared with those obtained from 36 age and sex matched untreated control animals.

For repeated treatment schedule the drug was fed with a single dose of 50 mg/kg/day for 3 consecutive days and the animals were killed at the end of 1, 2, 3, 4, 5 or 6 weeks after the last treatment. 3-4 male mice were used for each week. 5 age matched untreated males served the purpose of control for each week. We would like to mention here that these animals employed in repeated treatment schedule provided, besides bone marrow, the materials for spermatocyte chromosome analysis, sperm morphology assay and sperm count preparations.
The collection and processing of materials, and preparation and staining of slides were done as described earlier in Materials and Methods (General) (vide 2.1).

3.1.2.2 Spermatocyte chromosome analysis:

For analysis of spermatocyte chromosomes the adult males employed in the repeated treatment schedule for bone marrow chromosome analysis (see 3.1.2.1) were used, and their testes were collected and processed following the schedule described earlier in Materials and Methods (General) (vide 2.3).

3.1.2.3 Sperm test (sperm morphology and sperm count assay):

Here also control and treated individuals used for repeated treatment schedule for bone marrow chromosome analysis (vide 3.1.2.1) provided the materials. Details of processing of the material and scoring have been described earlier (vide 2.5.1 and 2.5.2).

3.1.3 Results

3.1.3.1 Bone marrow metaphase analysis:

Qualitative - The drug had in no case any effect on the behaviour of the animal.

As the aberrations induced by the drug following single and multiple treatment, and in the dose-response and time-response studies did not differ much qualitatively they are considered here together. In no case general type of effect like despiralization or centromeric stretching was noted. The structural chromosomal
aberrations encountered were mainly of chromatid type. Chromatid gaps and sub-chromatid breaks together constituted the most common type of aberrations (Figs. 1a, b). Among the break type aberrations chromatid breaks and fragments constituted the most frequently encountered aberrations. In case of chromatid breaks the acentric broken chromatid parts were found to lie either near to its place of origin with little displacement (Figs. 1c, d) or somewhere else in the metaphase plate (Figs. 1e-g). In some cases the break involved the secondary constriction region (Fig.1h). Though chromosome with multiple breaks was not encountered two aberrations; one break type and another gap type, involving the same chromatid or two chromatids of a chromosome were not rare. Small acentric fragments of untraceable origin was noted in a number of cases (Figs. 1i, j). A metaphase plate was found to contain paired fragments (Fig.1k) probably originating from a chromosome or iso-chromatid break. Iso-chromatid breaks and gaps were also available. A number of metaphases were also recorded to contain centric ring chromosome associated with (Fig.1l) or without (Fig. 1m) acentric fragment; it was assumed to have originated by the reunion of the broken ends of the sister chromatids. A number of exchange configurations most of which were of chromatid type forming dicentric chromosomes were noted (Figs. 1n, o) and some of them were accompanied with acentric fragments (Fig.1n). In one instance two chromosomes were found to be involved in the formation of a dicentric ring, but without any fragment (Fig.1p). Qualitatively aberrations encountered in the control individuals did not differ much from those obtained in metamizole treated ones. However, not a single iso-chromatid break, ring chromosome or exchange was noted in the former.
Explanation for Fig. 1

Photomicrographs of mouse bone marrow metaphases showing various types of structural chromosome aberrations induced by metamizole.

a. Chromosome showing a chromatid gap.

b. A chromosome with a sub-chromatid break.

c, d. Chromosomes showing chromatid breaks, the fragments remain near the place of origin.

e, f, g. Chromosomes showing chromatid breaks with displaced fragment.

h. A chromosome showing a chromatid break at secondary constriction region.
Explanation for Fig. 1 (Contd.)

i, j. Metaphase plates showing chromatid fragments of untraceable origin.

k. A metaphase plate with a paired-fragment of untraceable origin.

l. A metaphase showing a ring chromosome and a fragment.

m. A ring chromosome but without any fragment.

n. A metaphase with a dicentric chromosome and a fragment.

o. A dicentric chromosome but without any fragment.

p. A dicentric ring formed due to asymmetrical exchanges between two chromosomes but without any fragment.
During scoring we came across some polyploid and aneuploid cells. The frequency of these, however, never exceeded the control limit. Further, most of the aneuploid cells contained sub-diploid chromosome number which might be due to preparational shortcomings. Hence numerical changes of chromosomes were not taken under consideration.

Quantitative - Data on the structural chromosome aberrations induced by the drug in different hours following single treatment are presented in Table 1. A tendency of increase in the 'break type' aberrations (excluding gaps and sub-chromatid breaks) was clearly marked, compared to controls, among the treated individuals. The same picture was noted when major aberration types like chromatid breaks, fragments and gaps and sub-chromatid breaks were considered separately. The increases were statistically significant in comparison to the control values in all the sampling periods for 'break type' aberrations and in first four sampling periods (8-48 h) for 'total' aberrations (including gaps and sub-chromatid breaks). However, structural chromosome aberrations excluding or including gaps and sub-chromatid breaks failed to show any correlation with the post-treatment sampling periods (as revealed from analysis of correlation coefficient). The maximum response obtained at 24 h ('break type' aberration) was about 11 times the control value.

Table 2 summarizes the data on dose-response study following single treatment. Interestingly the intermediate dose (50 mg/kg) produced the maximum aberrations whether considered 'break type' aberrations or 'total' aberrations or individual
Table 2: Incidence of different types of structural chromosome aberrations in bone marrow cells of mice induced by single treatment of metamizole with different doses at 24 h post-treatment. Each iso-chromatid break, exchange or ring chromosome was counted as two breaks. Values in parentheses are mean per 100 metaphases ± S.E.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Metaphases scored/Animals</th>
<th>Break type aberrations</th>
<th>Gaps+Sub-chromat. break</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chromat. Iso-break</td>
<td>Frag. Exch. Ring</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>400/4</td>
<td>2</td>
<td>4</td>
<td>7 + 1 (2.00 ± 0.35)\textsuperscript{a}</td>
</tr>
<tr>
<td>50</td>
<td>400/4</td>
<td>9</td>
<td>16</td>
<td>26 + 1 (6.75 ± 1.14)\textsuperscript{a}</td>
</tr>
<tr>
<td>100</td>
<td>300/3</td>
<td>2</td>
<td>1</td>
<td>3 (1.00 ± 0.47)</td>
</tr>
<tr>
<td>0 (Cont.)</td>
<td>2600/36</td>
<td>5</td>
<td>10</td>
<td>15 (0.58 ± 0.16)</td>
</tr>
</tbody>
</table>

\textsuperscript{a, b} 't' test: significantly different from control at a = p < 0.001, b = p < 0.01

\textit{r} = -0.346 \qquad \textit{r} = -0.234
\textit{df} = 2 \qquad \textit{df} = 2
aberration types. Though the lower two doses elevated the aberration frequencies significantly over the control the highest dose level tested failed to do so. Analysis of correlation coefficient did not also reveal any influence of dose on either 'break type' or 'total' aberration frequencies.

The data on structural chromosome aberrations obtained in different post-treatment weeks following treatment for 3 successive days were compared week-wise with the respective control values (Table 3). The incidences of 'break type' aberrations showed significant increase over the respective control value at sampling weeks 1, 3 and 5, the 'total' aberrations showed significant increase at week 5 only. However, if the treated data were compared with the pooled control mean (0.72 for break type aberrations and 4.10 for total aberrations, as the control values obtained in different weeks did not vary from each other greatly an attempt was made to compare the treated data with the pooled control mean value) significant increases were marked at weeks 1, 3 and 5. The highest incidence of breaks (3.75%) obtained at weeks 1 and 3 was more than 5 times the pooled control mean value.

3.1.3.2 Spermatocyte chromosome analysis:

Qualitative - From the qualitative point of view the effects of the drug on meiotic chromosomes were analyzed under three general heads: structural changes, numerical changes and effects on the pairing behaviour.

So far structural changes were concerned the effects in the control and treated series as well as in different sampling
intervals were the same except presence of absence of certain aberration type(s) at certain sampling interval. The structural aberrations comprised chromosome and chromatid type breaks, fragments of untraceable origin and exchanges. Chromatid type breaks involved both autosomes (Figs. 2a, b) and X-chromosome. In the affected cells after chromosome break the fragments were seen placed near to their origin with a little displacement only (Fig. 2c). A number of cells were found to contain some extra fragments smaller than the smallest chromosome (Figs. 2d, e); because of their smallness their origin could not be traced and categorized as fragments of untraceable origin. In a number of cells translocation multivalents were noticed, however, in no case sex chromosome was involved. These multivalents were in the form of chain of three, chain of four and ring of four (Figs. 2f-h). Gaps in X chromosome (Fig. 2i) and autosomes were also noticed.

Though occurrence of polyploid diakinesis-metaphase I plates was common, but the incidence did not vary remarkably from that of control. In an extreme case a 12 n cell was noted. In mice the occurrence of such cells is common (Dym and Fawcett, 1971; Beatty et al., 1975; Kar and Das, 1981), and, hence the data are not presented. Though cells with less than 20 bivalents were very common only a few cells were recorded to contain one or two extra univalent(s) and/or bivalent(s) (Figs. 2j, k). No importance was given to cells with less than 20 bivalents as they might have originated due to technical shortcomings, and they are not considered for statistical analysis.

Both autosomes and sex chromosomes were involved in univalent formation. In several cases X and Y were found to lie
Explanation for Fig. 2

Photographs of diakinesis-metaphase I chromosomes showing structural, numerical and pairing anomalies induced by metamizole.

a, b. Autosomal bivalents showing chromatid breaks.

c. A bivalent with a chromosome break, the fragment lying near to its origin.

d, e. Plates showing chromosomal fragments of unknown origin.

f. A diakinesis-metaphase I plate showing a multivalent configuration - a chain of three (Ch. III).

g. A diakinesis-metaphase I plate showing a multivalent configuration - a chain of four (Ch. IV).
h. A cell showing a multivalent configuration - a ring of four (R-IV).

i. The X chromosome showing a chromosome gap.

j. A diakinesis-metaphase I plate with 20 bivalents and two extra univalents (20 II + 2 I).

k. A cell with 21 bivalents (21 II).

l. A plate showing early separation (univalent formation) involving sex chromosomes and a pair of autosomes.
wide apart (Figs. 21, m). Similar cases were also noted involving autosomes (Figs. 2b, l). Among the autosomal bivalents the smaller ones were involved frequently.

Quantitative - The data on the abnormalities of meiotic chromosomes induced by the treatment of metamizole at different sampling intervals following repeated treatment are presented in Table 4. In no week in the control series aberration type other than chromatid break and fragment was observed. In contrast, besides them, in the treated series a number of chromosome breaks and exchange cases were noted. The breakage frequencies obtained in all the post-treatment weeks revealed a tendency of increase when compared against the respective control values. However, the increase was statistically significant only at week 6, that too marginally. The control values in different weeks did not differ from each other; if those values were pooled and the treated value at week 6 was compared with that pooled control mean value (0.74 ± 0.22) the picture remained unchanged. Analysis of correlation coefficient did not reveal any correlation of the treated values with the sampling intervals (r = +0.108, df = 5). The individual aberration types did not also exhibit much variation in their frequencies in different weeks.

The frequencies of univalent formation, taking autosomes and sex chromosomes together increased significantly at weeks 4 and 6; here also the increases were marginal. The incidence of univalent formation involving sex chromosomes only also showed a marginal increase at week 6. But in no instance the autosomes exhibited significantly higher incidence.
3.1.3.3 Sperm morphology assay:

Qualitative - A population of sperm in both control and treated individuals exhibited abnormal morphology in their head as well as tail structures. As the head abnormalities were easily recognizable and much more common than tail abnormalities the author concentrated his studies on the former. It was not possible to group the abnormalities under some distinct categories. They were classed into some arbitrary 'types'. The 'types' like with flat base, 'axe' shaped, with acroosome spine abnormalities, sickle shaped and amorphous were very common in occurrence (Figs. 3a-i). The sperm heads with acrosomal spine abnormalities exhibited a high degree of variation in the spine morphology ranging from a total absence to a long hook-shaped one. In addition to the above 'types' spherical, giant, with vacuole, triangular, rectangular, semilunar, bifurcated acroosomal spine, notched, fusiform and balloon shaped sperm heads were also available, though rare, in both control and treated individuals (Figs. 3c, g, j, k). Some abnormal types like dichotomous, and 'spear-head' shaped were recorded, admittedly rare, in the treated individuals only. Sometimes a particular sperm head exhibited a combination of two or more 'types' of abnormalities (Fig. 3c).

Quantitative - As the grouping of abnormal 'types' was arbitrary it was not possible to have quantitative analysis 'type-wise'; all the morphological deformities were considered together as 'abnormal' for quantitative analysis. In the control series the frequencies of abnormal sperm heads obtained in different weeks did not differ much; they ranged from 3.58 - 4.40% (Table 5). Further, the correlation coefficient analysis did not
Explanation of Fig. 3

Photomicrographs of sperm showing abnormalities in head morphology in mice treated with metamizole.

a, b. Sperm heads with flat base.

c. An 'axe'-shaped sperm head with vacuole.

d. A sperm head with acrosomal spine abnormality.

e. A 'sickle'-shaped sperm head.

f-i. Photographs displaying various types of amorphous sperm heads.

j. A sperm head with vacuole.

k. A sperm with semilunar head.
Table 5: Effect of metamizole on the incidence of sperm head abnormalities in mice at different post-treatment sampling weeks after 3 days repeated treatment (oral, 50 mg/kg X 3). Values are mean per 100 sperm ± S.E. For each point 4-5 animals were employed and 1000 sperm heads were examined from each individual.

<table>
<thead>
<tr>
<th>Series</th>
<th>wk 1</th>
<th>wk 2</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 5</th>
<th>wk 6</th>
<th>r-value (df = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>7.94 ± 0.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.62 ± 1.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.90 ± 2.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.63 ± 2.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.45 ± 1.61</td>
<td>4.18 ± 0.97</td>
<td>-0.275</td>
</tr>
<tr>
<td>C</td>
<td>4.15 ± 0.94</td>
<td>4.30 ± 0.68</td>
<td>4.40 ± 1.13</td>
<td>4.28 ± 1.28</td>
<td>3.58 ± 0.52</td>
<td>4.23 ± 0.14</td>
<td>-0.124</td>
</tr>
</tbody>
</table>

T = Treated, C = Control

<sup>b, c</sup>: t-test: significantly different from control at <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.05
reveal any influence of 'weeks' on the values obtained. If those values were pooled the pooled control mean value would be 4.15% ± 0.36 which is very close to the value obtained by earlier workers (Bruce et al., 1974; Kar and Das, 1983) in the same strain of mice.

In the treated series the values obtained in different weeks fluctuated between 4.18 and 14.63%. Significant increase in the incidence of mis-shapen sperm was noted following treatment in the first four weeks (weeks 1 - 4) whether the treated values were compared with the respective control value or with the pooled control mean value (4.15%). Interestingly, in the treated series the values obtained in different weeks showed a gradual increase with the lapse of time reaching a peak at week 4, then there was a decline and at week 6 the value came down to the control level. The highest value (14.63%) recorded at week 4 following treatment of the drug was more than 3.5 times the pooled control mean value (4.15%).

3.1.3.4 Sperm count assay:

In the treated series the sperm counts fluctuated between 61.72 and 105.81 and in the control series between 77.26 and 114.65 (Table 6). Week-wise comparison of the treated and control data indicated a marginally significant decrease in count at week 3 only. As the control values for six different weeks did not differ remarkably from each other (F = 1.56, df: n₁ = 5, n₂ = 20) the pooled control mean value was also calculated (98.98 ± 5.18) and when that value was compared with the treated ones the latter obtained at week 2 and week 3 showed significant decrease. As a whole the differences between the control and
Table 6: Effect of metamizole on epididymal sperm count of mice in different post-treatment weeks after 3 days repeated treatment (50 mg/kg X 3). The values are mean number of sperm heads in one WBC chamber of haemocytometer ± S.E. To obtain total sperm count per epididymis each value should be multiplied with $8 \times 10^4$ (dilution factor). For each week 4-5 animals were employed.

<table>
<thead>
<tr>
<th>Series</th>
<th>wk 1</th>
<th>wk 2</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 5</th>
<th>wk 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>82.38 ± 17.52</td>
<td>61.72 ± 9.84</td>
<td>68.20 ± 8.16&lt;sup&gt;C&lt;/sup&gt;</td>
<td>69.21 ± 11.24</td>
<td>105.61 ± 8.57</td>
<td>89.78 ± 10.46</td>
</tr>
<tr>
<td>C</td>
<td>77.26 ± 8.02</td>
<td>87.29 ± 11.51</td>
<td>108.52 ± 9.08</td>
<td>101.54 ± 10.37</td>
<td>114.65 ± 10.14</td>
<td>112.95± 14.48</td>
</tr>
</tbody>
</table>

*T* = Treated, *C* = Control

* t* test: significantly different from control at *c* = *p* < 0.05
3.1.4 Discussion

3.1.4.1 Bone marrow metaphase analysis:

Both time-response and dose-response analyses of bone marrow metaphases following single treatment reveals significantly higher breakage frequencies in the treated series over the control ones; the 'total' aberrations which included both break and gap type aberrations also showed the same picture. Significantly higher frequencies of chromosome aberrations, including or excluding gaps, compared to controls, were also noted in some of the post-treatment weeks following 3 successive treatments. All this clearly indicates clastogenic capacity of the drug. It is interesting to note that neither dose-response study nor time-response study revealed any correlation of the aberrations obtained with the doses or post-treatment sampling intervals.

Qualitatively almost all the aberrations induced by metamizole are of chromatid type, even after repeated treatment. It seems the drug like the great majority of chemical chromosome-damaging agents requires replicative DNA synthesis in order to have the lesion it induces translated into structural aberrations.

Earlier metamizole was also tested for its potential clastogenicity in bone marrow system of mice using metaphase analysis (Anantha Reddy, 1983a); the report included single and multiple treatment studies, as well as time-response and dose-response studies. As in the present study, Anantha Reddy (1983a) noted occurrence of gaps to a large extent; however, in contrast....
to ours break-type aberrations were extremely rare in his study. The difference in the incidence of break-type aberrations between two studies may be attributed to high doses used in the earlier study; the minimum dose (3.9 mg/individual) tested by him comes to about 156 mg/kg (assuming that he used mice having body weight 25 g each). This drug has been demonstrated to have great mitodepressive effect by the same author in bone marrow system of mice (Anantha Reddy, 1983a) as well as in root tip cells of *Allium cepa* (Anantha Reddy, 1983b); the effect showed a dose-related increase and was more following repeated treatment. Even the lowest dose (156 mg/kg) inhibited the mitosis very greatly. Probably in the earlier study, due to marked inhibitory effect of the drug on cell division for high doses used, the affected cells did not come in the metaphase stage at the time of scoring, and consequently clastogenicity was not noticed. We did not do any study to demonstrate inhibitory effect of the drug on the cell division process. However, in our dose-response study the maximum response obtained with the intermediate dose, and no remarkable increase of the aberration yield over the control value with the highest dose can be explained in this line.

In the present investigation following single treatment a sharp decline in the aberration yield was found after 48 h. A similar situation was also noted by the earlier worker (Anantha Reddy, 1983a) (after 48 h sampling was done at 96 h in our case, while at 72 h in earlier case). In our study the yields in some post-treatment weeks following repeated treatment were high in comparison to the yield at 96 h after single treatment and this seems to be due to repeated administration
of the drug. The incidence of polyploidy in both the studies (earlier and present) remained in the control range. Thus, our results in many ways agree well with those of Anantha Reddy (1983a). So far the author is aware literature contains no other report on the potential clastogenic effect of the drug on somatic chromosomes.

3.1.4.2 Spermatocyte chromosome analysis:

The importance of germ line cells, particularly of mammals, in the evaluation of potential genotoxicity of a drug has already been discussed. Though the bone marrow cells demonstrated clearly the clastogenic efficiency of the drug, the breakage frequencies involving spermatocyte chromosomes in different post-treatment weeks did not exceed the control limit significantly except at week 6, that too marginally. Lack of appreciable clastogenic effect of the drug on spermatocyte chromosomes of mice was also reported earlier by Anantha Reddy and Subramanyam (1983) who tested three dose levels - 156, 312 and 624 mg/kg (approximately) and analyzed data at 24 h and 1 - 6 post-treatment weeks. The incidence of structural aberrations noted by earlier workers, compared to ours, was less. It might be due to high doses used by them and consequent mitotic inhibition. In neither study the frequencies of structural aberrations exhibited any correlation with the period of intervals between treatment and preparation of materials.

Thus, this drug demonstrates marked chromosome damaging effect in bone marrow system but not in spermatocytes. Such a differential response is not at all new in the field of chemical
mutagenesis. Chemicals like triethylenemelamine (TEM) and mitomycin C (MC) have been proved to be highly effective clastogens in bone marrow cells as well as in spermatogonia, but they produce very little aberrations in spermatocytes after treatment at spermatogonial stage (Adler, 1982a; Generoso, 1982). Very few chemicals are known to induce cytogenetic effects in stem cells that can be measured in meiotic cells. Adler (1982a, b) has discussed the probable reasons for this at length, but a definite answer is yet to be known. The minimum period of interval between treatment and fixation of the tissue in our experiment was 1 week; the diakinesis-metaphase I cells scored at that sampling week were expected to remain at pachytene stage at the time of treatment (Oakberg, 1957) assuming that the drug has no inhibitory effect on the cell cycle with the dose tested. Thus, it indicates that the mouse spermatocytes are non-responsive to the drug.

With regard to univalent formation the earlier workers (Anantha Reddy and Subramanyam, 1983) noted remarkable increase in the incidence of univalent formation particularly after chronic treatment for 3 days. On the other hand, in the present study the incidence of univalent formation increased significantly in two sampling weeks only - 4 and 6, that too marginally. There are several factors which may be accounted for differential results. In the earlier work (Anantha Reddy and Subramanyam, 1983) Novalgin tablet available in the market, not the base free sample, was tested. Other ingredients present in the tablets may be accounted for univalent formation. Further, the influence of high doses tested by them can not be ruled out for such phenomenon. The high doses of the drug as mentioned before have a great inhibitory
effect on the process of cell division, but it is not certain that it would not cause the univalent formation, particularly in view of the assumption of differential factors for clastogenicity and univalent formation based on the work on antirabies vaccine and tetanus toxoid (Das and Nayak, 1988).

The cause of univalent formation is not clear. It is assumed by many workers (Brewen and Preston, 1978) that the method of slide production is largely responsible for such phenomenon and the degree of spontaneous univalent formation varies from strain to strain (Lin et al., 1971; Schleiermacher, 1970; Beechey, 1973; Purnell, 1973).

In Swiss mice the univalent formation was very poorly marked also with benzodiazepine tranquilizers like diazepam (Kar and Das, 1981) and chlordiazepoxide (Kar and Das, 1987) and antileprosy drug, dapsone (Roy and Das, 1988), but highly marked with the antileprosy drug - clofazimine (Das and Roy, 1987), and antirabies vaccine and tetanus toxoid (Das and Nayak, 1988).

3.1.4.3 Sperm morphology assay:

Although the criteria of sperm morphology include 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 is concerned treated animals compared to control ones produced a few more types of abnormal sperm heads, though their frequencies were low. Similar occurrence of some particular abnormal types specifically for certain
chemicals were also noted earlier (Wyrobek and Bruce, 1975; Kar and Das, 1983). Significant increase in the incidence of mis-shapen sperm in first four post-treatment weeks clearly indicates positive effect of the drug; the peak effect was recorded, as noted earlier (Wyrobek and Bruce, 1975) for several other chemicals, at week 4. Such a highly marked effect was also noted with mitomycin C, thiotepa, myleran, vinblastin sulphate and other chemicals (Wyrobek and Bruce, 1975). In mice, as mentioned earlier, the shape of a normal sperm seems to be genetically controlled and induced abnormalities are suggested to be the result of genetic damage (Wyrobek and Bruce, 1975, 1978; Bruce and Heddle, 1979; Wyrobek et al., 1983a, b). Our results thus demonstrate genetic effect of the drug, and support the findings based on bone marrow chromosome analysis. The period of first four post-treatment weeks covered mainly the spermiogenesis step for the sperm under study. From significantly very high incidences of mis-shapen sperm in the first four weeks it seems the spermatids of different stages and matured sperm are susceptible to the drug for the induction of abnormality in sperm head morphology. Sensitivity of the sperm head abnormality assay for detecting deleterious effects of chemicals and other environmental agents on genetic material has been demonstrated by several workers (see Wyrobek et al., 1984). Our results although suffer from the limitation of having data for one dose level only, strengthen such findings.

3.1.4.4 Sperm count assay:

Though morphology of sperm is highly affected by metamizole its production seems to be influenced little. Marginally
significant decrease in count is found at week 3. A little suppression in count is documented in the entire treated series but it is not highly marked.

3.1.4.5 General:

Thus, our data on chromosome analysis in bone marrow cells reveal beyond doubt that metamizole has mild clastogenic effect particularly at early hours. Its deleterious effect on the sperm morphology is also remarkable; however, production of sperm was little affected. The mechanism of action of the drug, leading to the production of genotoxicity is not known. Our knowledge on the pharmacokinetics of the drug is far from complete, nor we know the mechanism of its action at the macromolecular level. This is due to the fact that, as mentioned before, this drug has not got a place in the British or Indian Pharmacopoeia in spite of its wide use. So far it is known 22.6% of the drug is excreted as 4-aminophenazone, 27.5% as 4-acetylamino phenazone and a small amount as rubazonic acid (Halberkann and Fretwurst, 1950). Less marked effect at 96 h following single treatment and in different weeks following repeated treatment in the present study may be explained from the point of gradual loss of the drug through excretion; loss is more with the increase of time between treatment and fixation of material.

The human therapeutic dose varies from 0.5 - 3 g daily in divided doses. Assuming an average dose of 2 g for a human of an average body weight 50 kg the human dose comes to 40 mg/kg.
The dose of 50 mg/kg tested here in time-response study in bone marrow cells is equivalent to 1.25 times the human dose on the basis of the body weight. But if it is calculated on the basis of the surface area of a mouse as usually done for most of the drugs and chemicals, the equivalent mouse-dose would be 12 times the human-dose (Freireich et al., 1966) on per kg basis i.e. 12 x 40 mg/kg; the dose 50 mg/kg would then be close to 1/10th the human-dose.
3.2  **Effect of Phenylbutazone**

3.2.1  Introduction

Phenylbutazone, as mentioned before, is also a pyrazolone compound. Chemically it is 1, 2- Diphenyl-3, 5-dioxo-4-n-butylpyrazolidine. Its structural formula is as follows:

![Structural formula of Phenylbutazone]

Phenylbutazone (1,2-Diphenyl-3, 5-dioxo- 4-n- butylpyrazolidine)

Phenylbutazone is a commonly prescribed drug having antiinflammatory, analgesic and antipyretic effect. It has also some uricosuric effect. This drug is particularly suitable for rheumatic disorder, and an effective alternative to colchicine in acute gout. Originally it was being employed as a solubilizing agent for aminopyrine; in 1949 it was first introduced for the treatment of rheumatoid arthritis. However, toxicity limits its use in the long term therapy.

Phenylbutazone is the generic name of the drug and is marketed under the trade names Butazolidin, Zolandin, Ticinil and Irgapirina. It is available both as tablet for oral consumption and as solution for injection.
In India alone total production of the drug was about 40.00 metric tons for the year 1986-87 (OPPI, 1987), which gives an idea about the wide spread use of the drug.

Some type of side effects are noted in 10-45% of patients. Nausea, vomiting, epigastric discomfort and skin rashes are the most frequently reported ontoward effects.

So far potential genotoxicity of phenylbutazone is concerned lot of works have been done in this line, but reports are contradictory, Stevenson et al. (1971) noted a significantly higher frequency of chromosome danages in lymphocytes of patients taking the drug than in the controls. However, other chromosome aberration studies in lymphocytes of patients under chemotherapy demonstrated either negative or weakly positive results (Walker et al., 1975; Crippa et al., 1976; Vormittag and Kolarz, 1979). Similarly lack of effect of the drug was noted in bone marrow cells of Chinese hamsters, rats and man (Muller and Strasse, 1971; Gebhart and Wissmuller, 1973). On the other hand, in vitro studies by Wissmuller and Gebhart (1970) and Ishidate (1981) yielded positive clastogenic effect of this pyrazolone compound in human lymphocytes and in a Chinese hamster lung fibroblast cell line respectively. But the DLT in mice at doses even 50 and 100 mg/kg failed to show any positive response (Machemer and Hess, 1971). The MNT conducted in mouse bone marrow system also revealed absence of clastogenic effect of the drug (Charles and Leonard, 1978). The frequency of non-disjunction and crossing over in Aspergillus nidulans remained in the control range following treatment of phenylbutazone, but hydroxyphenylbutazone did increase the frequency to a great extent (Bignami et al., 1974).
In view of widespread use of this non-narcotic analgesic and conflicting reports on its potential genotoxicity it was further evaluated in mouse in vivo system.

The details of the protocols followed for phenylbutazone are given below:

I. Bone marrow metaphase analysis
   A. Single treatment
      1. Time-response study
      2. Dose-response study
   B. Repeated treatment
      1. Time-response study

II. Bone marrow micronucleus test
    A. Dose-response study

III. Spermatocyte chromosome analysis
    A. Repeated treatment
       1. Time-response study

IV. Dominant lethal test
    A. Repeated treatment
       1. Time-response study

V. Sperm test
   A. Sperm morphology assay
      1. Repeated treatment
         a. Time-response study
   B. Sperm count assay
      1. Repeated treatment
         a. Time-response study
3.2.2 Materials and Methods

10-14 weeks old healthy adult mice, weighing 25-30 g were employed as the experimental animals. Base free sample of phenylbutazone (Batch No. BM-330R, C. No. 270317) was obtained from SG Chemicals and Pharmaceuticals Limited, Ranoli, India as gift. For all the protocols the drug was administered to the mice via oral route without any vehicle. With the help of two pairs of blunt forceps the drug was forcefully pushed into the throat of the animals. Age and sex matched untreated animals were employed as controls. Controls for some of the protocols were common to both metamizole and phenylbutazone.

3.2.2.1 Bone marrow metaphase analysis:

Mice of both the sexes were fed once with a dose of 100 mg/kg of phenylbutazone and killed at 4, 8, 16, 32 or 64 h post-treatment. This is referred to here as time-response study. Similarly for dose-response study the mice were treated orally with a dose of 50, 100 or 200 mg/kg of the drug and killed at 16 h post-treatment. Four mice were employed for each dose level and sampling period.

In another set of experiment healthy adult male mice were fed with the drug for seven successive days with a dose of 100 mg/kg/day and killed 1, 3, 4, 6 or 8 weeks after the last treatment. For each week 4-5 animals were employed. Age matched untreated males served the purpose of control. This is referred to here as repeated treatment set. Bone marrow preparations and staining of slides were done as described in the section 2.1.
3.2.2.2 Bone marrow micronucleus test:

Mice of either sex were administered orally with different doses (50, 100 or 200 mg/kg) of the drug twice at an interval of 24 hours and sacrificed at 30 h after the first treatment. 4 animals were employed for each dose level. Controls were age and sex-matched untreated animals. The bone marrow cells were processed and slides were prepared following the schedule described earlier (vide 2.2).

3.2.2.3 Spermatocyte chromosome analysis:

The males of the repeated treatment set for bone marrow metaphase analysis mentioned above provided the material for spermatocyte chromosome preparation also. So, the treatment schedule as well as sampling periods were the same as described above in repeated treatment set for bone marrow preparation (vide 3.2.2.2). Untreated males of the same age group were used as controls. Processing of testes for spermatocyte chromosome preparation have already been described in section 2.3.

3.2.2.4 Dominant lethal test:

Same as mentioned earlier in Materials and Methods (General) (vide 2.4).

3.2.2.5 Sperm test (sperm morphology and sperm count assay):

Vasa deferentia and caput epididymes provided sperm for sperm morphology assay and sperm count respectively. The materials were collected from the male animals used for spermatocyte chromosome preparation (vide 3.2.2.3). Details of the processing of the materials and scoring have been described earlier (vide 2.3).
3.2.3 Results

3.2.3.1 Bone marrow metaphase analysis:

Qualitative - No visible general type of effect on the behaviour of the animal was noted following single or repeated treatment of the drug. Numerical chromosome change was not also marked in the treated individuals. Structural chromosome damages encountered in the treated individuals were categorized under two main heads: gap-type and break-type aberrations. For convenience sub-chromatid type breaks were included along with gaps under gap-type aberrations. Gaps, both chromatid as well as chromosome or iso-chromatid types, were very common in occurrence (Fig. 4a). Among the break type aberrations chromatid breaks and fragments were very common. In most cases the broken acentric part showed little displacement (Fig. 4b); but cells containing broken fragments placed widely apart were not uncommon (Fig. 4c). Sometimes secondary constriction region of a chromosome was found to be involved in breakage. In no case a particular chromosome was found to contain two breaks. Though the fragments of untraceable origin were in general very small they varied in size from cell to cell (Figs. 4d, e). In one occasion a cell was noted to contain a paired fragment. Only a few exchange configurations were available; they were asymmetrical chromatid exchanges showing dicentric configuration, however, in no case the exchange was accompanied with a fragment. Qualitatively the chromosome aberrations obtained in the single and repeated treatment sets were the same.
Explanation for Fig. 4

Photomicrographs of bone marrow metaphases of mice showing structural chromosome aberrations induced by phenylbutazone.

1. A chromosome with a chromatid gap.

b. A chromosome with a chromatid break, the fragment placed near to its origin.

c. A chromosome with chromatid break, the acentric fragment is greatly displaced.

d,e. Metaphases showing chromatid fragments of unknown origin.
Quantitative - Time-response analysis of structural chromosome aberrations following single treatment (Table 7) exhibited a slight significant increase of the breakage frequencies at 16 h post-treatment; in other sampling intervals the incidence of breakage remained in the control range. Analysis of correlation coefficient did not reveal any correlation of the breakage frequencies with time (sampling intervals). Break and gap type aberrations when taken together (i.e. total aberrations) showed significantly higher incidence at 16 and 64 h post-treatment. However, the increases were not so highly remarkable, only about 2 times the control value. No correlation was also marked between the incidences of total aberrations and sampling intervals.

Table 8 summarizes the data on structural chromosome aberrations in the dose-response study following single treatment. As the time-response study mentioned above yielded the maximum response at 16 h, the dose-response study was conducted at that particular sampling interval. Interestingly here only the intermediate dose (100 mg/kg) elevated the breakage frequencies significantly over the control value; however, when total aberrations were considered the incidences for 100 and 200 mg/kg showed significantly higher increase over the control value. Neither break-type aberrations nor total aberrations showed any correlation with the doses tested.

The data on structural chromosome aberrations induced in bone marrow cells following repeated treatment are presented in Table 9. Here only chromatid breaks and fragments constituted
Table 7: Incidence of different types of structural chromosome aberrations in bone marrow metaphases of mice treated with a single dose of phenylbutazone (100 mg/kg) in different post-treatment sampling intervals. Each exchange was counted as two breaks. Values in parenthesis are mean per 100 metaphases ± S.E.

<table>
<thead>
<tr>
<th>Sampling period (hour)</th>
<th>Metaphases scored/Animals</th>
<th>Break type aberrations</th>
<th>Gaps + Sub-chromat. break</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chromat. break</td>
<td>Frag.</td>
<td>Exch.</td>
</tr>
<tr>
<td>4</td>
<td>300/4</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>300/4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>350/4</td>
<td>6</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>350/4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>64</td>
<td>350/4</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>(Cont.)</td>
<td>2600/36</td>
<td>5</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

r = -0.335
\[df = 4\]

²'t' test: significantly different from control at \( b = p < 0.01 \)
Table 8: Incidence of various types of structural chromosome aberrations in bone marrow metaphases of mice treated once with different doses of phenylbutazone at 16 h post-treatment. Each exchange was counted as two breaks. Values in parenthesis are mean per 100 metaphases ± S.E.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Metaphases scored/Animals</th>
<th>Break type aberrations</th>
<th>Gaps+Sub-chromat. break</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chromat. break</td>
<td>Exch.</td>
<td>Total</td>
</tr>
<tr>
<td>50</td>
<td>400/4</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.00±0.35)</td>
</tr>
<tr>
<td>100</td>
<td>350/4</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3.00±0.77)(^b)</td>
</tr>
<tr>
<td>200</td>
<td>350/4</td>
<td>-</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.25±0.54)</td>
</tr>
<tr>
<td>0 (Cont.)</td>
<td>2600/36</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.58±0.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3.46±0.36)</td>
</tr>
</tbody>
</table>

\( r = 0.075 \)
\( df = 2 \)

\( r = 0.345 \)
\( df = 2 \)

\(^b\), \(^c\) 't' test: significantly different from control at \( b = p < 0.01 \), \( c = p < 0.05 \)
the break type aberrations; neither exchange nor iso-chromatid break was noted. The frequencies of break-type aberrations ranged from 0.47 to 2.33%, and in no case the treated value exceeded the respective control value significantly. If the control values for break-type aberrations obtained in different weeks, which did not vary from each other markedly were pooled and that pooled control mean value (0.83%) was compared with the treated values, the picture remained unaltered. However, when total aberrations (including gaps) were considered the frequencies at weeks 1 and 4 in the treated series showed significant increase over their respective control values; had the treated data been compared with the pooled control mean value (4.16%) the data at weeks 1, 3 and 4 would show significant increase.

3.2.3.2 Micronucleus test:

Qualitative - The affected erythrocytes contained one micronucleus (Figs. 5a-e). The MN varied greatly in size and location in the cytoplasm (Figs. 5a-e). The MN recorded in the nucleated cells (Figs. 5f, g) were in general bigger in size while in the erythrocyte line they were small. Some mitotic figures with lagging chromosome(s)/chromatid fragment(s) were also encountered (Fig. 5h). Its incidence though not quantitatively estimated seemed to be close to the control value.

Quantitative - The incidences of micronuclei in erythrocytes and nucleated cells are presented in Table 10. The frequency of erythrocytes with MN obtained in the control series (0.30%) was very close to the value obtained earlier in the same
Explanation for Fig. 5

Cut-out photomicrographs of bone marrow smears displaying micronucleated erythrocytes and nucleated cells, and abnormal mitotic figures induced by phenylbutazone.

a-e. Poly-and normo-chromatic erythrocytes, each with one micronucleus (MN). The micronuclei vary in size and location.

f,g. Nucleated cells, each with one MN.

h. An anaphase with a lagging chromosome.
Table 10: Results of the micronucleus test in bone marrow cells of mice treated with different doses of phenylbutazone. Animals were treated twice at an interval of 24 hours and killed 6 hours after the second treatment. Values are mean per 100 cells ± S.E. Figures in parentheses are MN/cells scored. For each dose or control 4 animals were employed.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>PCEs with MN</th>
<th>NCEs with MN</th>
<th>PCEs + NCEs with MN</th>
<th>Nucleated cells with MN</th>
<th>PCE/NCE</th>
<th>RBC/100 nucleated cells</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.38 ± 0.02</td>
<td>0.23 ± 0.04</td>
<td>0.30 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>9.81 ± 0.27</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>(15/4, 000)</td>
<td>(9/4, 000)</td>
<td>(24/8, 000)</td>
<td>(6/8, 000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.60 ± 0.09C</td>
<td>0.35 ± 0.03</td>
<td>0.48 ± 0.05C</td>
<td>0.08 ± 0.01</td>
<td>0.79 ± 0.04</td>
<td>12.31 ± 0.89</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>(24/4, 000)</td>
<td>(14/4, 000)</td>
<td>(38/8, 000)</td>
<td>(6/8, 000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.45 ± 0.03C</td>
<td>0.20 ± 0.04</td>
<td>0.33 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>0.86 ± 0.02</td>
<td>10.13 ± 0.63</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>(18/4, 000)</td>
<td>(8/4, 000)</td>
<td>(26/8, 000)</td>
<td>(16/8, 000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.30 ± 0.04</td>
<td>0.30 ± 0.05</td>
<td>0.30 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.95 ± 0.07</td>
<td>10.38 ± 0.56</td>
<td>0.54</td>
</tr>
<tr>
<td>(Cont.)</td>
<td>(12/4, 000)</td>
<td>(12/4, 000)</td>
<td>(24/8, 000)</td>
<td>(9/8, 000)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r = +0.126  r = -0.371  r = -0.034
df = 2       df = 2       df = 2

'C' test: significantly different from control at  c = p < 0.05
MI = Mitotic index (% of dividing cells)
strain of mice by Chaubey et al. (1975), Van Went (1978), Das and Swain (1982) and Das and Kar (1986). The frequencies of micronucleated polychromatic erythrocytes (PCE) increased over the control for all the dose levels tested. However, the increases were significant for the higher two doses (100 and 200 mg/kg).

In no case the incidence of micronucleated normochromatic erythrocytes (NCE) as well as nucleated cells exceeded the control limit. When PCEs and NCEs were taken together the incidence of micronucleated cells showed significant increase only for the intermediate dose. The values for PCE/NCE ratio, RBC/100 nucleated cells and mitotic index (MI) obtained for three dose levels did not differ very much from the respective control values.

3.2.3.3 Spermatocyte Chromosome analysis:

Qualitative - Qualitatively the effects encountered in the control and treated series, as well as in different post-treatment weeks were the same except presence or absence of certain aberration type(s) in a particular week, which may be due to the low number of cells scored. The effects were analyzed mainly under two heads; structural changes and effect on pairing behaviour. An attempt was also made to examine potential effect on numerical changes of chromosomes. Although some polyploid cells and some cells with less than 20 bivalents (Fig. 6a) were recorded hyper-diploid cells were extremely rare; and the numerical changes were not considered for quantitative analysis.

The structural changes were manifested mainly in the form of chromatid breaks and fragments (Fig. 6b). In one occasion
Explanation for Fig. 6

Photomicrographs of diakinesis-metaphase I chromosomes of mice showing structural, numerical and pairing anomalies induced by phenylbutazone.

a. A diakinesis-metaphase I plate with 19 bivalents (19 II).

b. A chromosomal fragment of unknown origin.

c. Univalent formation (early separation) involving one autosomal pair.

d. A polyploid (or fused) diakinesis-metaphase I cell showing univalent formation (early separation) of sex chromosomes.
in the treated series a chromosome type or isochromatid break was recorded. Not a single exchange configuration was noted.

Univalent formation involving autosomes and sex chromosomes was a common phenomenon (Figs. 6c, d). Among autosomes mostly the smaller ones were involved. The homologues were variously placed in the field.

Quantitative - Table 11 summarizes the data on structural chromosome changes and univalent formation induced after repeated treatment of phenylbutazone. The structural aberrations in the treated series ranged from 0.50-1.50%, while the control values ranged from 0.50-1.33%. Thus, in no week there was perceptible difference between the control and treated values.

Regarding univalent formation, when the treated values were compared with the respective week-wise control values significant increases were noted at weeks 1, 3 and 8 for autosomes, and at weeks 1 and 4 for sex chromosomes. For autosomes the control values fluctuated between 1.33 and 5.33% and the maximum treated value obtained at week 3 (11.50%) was more than 3 times the pooled control mean value (3.27%). In case of sex chromosomes the control values also ranged from 1.33-5.33% with a mean 3.03%, while the treated values ranged from 4.00-9.20%; the maximum treated value noted at week 8 was also more than 3 times the pooled control mean value (3.03%). Autosomes and sex chromosomes taking together the incidences in the treated series showed significant increases over the respective week-wise control values at weeks 1, 3 and 8.
3.2.3.4 Dominant lethal test:

The total number of implants in the treated and the control series varied from $2^{12}$ and $2^{11}$ respectively. In most of the cases, the affected female was found to contain only 1 dead implant; in no case the number of dead implants exceeded two. Further, the two horns of an uterus did not show any preference with regard to the distribution of dead implants. In the present investigation no differentiation was made for early and late embryonic death, all the dead implants were taken under the same heading 'dead'. However, in most of the cases intrauterine death occurred at an early stage.

The data on the dominant lethal test are presented in Table 12. The mating indices recorded in the treated series (46.43-75.00) were very close to those recorded in the control series (40.00-70.00). Statistical analysis ($\chi^2$ test) revealed no significant difference between the control and treated values in any week.

When the incidences of dead implants per pregnant female were analyzed the value obtained in any week in the treated series differed from neither the respective control value nor the pooled control mean value (0.50) significantly. When the incidences of living implants per pregnant female were considered the treated values except at week 1 remained very close to their corresponding control values. At week 1 the frequency of living implants per pregnant female decreased significantly from its respective control value as well as from the pooled control mean value (7.04). Similarly for total implants per pregnant
female the treated value at week 1 only decreased significantly from the corresponding control value; in all other weeks the treated values remained close to the control values. Not a single parameter in the present study showed any correlation with the post-treatment mating weeks.

3.2.3.5 Sperm morphology assay:

Qualitative - Qualitatively the abnormalities induced in the sperm head following phenylbutazone treatment were of varied types; among them with flat base, 'sickle' shaped, 'axe' shaped, fusiformed, with acrosome spine abnormalities and amorphous were very common (Figs. 7a-l). Here also a high degree of variation in spine morphology was noted in sperm heads with acrosomal spine abnormalities, it ranged from a total absence to a long hook shaped one (Figs. 7c, e-i). Abnormal types like notched, giant size, spherical, triangular, rectangular, L-shaped, invaginated, 'balloon' shaped and with vacuole were also available though not so common. All the types mentioned above were also recorded in the control series. In the treated series a few pentagonal sperm heads were noted, it was available neither in the control nor in the metamizole treated individuals. However, dichotomous and 'spear-head' shaped recorded in the metamizole treated series were not found here. Sometimes a particular sperm head was found to exhibit a combination of two or more types of abnormalities.

Quantitative - Here also all the types of abnormal sperm heads were taken together under the heading 'abnormal' for
Explanation of Fig. 7

Photomicrographs of sperm showing abnormalities in head morphology in mice induced by phenylbutazone.

a, b. Sperm heads with flat bases.

c, d, f-i. Sperm heads with flat bases and acrosomal spine abnormalities.

e. Sperm head with acrosomal spine abnormality.

j-l. Various types of amorphous sperm heads.
convenience in quantitative analysis. The frequency of sperm with mis-shapen heads in the control series ranged from 3.73-4.40% (Table 13); the values obtained in different weeks thus did not differ much. Analysis of correlation coefficient did not also reveal any influence of 'weeks' on the values obtained in different weeks. If the values for different weeks in the control series were pooled the mean value would have been 4.16% which is very close to the value obtained, by earlier workers (Bruce et al., 1974; Kar and Das, 1983).

In the treated series the lowest value (3.70%) which was close to the lowest value obtained in the control series was recorded at week 3; while the highest value (8.35%) was noted at week 6 (Table 13). Though there was a tendency of increase in the frequency of sperm with mis-shapen head at weeks 4, 6 and 8 the value at week 6 only showed significant increase whether compared with the corresponding control value or compared with the pooled control mean (4.16%). Lack of influence of 'weeks' on the values obtained in different weeks in the treated series was also greatly marked.

3.2.3.6 Sperm count assay:

The data on epididymal sperm counts are presented in Table 14. If the treated values obtained in different post-treatment weeks were compared with the corresponding control values, in general, there was decrease in count in the treated series except at week 6. However, the decrease was statistically significant only at week 4 when the data were compared with the
Table 13: Effect of phenylbutazone on the incidence of sperm head abnormalities in mice in different post-treatment sampling weeks after 7 days repeated treatment (oral, 100 mg/kg X 7). Values are mean per 100 sperm ± S.E. For each week 4-5 animals were employed and 1000 sperm heads were examined from each individual.

<table>
<thead>
<tr>
<th>Series</th>
<th>wk 1</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 6</th>
<th>wk 8</th>
<th>r-value (df = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>4.03 ± 0.12</td>
<td>3.70 ± 0.56</td>
<td>5.45 ± 0.49</td>
<td>8.35 ± 0.91b</td>
<td>4.82 ± 0.91</td>
<td>0.48</td>
</tr>
<tr>
<td>C</td>
<td>4.15 ± 0.94</td>
<td>4.40 ± 1.13</td>
<td>4.28 ± 1.28</td>
<td>4.23 ± 0.14</td>
<td>3.73 ± 0.63</td>
<td>-0.65</td>
</tr>
</tbody>
</table>

T = Treated, C = Control

b 't' test: significantly different from control at b = p < 0.01
Table 14: Effect of phenylbutazone on epididymal sperm count of mice in different post-treatment weeks after 7 days repeated treatment (100 mg/kg \times 7). The values are mean number of sperm heads in one WBC chamber of haemocytometer ± S.E. To obtain total sperm count per epididymis each value should be multiplied with \(8 \times 10^4\) (dilution factor). For each week 4-5 animals were employed.

<table>
<thead>
<tr>
<th>Series</th>
<th>wk 1</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 6</th>
<th>wk 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>67.62 ± 17.44</td>
<td>87.28 ± 4.20</td>
<td>41.44 ± 12.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>126.65 ± 18.05</td>
<td>90.00 ± 15.08</td>
</tr>
<tr>
<td>C</td>
<td>77.26 ± 8.02</td>
<td>108.52 ± 9.08</td>
<td>101.54 ± 10.37</td>
<td>112.95 ± 14.48</td>
<td>111.15 ± 7.83</td>
</tr>
</tbody>
</table>

\(T = \) Treated, \(C = \) Control

<sup>b</sup> 't' test: significantly different from control at \(b = p < 0.01\)
corresponding control one. The picture would remain unaltered if the treated values were compared with the pooled control mean value (101.06 ± 5.43).

3.2.4 Discussion

3.2.4.1 Bone marrow metaphase analysis:

Our data on bone marrow chromosome analysis clearly reveal very little clastogenic effect of the drug. At 16 h post-treatment only for 100 mg/kg the breakage frequency showed a significant increase, the highest dose tested (200 mg/kg) even failed to enhance the breakage frequency significantly; repeated treatment schedule did not also elevate the breakage frequency remarkably in any post-treatment week. However, the incidence of gaps increased considerably in treated individuals, which led to the marked increase of total aberrations (break-type + gap and sub-chromatid type aberrations) in several post-treatment sampling periods. In view of high frequencies of gaps it is not unreasonable to assume that the drug affects, whatever little it may be, the G_2 or prophase chromosomes (Bender et al., 1974; Brewen and Stetka, 1982). As the significance of the origin of gaps and sub-chromatid breaks is yet to be known clearly we do not want to attach much importance on the total aberrations.

Bone marrow metaphase analysis in other mammals like Chinese hamsters rats and man (Müller and Strasse, 1971; Gebhart and Wißmuller, 1973) also failed to demonstrate clastogenic capacity of the drug. The dose-range tested by Müller and Strasse (1971) in Chinese hamsters included the doses used by us
(50, 100 and 200 mg/kg); and they treated the animals twice at an interval of 24 h and sampling was done at 30 h after the first treatment. Less effects obtained by them (Muller and Strasse, 1971), compared to ours, may be due to differential response of two species.

Metaphase chromosome analysis in bone marrow cells as well as in lymphocytes of the peripheral blood of patients under chemotherapy also exhibited either negative or weakly positive results (Walker et al., 1975; Crippa et al., 1976; Vormittag and Kolarz, 1979). However, literature provides some positive reports too; in a study of fifty patients who had been taking phenylbutazone for at least three months it was found that the incidence of chromosome damage including dicentric chromosome in lymphocytes was significantly higher than in controls (Stevenson et al., 1971). Human lymphocytes exposed to the drug in vitro also exhibited significantly enhanced chromosome damage (Wissmuller and Gebhart, 1970). Thus, the previous studies based on metaphase chromosome analysis record, save a few, negative or very weakly positive effect of the drug, and our results are in good agreement with those of the earlier works.

3.2.4.2 Micronucleus test:

The MNT is now considered as sensitive as, or more sensitive than, the metaphase analysis for recognizing potential genotoxicity of an agent. Two higher doses tested here increased the frequency of MN in PCEs significantly; however, the increases were significant marginally only. Thus, this assay system
demonstrates weak clastogenicity of the drug, and the finding is in accordance with that based on our metaphase analysis. As the incidence of micronucleated NCEs remained at the control level even at 30 h post-treatment it is reasonable to assume that the drug has no spindle poisoning effect (Schmid, 1976; Das and Kar, 1986); the values for PCE/NCE ratio as well as mitotic index (MI) in the treated series which remain close to the control value strengthen the assumption. So the little positive effect encountered in PCEs may be explained from the point of a little clastogenic effect of the drug.

So far as the author is aware, literature provides only one report on the assessment of potential clastogenicity of phenylbutazone using the micronucleus test. Charles and Leonard (1978) noted no significant increase in the incidence of MN in the bone marrow cells of laboratory mice treated with an acute intraperitoneal dose of 100 mg/kg of the drug. In the present experiment 3 dose levels were tested; the two higher dose levels (100 and 200 mg/kg) showed significantly higher, though very marginally, incidence of MN in PCEs. The differential results obtained in two studies for the same dose (100 mg/kg) may be attributed to the treatment schedule, time between treatment and fixation of material, and route of administration of the drug (see Heddle et al., 1983). The earlier workers (Charles and Leonard, 1978) treated the mice ip once only and fixed the bone marrow cells at 36 h post-treatment. On the other hand, in the present study the animals were treated via oral route twice at an interval of 24 h and killed for fixation of the
bone marrow cells at 30 h after the first treatment. Differential results due to different route of administration have been noted by several workers in connection with several other chemicals (Bhunya and Pal, 1986; Bhunya and Behera, 1987; Bhunya and Pati, 1988; Behera and Bhunya, 1987). Slightly higher incidence of MN in our case may also be due to double treatment; since enucleation takes place about 6 h after the final mitosis the PCE population sampled at 30 h post-treatment may represent PCEs resulted from enucleation following both 1st and 2nd treatment. As evidenced from the mataphase analysis in bone marrow cells phenylbutazone causes an early effect whatever a little it may be. With the increase of sampling period (time between treatment and fixation of cells) beyond 16 h the effect gets reduced. So low incidence of micromucleated PCEs with the increase of sampling period is not quite impossible. Over and above there is question of individual sensitivity.

The control value for PCEs obtained by us (0.3%) was very close to the value obtained by earlier workers in this strain of mice (Chaubey et al., 1975, 1977; Van Went, 1978; Kar and Das, 1979; Das and Swain, 1982; Das and Kar, 1986).

3.2.4.3 Spermatocyte chromosome analysis:

Lack of clastogenic effect of the drug was documented from spermatocyte chromosome analysis which failed to reveal any significant increase in the incidence of structural changes over the controls following treatment. Our study covers a period of 8 post-treatment weeks. Absence of any effect at week 1 is
indicative of the fact that the meiotic chromosomes are resistant to the drug. However, we have certain reservations for such an assumption for spermatogonial chromosomes, because, as it has been mentioned earlier, only a few chemicals are known to induce cytogenetic effect in spermatogonia that can be translated in meiotic cells (Adler, 1982a).

With regard to univalent formation the drug seems to have a little effect on the pairing behaviour of the chromosomes. Though the frequencies of univalent formation increased significantly in certain cases only, a tendency of increase is clearly marked in almost all the sampling weeks. Comparison of the treated values with the pooled control mean values does not also alter the picture. From our data it is not known if factors for clastogenicity and univalent formation are the same.

3.2.4.4 Dominant lethal test:

The DLT has been used extensively particularly in the mouse during the last 15 years or so to assess mutagenicity of a wide variety of substances. Owing to its involvement with the germ cells it has become one of the important screening systems currently used in mutagenicity testing. The principle, importance and sensitivity of this test has been discussed earlier in the general 'Introduction' (vide 1).

This test in the present investigation had some limitation, only one dose level (100 mg/kg), - the intermediate dose tested in bone marrow metaphase analysis and MNT, was employed. The frequency of total implants per female in the
control series ranged from 5.60 to 8.57 with a mean value of 7.54 which is close to the value obtained earlier in mice by other workers (Ehling et al., 1968, 1972; Generoso et al., 1978; Kar et al., 1984; Kar and Das, 1979, 1987). Again, our data on the incidence of dead implants per pregnant female in control groups, which varied between 0.20 and 1.00 with a mean of 0.50 were also at par with those of several other workers (Lorke and Machemer, 1974; Petersen and Legator, 1973; Kar and Das, 1979, 1987; Kar et al., 1984).

Among the treated individuals in no mating week the incidence of dead implants differed significantly either from the respective control value or from the pooled control mean, which indicates lack of dominant lethal effect of the drug. The cases of resorption (deciduomata) have not also been marked.

Significant decrease in the frequency of live implants per dam at week 1 following treatment can be ascribed to the fact that during this period the control group displayed the highest rate of implantation and the treated group displayed the lowest value. This is certainly not the result of a greater loss of implants as evidenced from the data on dead implants per dam which remained close to the control value. Identical situation was noted for total implants at week 1 also. The present study does not account the pre-implantation loss, it deals with the post-implantation loss only. Obviously we are not in a position to say if pre-implantation loss has any role for decrease of total implantation in the treated animals; however, here involvement of pre-implantation loss seems to be very unlikely because the
decline in total implantation is not marked even at week 2. Our data are very limited to come to any conclusion with regard to this aspect. Thus, our results clearly reveal lack of dominant lethal effect of phenylbutazone and support the earlier findings of Machemer and Hess (1971) who tested 2 dose levels (50 and 100 mg/kg) in the CFLP mouse and failed to obtain any pre-implantation as well as post-implantation dominant lethality. In the earlier experiment the data were collected through 8 consecutive post-treatment weeks.

3.2.4.5 Sperm morphology:

So far our knowledge goes, phenylbutazone has been studied neither in patients taking the drug nor in any other mammal for its potential effect on sperm morphology and sperm production. As found with various other chemicals (Wyrobek and Bruce, 1975; Kar and Das 1983; Roy 1987) qualitatively phenylbutazone produced some specific type of head shape abnormality like 'pentagonal' head which was noted neither in the controls nor in metamizole treated mice. The frequency of this type was, however, very low. Naturally, it will not be unreasonable to assume that they are not typical for the drug.

The frequency of mis-shapen sperm following treatment increased significantly at week 6 only. So, we can compare the abnormal sperm-head producing capacity of phenylbutazone with that of diazepam (Kar and Das, 1983) which has also been shown to elevate significantly the sperm head abnormalities at week 6 only. Similar less marked effect on sperm morphology was also noted with
hydroxyurea, imuran, 5-iododeoxyuridine, aminopterine, etc. (Wyrobek and Bruce, 1975). However, in all those cases maximum effect was noticed at week 4.

Sperm morphology assay like all other assays reveals very mild effect of phenylbutazone, only at week 6 it shows high effectiveness. Lack of any effect from weeks 1-4 clearly indicates absence of effect of the drug on meiotic and post-meiotic cells. At present we are not in a position to put forward exact explanation for high effectiveness at week 6 only. The sperm studied at week 6 are expected to remain at differentiating spermatogonial stages at the time of treatment. So susceptibility of differentiating spermatogonial stages may well be the reason for higher effect at week 6. The sperm scored at week 8 remained at spermatogonial stem cell stage at the time of treatment. Lack of effect at week 8 may be assumed to be due either to resistance of the spermatogonial stem cells to the drug or to selective elimination of the affected stem cells.

3.2.4.6 Sperm count:

Among the treated values obtained, the value at week 4 only exhibited statistically significant decrease, compared to the control. However, the decrease seems to have little biological significance as the value was the lowest among the treated ones recorded and far below the general range. Further, the treated values do not display a particular pattern with respect to time (weeks of interval between treatment and fixation of the material).
The data on counting of epididymal sperm, which may be considered as an ancillary experiment to dominant lethal test are, therefore, in good agreement with those of dominant lethal test. On the basis of the data obtained from X-irradiated mice it has been reported by Searle and Beechey (1974) that if the sperm count comes below 10% level of the normal count complete sterility results. Our dominant lethal test did not account for complete sterility in any test week.

3.2.4.7 General:

Our results, thus, reveal a very mild clastogenic effect of the drug. So far pharmacokinetics of phenylbutazone is concerned, it is rapidly and completely absorbed from the gastrointestinal tract or the rectum, and the peak plasma level reaches in 2 hours. Following therapy 95% of the drug is bound to plasma protein. The half-life of the drug is 50-65 hours (Flower et al., 1985). Only a trace of unchanged phenylbutazone is excreted in the urine; it is known to undergo extensive metabolic transformation. The most important and main metabolite is oxyphenylbutazone which has antirheumatic and sodium retaining activities similar to those of the parent drug. The metabolite has a half life in plasma of several days (Flower et al., 1985). So a slight increase in cytogenetic effect noted at early hours (to be precisely at 16 h post-treatment) following single treatment in bone marrow cells and absence of any effect in late hours and in different post-treatment weeks may be accounted for shorter half-life of the drug. Lack of any noticeable effect at still earlier sampling
periods, i.e. at 4 and 8 h, may be explained from the point of time taken by the drug to reach to the bone marrow cells and causing effect. In the MNT significant increase in the incidence of micronucleated PCEs, but not NCEs, also documents that the drug takes a little time to reach to and affect the bone marrow cells. Lack of effect at later sampling hours also indicates ineffectiveness of exyphenylbutazone the half-life of which is several days.

The human therapeutic dose of phenylbutazone varies, the maximum therapeutic effect is provided by a dose of 300-600 mg daily for a brief period (Flower et al., 1985). Assuming an average daily dose of 500 mg for a man of 50 kg body weight our dose level 100 mg/kg comes to 10 times the human dose, this is the calculation based on body weight. However, if one calculates on the basis of the surface area for absorption, which is the usual practice for drugs and chemicals, for conversion of human dose to some other animal dose the mouse-dose would be 12 times the human dose (Freireich et al., 1966), that means here 120 mg/kg; so 100 mg/kg dose tested here would be almost equivalent to the human therapeutic dose.