5 EFFECT OF SULFAMETHOXAZOLE

5.1 Introduction

Sulfamethoxazole (SMX) is a non-antibiotic antimicrobial agent. It is a sulfa drug belonging to the group sulfonamide and very commonly used for prevention and cure of bacterial infections in man. It would not be inappropriate to present here a brief introduction on sulfa drugs and a review on their potential genotoxicity. Penicillin - the wonder drug, was mass-produced and became commercially available in 1940s; before that sulfonamides constituted the mainstay of antibacterial chemotherapy. The modern era of chemotherapy of infection in fact started with clinical use of sulfanilamide in 1936. The term sulfonamide is usually used as a generic name for derivatives of para-aminobenzene sulfonamide (sulfanilamide). No doubt the advent of penicillin and subsequently other antibiotics diminished the usefulness of the sulfonamides. However, widespread use of antibiotics has led to misuse of them and emergence of antibiotic-resistant pathogens, which is a great human concern and in turn has created an ever-increasing need for new drugs. The introduction of the combination of trimethoprim (TMP) and sulfamethoxazole in mid-1970s has resulted an increased use of sulfonamides for the treatment of specific microbial infections. Now-a-days the importance of sulfonamide is increasing at an alarming rate. Since 1936 more than 5000 congeneric substances were synthesized and tested for their antimicrobial property; however, only a few have attained therapeutic importance. Sulfonamides have a wide range of antimicrobial activity against both gram-positive and gram-negative microorganisms. Except in a few cases their efficacies in vitro and in vivo hardly differ.
They act as bacteriostatic agents. Chance of developing sulfonamide resistant organism is also there but less.

In India alone in 1986-87 a total of 1257.5 metric tons of sulfa drugs (which comprise 9 separate drug species including sulfamethoxazole) was produced (OPPI, 1987).

Sulfonamides have extensively been studied earlier in root tip cells of onion and shown to produce a variety of cytotoxic effects like spindle inhibition, induction of polyploidy and binucleate cells, and contraction of chromosome, etc. (Peters, 1946; Fuller, 1947; Nehra, 1949). Sharma (1971) described chromosome aberrations and other mitotic abnormalities in root tip cells of Allium treated with sulfanilamide. While in D. melanogaster sulfanilamide has been reported to produce an antimutagenic activity (Goncharova and Turbin, 1965). Trisulfamide and sulfaguanidine are shown to induce chromosome aberrations and radiosensitizing effect in V. faba (Lazanyi et al., 1963; Lazanyi, 1966). In the Ames Salmonella/microsome assay system, however, sulfaguanidine is shown to have no mutagenic effect (Mc Cann et al., 1975). Bignami et al., (1974) evaluated the genetic effect of 6 sulfanilamide drugs viz. sulfameter, sulfalene, sulfaphenazole, sulfamethizole, sulfamethoxypyridazine and sulfanilamide using non-disjunction and crossing over as criteria in Aspergillus nidulans, and three of them (sulfalene, sulfamethizole and sulfanilamide) enhanced crossing over frequency significantly, maximum being 45% of the plated conidia. Comparatively our knowledge on the potential cytotoxic and genotoxic effect of sulfa drugs in mammalian system, in vivo in particular, is scanty. Murthy and
Subramanyam (1981) tested sulfadiazine in meiotic cells of the mouse and noted frequent occurrence of univalent formation, but structural chromosome aberrations were very low. Cytogenetic effects of sulfamethoxazole which the present chapter is concerned with have been studied in man in vivo and in vitro and the works have been reviewed in subsequent paragraphs.

Sulfamethoxazole (GANTANOL) is a close congener of sulfisoxazole. But its rate of enteric absorption and urinary excretion are slower than those of sulfisoxazole. Its important feature is that sulfur is directly linked to the benzene ring; the structural formula of the drug is as follows:

\[
\begin{align*}
H_2N & \quad \text{SO₂NH} \\
& \quad \text{NH} \\
& \quad \text{CH₃}
\end{align*}
\]

Sulfamethoxazole

Sulfamethoxazole is employed for both systemic and urinary tract infections. It is available for oral use as tablets and also as a suspension. The clinical uses of sulfamethoxazole are the same as those for sulfisoxazole. It is presently marketed in fixed-dose combination with phenazopyridine (AZO GANTANOL) as an urinary antiseptic and analgesic. The introduction of trimethoprim (a compound related to pyrimethamine) in combination with sulfamethoxazole constitutes an important advance in the development of clinically effective antimicrobial agents. It represents the practical application of a theoretical consideration,
that is, the two drugs act on sequential steps in the pathway of an obligate enzymatic reaction in bacteria in a synergistic manner (see Hitchings, 1961). The combination of trimethoprim and sulfamethoxazole is known as co-trimoxazole which is sold in the market under various trade names like Bactrim, Septran, etc. The sulfonamide-trimethoprim combination which provides a bactericidal mixture from two bacteriostatic drugs has largely replaced the use of sulfonamides alone. But a sulfonamide alone still finds use in some cases of urinary infection, meningitis and eye infections. Similarly sulfamethoxazole in combination with other compound(s) forms more than a dozen of combination drugs which are sold in the market under various trade names (to name a few Cidal Forte, Infectra, Methoxaprim, Otrim, Servoprim, Sugaprim-S, Sumetrol, Trisoprim, etc.) and used widely as antimicrobial agents. The total production of sulfamethoxazole for the year 1986-87 in India alone was 500 metric tons (OFPI, 1987), which gives an idea of wide-use of the drug.

SMX is known to interfere with the final stages of folic acid synthesis; folic acid is essential for purine and hence nucleic acid synthesis (Hitchings and Burchall, 1965). As an inhibition of folate metabolism and, thereby, of DNA synthesis it seems to be a possible mutagen and chromosome breaking agent. The chromosome breaking capacity of other folic acid antagonists like methotrexate and pyrimethamine has been established (Bottura and Coutinho, 1965; Maier and Schmid, 1976; Jensen and Nyfors, 1979). The drug has some haematological side effects like neutropenia, plastic anemia, thrombocytopenia and megaloblastosis (Frisch, 1973). Depression of spermatogenesis following treatment
of SMX + TMP has also been reported in man (Murdia et al., 1978).

Inspite of wide use of this sulfa drug (sulfamethoxazole) and its possible genotoxic effects it has not drawn proper attention of geneticists and cytogeneticists for some detailed study. The reports so far made are very limited, that too are contradicting. In early 1970s some works were performed to test clastogenic efficiency of SMX in vivo and in vitro. Stevenson et al. (1973) and Gebhart (1975) could not find any chromosomal damage in lymphocytes of patients taking regularly the drug in combination with TMP. Similarly negative result was obtained in lymphocytes exposed to the drug alone or to the combination of SMX and TMP in vitro (Stevenson et al., 1973). Gebhart (1973), however, noted poor clastogenic effect of Bactrim (SMX + TMP) in bone marrow cells of patients under therapy. In contrast, Sorensen and Jensen (1981) recorded a significant increase in the incidence of micronucleated erythroblasts in bone marrow of patients taking SMX along with TMP, though the frequency of structural changes of metaphase chromosomes remained at the control level.

From the foregoing paragraph it is clear that no work has so far been done with SMX alone on its probable genotoxic effect in mammalian in vivo system. Most of the works mentioned above were concerned with co-trimoxazole, i.e. the combination of SMX and TMP. It is not known if the positive cytogenetic effect when noted was due to SMX or TMP or both. We have studied the potential effect of SMX alone on chromosomes as well as mitosis process in mouse in vivo system.

Only metaphase analysis and MNT were conducted for this drug, and the following parameters were adopted.
I. Bone marrow metaphase analysis
   A. Single treatment
      1. Time-response study
   B. Double treatment
      1. Dose-response study

II. Micronucleus test
   A. Double treatment
      1. Dose-response study

5.2 Materials and Methods

Healthy adult mice of both the sexes and of the age group 10-12 weeks having body weight 20-25 g constituted the experimental animals. Base free sample of sulfamethoxazole (A. No. 41379) in the form of powder was donated by its manufacturer Burroughs Wellcome (India) Limited, Bombay. The drug was administered 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.

5.2.1 Bone Marrow Metaphase Analysis

For time response study mice were orally administered with a single dose of 40 mg/kg of the drug and sampling was done at 8, 24 and 48 h post-treatment. For each period 3 animals of both the sexes were utilized. For dose-response study the animals of either sex were treated with the drug twice at an interval of 24 h and killed 24 hours after the second treatment; the doses tested were 20, 40 and 30 mg/kg. 4 animals (2 males and 2 females)
were employed for each dose level. As the drug was treated without any vehicle age and sex matched untreated animals were used as controls, and the same control data were used both for time-response and dose-response studies. The procedure followed here for bone marrow metaphase preparation was almost the same as described in section 2.1 except that no colchicine pretreatment was done in control and treated series. In a pilot experiment we noted some colchicine-like effect of the drug which prompted us to drop colchicine pretreatment.

5.2.2 Micronucleus Test

For micronucleus test in fact no separate treatment was done. Animals used for dose-response study in the metaphase analysis provided the material for MNT also. The bone marrow cells from one femur were processed for metaphase analysis, and the cells from the other for MNT. Similarly the same control animals were used here. As mentioned above for each dose level 4 animals (2 males and 2 females) were employed and there was no colchicine pretreatment. Details of the preparation was given in section 2.2.

5.3 Results

5.3.1 Bone marrow Metaphase Analysis

No effect on behaviour of the animals was marked following treatment (single or double).

Qualitative - As qualitatively the cytogenetic effects encountered in single and double treatment series, as well as in dose-response and time-response series were more or less the same they are considered here together. The effects observed were
grossly categorized under two heads: (i) effect on the structural integrity of chromosomes and (ii) effect on cell division (or in other words, mitotic poisoning effect).

Though there was no colchicine pretreatment a lot of separable metaphases were available; however, the chromosomes in general looked slightly longer. Besides gap-type aberrations (Figs. 10a, b) which constituted the major bulk of the structural changes of chromosomes, chromatid breaks (Figs. 10a-e) and fragments of unknown origin (Fig. 10f) were of common occurrence. The acentric chromatid fragments following breaks were placed either near to their origin (Figs. 10a-c) or slightly displaced (Fig. 10d). In several metaphases both the chromatids of the affected chromosome were found to be involved with breaks and/or gaps at the same locus (Figs. 10a, b, g).

Data were also collected on stretching of or separation at secondary constriction regions, and on centomeric separation. In the pilot experiment we came across a number of cases of stretching of secondary constriction regions and centomeric separation of chromatids which led us to collect data on them from metaphase preparations. In general the secondary constriction region of the chromosomes became very prominent and clearly visible (Figs. 10h-j, vide infra); in certain extreme cases the secondary constriction regions showed a distinct dislocation leading to the formation of a centric fragment placed a little away (Figs. 10i, j); those cases were considered as iso-chromatid breaks at secondary constriction region. Breaks at the secondary constriction region involving one chromatid were also recorded (Fig. 10k).
Explanation for Fig. 10

Photomicrographs of metaphases showing structural chromosome aberrations and anaphases in bone marrow cells of mice treated with sulfamethoxazole.

a. A metaphase plate with a chromatid break and a chromatid gap at the same locus of a chromosome.

b. Cut-out photograph of a polyploid cell showing a chromatid break and a chromatid gap at the same locus of a chromosome.

c. Cut-out photograph of a polyploid metaphase showing a chromatid break in a chromosome, fragment not displaced.

d,e. Metaphase chromosomes showing chromatid breaks fragments slightly displaced.

f. A metaphase showing centromeric separation of all chromosomes and a fragment of unknown origin.

g. A chromosome showing one iso-chromatid break.

h. A metaphase plate showing stretching at the secondary constriction regions of some chromosomes.
Explanation for Fig. 10 (Contd.)

1. Metaphase chromosomes showing breaks at the secondary constriction regions involving both the chromatids.

k. Break at the secondary constriction region involving one chromatid only.

l. Metaphases showing centromeric separation of all chromosomes leading to the formation of c-metaphases.

m. Normal anaphases observed in the bone marrow preparations prepared by conventional flame-drying method without colchicine pretreatment.

n. A normal metaphase showing the polar view.
Centromeric separation of the sister chromatids leading to the formation of c-metaphases was not rare in the preparation (Figs. 10f, l, m). Metaphases were also found with centromeric separation involving a few chromosomes only, and the number varied from 1 to 7. Normal anaphases (Figs. 10n, o, 11a) and metaphases showing polar view (Figs. 10p) were also available in plenty.

To evaluate potential mitotic poisoning effect anaphase abnormalities (Figs. 11b-d), numerical changes of chromosomes and mitotic indices were analyzed. Anaphase abnormalities were studied both from smear preparations used for MNT and from preparations used for metaphase analysis and included lagging and multipolar spindle.

Quantitative - Data on structural changes of chromosomes following single treatment of the drug in time-response study are summarized in Table 18. Gaps and sub-chromatid breaks together constituted the major bulk of the aberrations. The incidence of break and gap-type aberrations taken together remained in the control range; but the break-type aberrations which included chromatid breaks, iso-chromatid breaks and fragments, alone increased significantly in all the sampling hours. Correlation coefficient analysis failed to show any correlation of the aberration frequencies with the time intervals between treatment and sampling.

Similarly dose-response study revealed a highly significant increase of break-type aberrations for all the
Explanation for Fig. 11

Cut-out photomicrographs of bone marrow smears of mice showing normal and abnormal mitotic figures, and micronucleated erythrocytes and nucleated cells induced by sulfamethoxazole.

a. Photograph showing normal anaphases.

b. An abnormal anaphase with an anaphase bridge.

c,d. Abnormal anaphases, each with lagging chromosome/chromosome fragment.

e-j. Poly- and normo-chromatic erythrocytes, each with one micronucleus.

k,l. Nucleated cells, each with one micronucleus.
Table 18: Incidence of structural chromosome aberrations induced by sulfamethoxazole (SMX) in bone marrow metaphases of mice in different post-treatment sampling hours. The animals were treated once with a dose of 40 mg/kg of the drug. Each iso-chromatid break was counted as two breaks. Values in parentheses 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>Iso-chromat. break</td>
<td>Frag.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>150/3</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>150/3</td>
<td>3</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>150/3</td>
<td>5</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>-</td>
<td>200/4</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>(Cont.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ r = -0.62 \]
\[ df = 2 \]
\[ r = -0.775 \]
\[ df = 2 \]

b, c 't' test: significantly different from control at  \( b = p < 0.01 \),  \( c = p < 0.05 \)
dose-levels tested (Table 19). Interestingly, the values exhibited a very good negative correlation (Significant at $p<0.01$) with the doses. However, the frequencies of chromosomal aberrations including gap-type ones showed no such correlation with the doses though they increased significantly over the control value.

Centromeric separations were categorized under 2 heads: involving all chromosomes, and involving a few chromosomes. For both the cases the frequencies following single treatment (Table 20) did not differ remarkably from the respective control values. However, following 2 consecutive treatments at an interval of 24 h the frequencies of involving-not-all chromosomes increased significantly for all the dose-levels and the values were very close to each other, though the frequencies of the category involving-all-chromosomes remained in the control range (Table 21).

An attempt was also made to analyze quantitatively the data on stretching of the secondary constriction regions (Tables 20 and 21). In an extreme case as many as 7 chromosomes exhibited stretching. In time-response study (Table 20) the maximal effect was obtained at 3 h when the value showed a 3 fold increase over the control, and the effect decreased gradually with the increase of time between treatment and sampling hour. The values in all three sampling hours showed significant increase. In dose-response study with double treatment (Table 21) the maximum effect which was less than 2 times the control value was noted with the lowest dose. The values obtained with different doses did not differ remarkably.
Table 19: Incidence of structural chromosome aberrations in bone marrow metaphases of mice treated with different doses of sulfamethoxamole (SMX). The animals were treated twice at an interval of 24 hours and killed 24 hours after the second treatment. Each of the iso-chromatid breaks and exchanges 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. break</td>
<td>Iso-chromat. Frag.</td>
<td>Exch.</td>
</tr>
<tr>
<td>20</td>
<td>190/4</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>210/4</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>80</td>
<td>200/4</td>
<td>3</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>(Cont.)</td>
<td>200/4</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

\[ r = -0.996^d \]  \[ r = +0.812 \]

\[ df = 2 \]  \[ df = 2 \]

a, b, c 't' test: significantly different from control at: \( a = p < 0.001, b = p < 0.01, c = p < 0.05 \)

d 'r' test: significantly different from control at \( d = p < 0.01 \)
6 multipolar (tri-/quadri-) anaphases out of a total of 130 anaphases were recorded in the treated series. In contrast, out of 45 anaphases scored in the control series not a single showed multipolar configuration. In most of the multipolar anaphases the poles were asymmetrical with respect to the number of chromosomes they contained. No multipolar spindle was found in single treatment series. 12 out of 130 anaphases scored exhibited lagging chromosomes, but not a single case was marked in the controls (Table 22).

So far numerical change of chromosomes is concerned the mouse system is not at all good for study of unequal distribution of chromosomes to daughter nuclei during anaphase. Though a number of aneuploid and polyploid metaphases were recorded in the treated individuals the frequencies of them, taking separately or together, did not increase markedly from the respective control values (Table 23). A number of metaphases with hypo and hyper-diploid chromosomes were available in the preparation. But we did not put much importance on the hypodiploid cells which might have originated due to technical short-comings and were comparatively in plenty. With regard to hyper-diploid cells only 3 metaphases with 41 chromosomes were recorded in the treated individuals out of a total of 600 metaphases scored. No hyper-diploid cell was noted in the controls.

For analysis of mitotic index (MI) (number of dividing cells per 100 cells) cells were scored from smear preparations used for MNT. The MI increased with the increase of the dose levels (Table 22). A ten fold increase in MI was noted with the highest
Table 22: Incidence of mitotic disturbances in bone marrow cells of mice treated twice with different doses of sulfamethoxazole (SMX). The animals were treated twice at an interval of 24 hours and killed 24 hours after the second treatment. For each dose or control 4 animals were employed.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>MI</th>
<th>A/M</th>
<th>Anaphases*</th>
<th>Total</th>
<th>Bi- and tri- nucleate cells</th>
<th>Total nucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
<td>With laggard</td>
<td>Multipolar</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24</td>
<td>1</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>40</td>
<td>1.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.053&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39</td>
<td>2</td>
<td>1</td>
<td>42</td>
</tr>
<tr>
<td>80</td>
<td>2.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.055&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49</td>
<td>9</td>
<td>4</td>
<td>62</td>
</tr>
<tr>
<td>0 (Cont.)</td>
<td>0.29</td>
<td>0.105</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
</tbody>
</table>

\[ r = +0.998^d \]
\[ df = 2 \]

\( MI = \text{Mitotic index (\% of dividing cells), analyzed from MN preparation} \)

\( A/M = \text{Anaphase to metaphase ratio, analyzed from metaphase preparation} \)

* = Scored from both MN and metaphase preparations

<sup>a, b, c</sup> t-test: significantly different from control at \( a = p < 0.001, b = p < 0.01, c = p < 0.05 \)

<sup>d</sup> 'r' test: significantly different from control at \( p < 0.01 \)
Table 23: Incidence of numerical changes of chromosomes in bone marrow metaphases of mice treated with different doses of sulfamethoxazole (SMX). The animals were treated twice at an interval of 24 hours and killed 24 hours after the second treatment. Values in parentheses are mean per 100 metaphases ± S.E.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Metaphases/schred/Animals</th>
<th>Aneuploid cells</th>
<th>Polyploid cells</th>
<th>Total numerical aberrations (Aneu.+ Poly)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>38</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>20</td>
<td>190/4</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>210/4</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>200/4</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>200/4</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>(Cont.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No treated value differs significantly from the control ("t" test)
dose tested (80 mg/kg). Analysis of correlation coefficient shows a very good positive correlation of the MI values with the doses.

5.3.2 Micronucleus Test

Qualitative - As found with other drugs mentioned earlier the micronuclei encountered were round in shape and varied in size (Figs. 11e-l). In general the affected cells were found to contain one MN. In certain cases, however, more than one MN was also noted.

Quantitative - Data on the incidence of micronuclei induced by SMX in bone marrow cells of mice are presented in Table 24. Dose-response analysis revealed significant increases in the frequencies of MN in both polychromatic and normochromatic erythrocytes, taking separately or together, at all the dose levels tested. The values obtained at different dose levels, however, did not vary remarkably. In general the frequencies of MN were more in PCEs than in NCEs. The nucleated cells also exhibited higher incidence of MN. Polychromatic to normochromatic (PCE/NCE) ratios showed a tendency of decrease, the decrease being maximum with the intermediate dose. Similarly, decrease in the RBC population as noted from the data on total RBC/100 nucleated cells was also marked in the treated individuals, the decreases being highly significant for higher two doses.

5.4 Discussion

Higher incidence of break-type aberrations as marked in both time-response and dose-response analyses of metaphase chromosomes clearly reveals clastogenic capacity of the drug.
Table 24: Results of the micronucleus test in bone marrow cells of mice treated with different doses of sulfamethoxazole (SMX). The animals were treated twice at an interval of 24 hours and killed at 24 hours after the 2nd treatment. Values are mean per 100 cells ± S.E. Figures in parentheses are MN/cells scored. For each dose level 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 ratio</th>
<th>RBC/100 nucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.36 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.30 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.34 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.82 ± 0.03</td>
<td>10.75 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>(15/4, 000)</td>
<td>(12/4, 000)</td>
<td>(27/8, 000)</td>
<td>(9/8, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.75 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.53 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.03</td>
<td>5.50 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(30/4, 000)</td>
<td>(12/4, 000)</td>
<td>(42/8, 000)</td>
<td>(17/8, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.55 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.04</td>
<td>0.88 ± 0.10</td>
<td>6.81 ± 0.92&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(22/4, 000)</td>
<td>(17/4, 000)</td>
<td>(39/8, 000)</td>
<td>(18/8, 000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.10 ± 0.04</td>
<td>0.10 ± 0.04</td>
<td>0.10 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.99 ± 0.09</td>
<td>11.69 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>(4/4, 000)</td>
<td>(4/4, 000)</td>
<td>(8/8, 000)</td>
<td>(2/8, 000)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> 't' test: significantly different from control at  
<sup>a</sup> = p < 0.001,  
<sup>b</sup> = p < 0.01,  
<sup>c</sup> = p < 0.05
Most of the aberrations even after 48 hours of treatment are of chromatid type; it seems for this drug, as in the case of most of the chemical agents, the affected cells need to be passed through the S-phase to translate the lesions into breaks. High frequencies of breaks at 8 h and of gap-type aberrations particularly following double treatment are indicative of the fact that the drug affects late S- and G₂-phase chromosomes.

SMX is known to interfere with the final stages of folic acid synthesis; folic acid is essential for purine and thereby DNA synthesis (Hitchings and Burchall, 1965). So chromosome damage through inhibition of DNA synthesis is not unexpected. Many DNA synthesis inhibitors are established chromosome breakers (Kihlman, 1966; Matter and Schmid, 1971; Manna and Das, 1975; Dubinin et al. 1986). Other folic acid antagonists like methotrexate and pyrimethamine have also been proved to be chromosome breakers (Jensen and Nyfors, 1979, Yamamoto and Kikuchi, 1981).

Our finding on clastogenic capacity of the drug tested in mouse in vivo system is in good agreement with those of Gebhert (1973) who also noted positive, though mild, clastogenic effect in bone marrow cells of patients treated with SMX in combination with TMP. Similar positive report was recorded by using the MNT in bone marrow cells of patients taking SMX along with TMP for 10 or more days (Sorensen and Jensen, 1981). But Sorensen and Jensen (1981) who also analyzed metaphases in bone marrow cells of the patients failed to note any deviation in the incidence of chromosome aberrations from the control level. Earlier studies based on peripheral blood lymphocytes exposed to the combination of SMX and TMP in vivo or in vitro also
exhibited negative results (Stevenson et al., 1973; Gebhart, 1975). Thus, the reports so far made are contradictory. Absence of any remarkable effect in the peripheral blood lymphocytes exposed in vivo may be attributed to the antifolate effect of the drug. Chromosome damage due to antifolate effect is, however, not expected in G1 but in the S-phase of the cell cycle. As the lymphocytes in the peripheral blood have long half-life (about 3 years) and divide rarely in vivo no structural changes of chromosomes would be expected in a sample of them. But the reason(s) for differential results obtained in bone marrow cells in human as well as for lack of effect in lymphocytes exposed in vitro remains unknown.

Interestingly our dose-response study showed significantly negative correlation of break type aberrations with the doses. This negative correlation may also be attributed to the antifolate effect of the drug. With the increase of the dose the highly affected cells due to inhibition of DNA synthesis probably failed to come to metaphase stages and thus escaped scoring. An inhibitory effect of the drug in combination with TMP on DNA synthesis has earlier been noted in Friend erythroleukemia cells, even with doses below antibacterial level; at higher doses cell growth was arrested (Steinberg et al., 1980).

The time-response study following single treatment revealed the highest frequency of break type aberrations at the earliest sampling period (8 h). The early clastogenic effect may well be explained from the pharmacokinetic behaviour of the drug. After an oral dose the drug is rapidly absorbed reaching the peak plasma concentration in 4 hours. Its half-lives in babies and
adults differ; in babies during the first 10 days it is considerably longer than in adults, with the aging of the babies it falls rapidly, it then increases towards the half-life characteristic for adults, nearly 6-12 hours (Mandell and Sande, 1985). About 65% of SMX is bound to plasma protein. Further, 25-50% of the administered drug is excreted in urine in 24 h, two-thirds of which is unconjugated. Shorter half-life and high excretion rate are believed to be the reasons for less effect at late hours.

The most important point to be noted in the present study is the mitotic poisoning effect of the drug. In the entire study colchicine pretreatment was avoided, but it did not put us into much trouble to analyze metaphases. In fact in our pilot study following colchicine pretreatment the incidence of scorable metaphases in the preparation from treated mice was found to be excessively higher than that recorded for years together in our laboratory in control individuals receiving colchicine pretreatment alone. This led us to drop colchicine pretreatment in this series. The frequency of dividing cells as judged from MI values increases greatly in individuals receiving the drug; the MI value obtained with the highest dose was about 10 times the control value. Further, the MI values show a highly significant dose-related increase. All this clearly indicate induction of mitotic arrest by the drug. Such a mitotic arresting effect of sulfamethoxazole was not recorded, so far as the author is aware, in any earlier work. To know the extent of mitotic arrest the anaphase to metaphase (A/M) ratios were analyzed from bone marrow metaphase preparations by counting the number of anaphases.
present for every 400 metaphases scanned. Interestingly the A/M ratios declined greatly in all the treated individuals; the values for three doses were very close to each other and to half the control value (Table 23). This reduction in A/M ratio with simultaneous elevation of the MI values reveals that the drug arrests metaphases. But the arrest is not complete, only partial, in no case the A/M ratio reduces to zero. If an agent completely arrests mitosis at metaphase, the A/M ratio would be zero. Occurrence of typical C-metaphases with condensed and separated sister chromatids was not so frequent, suggesting also partial arrest at metaphase.

As SMX is found to induce partial arrest at metaphase abnormal anaphases with laggards and/or multipolar spindle, as well as bi- and tri-nucleate cells were frequent. Similar partial metaphase block being accompanied with anaphase abnormalities was noted with low concentration of benzene in grasshopper embryo system (Liang et al., 1983), of halothane in root tip cells of V. faba (Nunn et al., 1971) and several other chemicals (see Liang et al., 1983). Rare occurrence of aneuploidy and polyploidy can easily be explained from the point of failure of the drug to induce complete metaphase arrest. Following complete arrest at metaphase if the arrested cells are allowed to recover the incidence of aneuploidy and polyploidy usually goes up. The reason(s) for failure of SMX to induce complete metaphase block is not clear. It is not known if dose higher than 80 mg/kg (the highest dose used by us) can induce complete metaphase arrest. Besides, in our study scoring was done 24 hours after the second or last treatment. As the half-life of the drug is very short it is not quite impossible
that the block was lifted by the time of scoring. Over and above, there is a question of response of cell system, halothane induced metaphase block is complete in root tip cells of *V. faba* (Grant et al., 1977), but partial only in cultured mammalian cells (Sturrock et al., 1975). Anyway, more works are needed with different other doses and shorter period of treatment.

The MNT is considered as a sensitive and important assay system to detect clastogenic efficiency of chemical agents. Significantly high incidences of MN in PCEs and NCEs, taken separately or together, at all the dose-levels clearly demonstrate clastogenic effect of the drug. However, there is no indication of dose-related effect, neither in PCEs nor in NCEs. Micronuclei may result from clastogenic as well as spindle poisoning effect (Schmid, 1976; Heddle et al., 1983; Das and Kar 1986). Spindle poisoning efficiency of this drug resulting mitotic arrest and formation of laggards and multipolar spindles has already been documented. But incidence of laggards and multipolar spindles alone was not as high as to account high incidence of MN noted here. Besides, the MN encountered in the preparations were in general small in size. All these are in line of assumption that SMX has chromosome breaking capacity too; and, thus, the results of MNT support our finding based on metaphase analysis. Our results on MNT are also in agreement with those of Sorensen and Jensen (1981) who recorded increased number of MN, compared to controls, in bone marrow cells of patients taking SMX along with TMP for 10 days for urinary tract infection. Both PCE/NCE ratios and ratios of RBC to nucleated cells showed a tendency of decrease following treatment particularly at higher doses. This is an
indication of the fact that the production of PCEs and thereby the total RBCs from erythroblasts is slowed down, which is in accordance with our observation of mitotic arresting capacity of the drug discussed earlier.

Thus, sulfamethoxazole induces both clastogenic and mitotic poisoning effect. Our finding that the drug causes mitotic poisoning effect is consistent with those obtained earlier with other sulfonamides in root tip cells (Peters, 1946; Fuller, 1947; Mehra, 1949; Sharma, 1971). A variety of environmental agents are known to affect cell division and arrest cells at metaphase, and this aspect has nicely been reviewed by Hsu and Liang (1983) and Liang and Satya-Prakash (1985).

85% of SMX is known to remain unmetabolized in man (Beck, 1971). There is evidence that the bacteriostatic action of the drug is exerted by the drug itself, not by its metabolites, suggesting that any action on DNA synthesis would also be by the unmetabolized drug, and therefore our positive finding is assumed to be due to the drug itself. The early effect of the drug as noted in time-response metaphase analysis also supports the assumption.

The usual human dose or SMX for an adult is 800 mg every 12 hours for 10-14 days (however, it is given in combination with TMP). So the daily dose, 1600 mg, comes to 32 mg/kg assuming the average body weight of an adult human is 50 kg. The intermediate dose tested here (40 mg/kg) is, therefore, close to the human therapeutic dose on per kg basis.
But the general practice to determine a dose for an animal from human dose is to calculate it on the basis of the body surface, dose on per kg basis may not work; accordingly a mouse dose is determined as 12 times the human dose (Freireich et al., 1966). So a mouse-dose equivalent to a human daily dose would be 384 mg/kg, and the highest dose in our present study is slightly more than 1/5th the mouse dose.