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16. Abstracts: The mutagenic potential of long-term exposure of 120 male Sprague-Dawley rats to nitrous oxide is measured by observing aberrations in bone marrow cell chromosomes. Forty rats serve as controls, 40 are exposed to 50 ppm nitrous exide plus 1 ppm halothane, and 40 are exposed to 500 ppm nitrous oxide plus 10 ppm halothane, for 7 hours a day, 5 days a week for 52 weeks. The most frequently found aberration is the chromatid gap. There is a significant increase in the number of gaps and this increase is dose-related. Chromatid breaks are the next most frequent aberrations observed. Chromosomal breaks are comparable among all groups. Chromosomal markers are significantly increased in the group receiving 500 ppm nitrous oxide. The incidence of polyploidy is comparable among control and treated groups. Fifty-nine percent of the animals in the first treatment group show aberrant cells; 68% in the second treatment group. It is concluded that exposure to 500 ppm nitrous oxide plus 10 ppm halothane can cause chromosomal damage to bone marrow cells in the rat.

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CYTOGENETIC EVALUATION OF BONE MARROW CELLS IN THE RAT FOLLOWING LONG-TERM INHALATION EXPOSURE TO NITROUS OXIDE PLUS HALOTHANE

FINAL REPORT

CDC 99-74-4C

Submitted to

National Institute for Occupational
Safety and Health/HSMA
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Cincinnati, Ohio

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HAZLETON LABORATORIES AMERICA, INC.

SPONSOR: National Institute for Occupational Safety & Health **DATE:**

MATERIAL: Nitrous Oxide and Halothane

SUBJECT: FINAL REPORT
Cytogenetic Evaluation of Bone Marrow Cells in the Rat Following Long-Term Inhalation Exposure to Nitrous Oxide Plus Halothane

Project 785-200

I. OBJECTIVE

This study was designed to assess the mutagenic potential of long-term exposure of male rats to nitrous oxide (N_2O) plus halothane as measured by aberrations in bone marrow cell chromosomes.

II. MATERIALS AND METHODS

A. Animals and Animal Groups

One hundred twenty sexually mature Sprague-Dawley strain male albino rats (Charles River COBS) were randomly assigned to the following groups:

Group No.	No. of Male Rats	Treatment
1	40	Air Control
2	40	50 ppm N_2O + 1 ppm halothane
3	40	500 ppm N_2O + 10 ppm halothane

B. Exposure Conditions

The animals in the treatment groups were exposed under dynamic conditions at 1200 liters/minute airflow to the respective N_2O and halothane mixtures in 6000-liter glass and stainless steel Rochester-type inhalation chambers.

Exposures were conducted for seven hours per day, five days per week, for a total exposure period of 52 weeks. The control animals were exposed to filtered room air in a similar chamber with flow characteristics identical to that of the treatment groups. After 12 weeks on study, all animals were removed from their exposure cages for use in a 21-day mating period and were then returned to their cages and maintained on the appropriate control or exposure level for the remainder of the 52-week period.

The animals were individually numbered with ear tags and housed on one tier, five to a cage, in stainless steel mesh cages with stainless steel top loading feeders and demand-type water valves. Water and basal laboratory diets (Purina Rat Chow) were available ad libitum. The cages were changed and washed weekly and the water bottles were changed and sterilized twice weekly.

C. Exposure Method

Input of chamber air was delivered via a tangential pipe into a cylindrical turret at the apex of the pyramidal top and the chamber exhausted via a goose-necked duct at the bottom above the drain pipe.

Nitrous oxide (N_2O) was supplied from cylinders containing certified 98% pure nitrous oxide anhydride. The gas was passed under positive pressure through a flowrater and critical orifice into a 3-necked mixing flask prior to being inserted via the input duct into the chamber turret.

The halothane used was the Fluothane® brand of 2-bromo-2-chloro-1,1,1,-trifluorothane. Nitrogen was passed via a flowrater and critical orifice through the headspace of a glass flask containing liquid halothane and then passed into the mixing flask to mix with the N_2O prior to delivery into the chamber input duct.

D. Chamber Monitoring

Chamber concentrations of N_2O and halothane were determined from samples pulled via a standard Teflon® probe located just above the middle cage in the chamber prior to exposure, at hourly intervals during the first five days of exposure, and at least daily during the remainder of the exposure period after chamber equilibration (T_{99} = 23 minutes). Analyses of chamber concentrations of nitrous oxide were determined by on-line infrared spectrophotometry validated against gas chromatography (1). Halothane concentrations were determined by peak height analysis on a gas chromatograph with an electron capture detector. Gas samples were taken by syringe from a septum in the sample line and injected into a vacutainer containing a measured volume of 2,2,4 trimethyl pentane, and then an aliquot of the solution was injected into the gas chromatograph.

E. Observations

All male rats were observed daily for mortality. Terminal body weight was recorded for each animal prior to sacrifice.

F. Sacrifice

Following the 52-week exposure period, the animals were administered an intraperitoneal injection of Colchicine (2 mg/kg) in distilled water in order to arrest mitosis in dividing cells. Approximately five hours post-injection, the rats were sacrificed via a brief exposure to chloroform followed by cervical dislocation.

G. Preparation of Chromosome Slides

Immediately following sacrifice, bone marrow cells were collected from both femurs of each rat by aspiration into pre-warmed (37°C) Hank's balanced salt solution at pH 7.4. After washing with one change of Hank's solution, the cells were treated with hypotonic KCl (0.055M) for 30 minutes. The cells were then fixed in a 3:1 methanol-acetic acid fixative for a minimum of one hour. Following fixation, the cells were centrifuged and resuspended in fresh fixative. Three to four drops of this suspension were dropped onto a clean glass slide. The slide was passed through a flame until dried. Coded slides were numbered chronologically with no reference to test groups.

H. Stain Preparation

Stock Giemsa stain was prepared from 0.38 gram Giemsa powder (Polyscience) added to 25 ml of glycerin and 25 ml of absolute methanol.

Four ml of this stock solution was then added to 3.0 ml of acetone and 33 ml of buffered H_2O (1.0 Harleco buffer salts per 100 ml distilled water).

I. Slide Staining

Upon completion of all preparations, the slides were stained for eight minutes in the Giemsa stain described above. Slides were rinsed in tap water, air-dried, and subsequently mounted in Permount®.

J. Chromosome Analysis

At least three slides were prepared for each animal. Twenty-five metaphases were evaluated from each bone marrow preparation that was analyzable and the vernier location was noted for each metaphase. The technical quality of the slides was recorded at the time of evaluation. Chromatid breaks (involving one chromosomal arm) were scored as either with a visible fragment or without a fragment. Chromosome breaks (involving both arms of the chromosome) were also scored with a visible fragment or without a fragment. All markers including exchanges, dicentrics, rings, and other abnormal chromosomes were scored separately. Where more than one type of aberration was observed in any one metaphase, the cell was also scored as having multiple aberrations. Those cells with more than nine aberrations were scored as having 10 or more aberrations. Unique markers were tabulated in these cells as well, since markers are considered to be extremely important in cytogenetic evaluation. An estimate of polyploidy was made for each cell at the time of evaluation.

Representative photographs were made on each slide showing characteristic aberrations as well as normal cells. Karyotype analysis was not made since this type of evaluation is not appropriate in the rat because most of the chromosomes are morphologically similar (telocentric) and pairs are not easily identified without more sophisticated staining procedures.

In addition, the number of gaps (regions of chromatid arms with stained areas less than the width of the chromatid arm) were scored.

All slides were scored blindly with no reference to group identification. Upon completion of all scoring, the data sheets were placed into the appropriate group and statistical analyses were performed.

K. Statistical Analysis

Each category of aberrations was recorded in two ways: (1) the number of aberrations per animal and (2) the number of cells showing a particular aberration per animal. Thus, it could be determined how wide-spread the effects observed were within each animal as against intensity of effect within the animal. Unless otherwise indicated, all analyses were made comparing the mean number of affected cells or the mean number of aberrations for each treatment group to similar means of the control group by analysis of variance (F-test) and by Student's t-test.

When variances differed significantly, Student's t-test was appropriately modified (t') and Cochran's approximation utilized (2). Terminal body weights were similarly analyzed. Finally, the proportions of animals showing aberrations and the proportions of examined cells showing aberrations in each group were analyzed by the Chi-square test. The level of probability chosen for rejecting the null hypothesis was ≤ 0.05.

III. RESULTS

A. Chamber Analyses

The means ± S.D. for all analytical determinations of N_2O and halothane generated during the 52-week exposure period were 49.7 ppm N_2O ± 5.1 with 1.1 ppm halothane ± .2, and 498.6 ppm N_2O ± 14.6 with 10.6 ppm halothane ± 1.8 for Groups 2 and 3, respectively.

B. Survival

Of the original 120 male rats, 114 survived the 52-week exposure period. The survivors comprised 39/40 in Group 1 (Control), 37/40 in Group 2, and 38/40 in Group 3.

C. Body Weight

Terminal body weight data are presented in Table 1.

Table 1 - Mean terminal body weight data ± S.D.

Group No.	Mean Terminal Body Weight g ± S.D.	Body Weight Range g
1 (Control)	855 ± 133	500 - 1275
2	843 ± 160	460 - 1070
3	841 ± 118	630 - 1075

Although there was considerable weight range variation between groups, the mean terminal body weights were similar among the control and treated groups.

D. Cytogenetic Evaluations

There were 39 animals to be evaluated in Group 1 (Control). Slide No. 100 was eliminated from Group 1 statistical analysis since only 11 metaphases were analyzable. Hence, data presented for Group 1 are based on 38 rats which represents a total of 950 bone marrow cells analyzed for chromosomal aberrations.

Data presented for Group 2 were collected from 37 rats which represent 925 bone marrow cells evaluated. Group 3 data are from 38 rats representing 950 cells.

The mean number of bone marrow cells per animal which revealed chromatid aberrations is given in Table 2.

Table 2 - Mean number of analyzed bone marrow cells per animal showing chromatid aberrations.

Group No.	Mean No. of Cells with Gaps ± S.D.	Mean No. of Cells with Breaks with Fragments ± S.D.	Mean No. of Cells with Breaks w/o Fragments ± S.D.
1 (Control)	1.13 ± 0.99	0.18 ± 0.46	0.03 ± 0.16
2	1.89* ± 1.49	0.24 ± 0.43	0.27* ± 0.56
3	2.84** ± 1.55	0.53* ± 0.69	0.24** ± 0.43

* Significantly higher than control at $p<0.05$.
**Significantly higher than control at $p<0.01$.

The data in Table 2 indicate that the number of cells with gaps in both Groups 2 and 3 was significantly increased over the control group. Little biological significance is attributed to the presence or absence of gaps in cytogenetic analysis; therefore, they are not here included in the calculation of total aberrations.

Cells in Group 2 which displayed chromatid breaks where a fragment was also visible were more numerous but not significantly so, than in the control group. The incidence of these aberrant cells was significantly elevated in Group 3.

Cells in both Groups 2 and 3 which displayed chromatid breaks where fragments were not visible were significantly increased over the control group.

It is important to note that all three chromatid parameters indicated significant increases in number of cells affected at the high exposure level.

Table 3 presents the mean number of chromatid aberrations observed per animal.

Table 3 - Mean number of chromatid aberrations per animal in analyzed bone marrow cells of rats.

Group No.	Mean No. of Gaps ± S.D.	Mean No. of Chromatid Breaks w/Fragments ± S.D.	Mean No. of Chromatid Breaks w/o Fragments ± S.D.
1 (Control)	1.26 ± 1.22	0.18 ± 0.46	0.03 ± 0.16
2	2.49** ± 2.18	0.24 ± 0.43	0.30* ± 0.66
3	3.45** ± 2.05	0.58** ± 0.76	0.24** ± 0.43

* Significantly higher than control at $p < 0.05$.
**Significantly higher than control at $p < 0.01$.

The data in Table 3 indicate that the total number of gaps was significantly increased in both Groups 2 and 3 when compared to the controls. As indicated previously, this finding is of little relevance in determining genetic damage.

Chromatid breaks with fragments in Group 2 were comparable to similar breaks observed in the control group. The incidence of these chromatid breaks with fragments in Group 3 was significantly increased over the controls.

Both Groups 2 and 3 showed significant increases in the incidence of chromatid breaks without fragments.

Table 4 presents the mean number of cells per animal in which chromosomal aberrations were observed.

Table 4 - Mean number of analyzed bone marrow cells per animal showing chromosomal aberrations.

Group No.	Mean No. of Cells with Breaks with Fragments ± S.D.	Mean No. of Cells with Breaks without Fragments ± S.D.
1 (Control)	0.16 ± 0.37	0.00
2	0.11 ± 0.31	0.00
3	0.21 ± 0.47	0.03 ± 0.16

The mean number of cells containing chromosomal breaks with fragments and without fragments in both test groups were comparable to the control group.

Table 5 presents the mean number of chromosomal aberrations with and without fragments per animal. No significant differences were indicated among the groups.

Table 5 - Mean number of chromosomal aberrations per animal.

Group No.	Mean No. of Chromosomal Breaks with Fragments ± S.D.	Mean No. of Chromosomal Breaks without Fragments ± S.D.
1 (Control)	0.24 ± 0.59	0.00
2	0.11 ± 0.31	0.00
3	0.24 ± 0.59	0.03 ± 0.16

Table 6 presents the mean number of cells per animal with 10 or more aberrations and mean number of cells with multiple aberrations for each group. No significant differences were indicated among the groups.

Table 6 - Mean number of analyzed bone marrow cells per animal showing 10 or more aberrations and/or cells with multiple aberrations.

Group No.	Mean No. of Cells with >10 Aberrations ± S.D.	Mean No. of Cells with Multiple Aberrations ± S.D.
1 (Control)	0.03 ± 0.16	0.11 ± 0.31
2	0.03 ± 0.16	0.19 ± 0.40
3	0.05 ± 0.23	0.34 ± 0.63

The mean number of cells per animal with marker chromosomes are presented in Table 7. The Group 3 mean was significantly higher than the mean of the control group.

Table 7 - Mean number of analyzed bone marrow cells per animal showing marker chromosomes (exchange figures, rings, and miscellaneous markers).

Group No.	Mean No. of Cells with Markers ± S.D.
1 (Control)	0.16 ± 0.44
2	0.32 ± 0.63
3	0.76** ± 1.17

*Significantly higher than control at $p<0.01$.

The mean number of marker chromosomes per animal is presented in Table 8.

Table 8 - Mean number of marker chromosomes (exchange figures, rings, and miscellaneous markers) per animal in bone marrow cells.

Group No.	Mean No. of Marker Chromosomes ± S.D.
1 (Control)	0.29 ± 0.87
2	0.49 ± 0.96
3	1.32* ± 2.43

*Significantly higher than control at $p< 0.05$.

The mean number of marker chromosomes in Group 3 was significantly higher than the value obtained for the control group.

Of the 950 cells analyzed in the control group, six cells revealed markers. Five of these six cells showed a total of 10 exchange figures and one cell displayed one miscellaneous metacentric marker. Because the animals were ≈ 65 weeks of age at the time of sacrifice, this relatively high marker incidence was not unexpected.

In Group 2, 12 cells out of the 925 analyzed revealed a total of 18 exchange figures. No rings or metacentric markers were observed. Statistically, this group was comparable to the control group.

In Group 3, however, there were 28 cells which revealed a total of 50 marker-type aberrations. One of these cells showed a single ring chromosome and two others showed a metacentric marker similar to that observed in the controls. These aberrations were significantly increased over similar findings in the controls. The increase was significant in terms of mean numbers of aberrations produced as well as mean number of cells affected. Of the 38 animals analyzed, 15 showed marker-type aberrations. Thus, the aberrations were not localized to one or two animals, but they were dispersed over approximately 38% of the group.

The data in Table 9 indicate that the mean number of cells showing all types of aberrations (excluding gaps) for Groups 2 and 3 was significantly higher than the value obtained for the control group.

Table 9 - Mean number of analyzed bone marrow cells per animal showing all types of aberrations (excluding gaps).

Group No.	Mean No. of Cells with Aberrations ± S.D.
1(Control)	0.39 ± 0.72
2	0.81* ± 0.88
3	1.32** ± 1.32

* Significantly higher than control at $p < 0.05$.
**Significantly higher than control at $p < 0.01$.

Table 10 presents the mean number of aberrations per animal excluding gaps.

Table 10 - Mean number of all types of aberrations (excluding gaps) per animal in bone marrow cells.

Group No.	Mean No. of All Aberrations ± S.D.
1 (Control)	0.74 ± 1.69
2	1.14 ± 1.36
3	2.37**± 3.13

**Significantly higher than control at p <0.01.

The data in Table 10 indicate that the mean number of all aberrations in Group 3 was significantly higher than the control group.

Table 11 presents the dispersion of aberrations within each group by number of animals affected and by number of cells affected.

Table 11 - Incidence of animals and bone marrow cells showing aberrations (excluding gaps) in chromosomal structure.

Group No.	No. of Animals with Aberrant Cells	No. of Animals Examined	No. of Cells with Aberrations	No. of Cells Examined
1 (Control)	11	38	15	950
2	22**	37	30*	925
3	26**	38	50**	950

* Significantly higher than control at p <0.05 via Chi-square test.
**Significantly higher than control at p <0.01 via Chi-square test.

Group 1 (Control) exhibited a relatively high background frequency of aberrations. Twenty-nine percent of the 38 animals in this group showed aberrations. Of the 950 cells examined, 15 or 1.6% had aberrations. The elevated aberration rate in these control animals is attributed to the age of the animals (≈65 weeks) at sacrifice.

Fifty-nine percent of the Group 2 animals (low level exposure) had aberrant cells. Of the 925 cells examined in this group, 30 or 3.2% revealed aberrations. Although the mean number of cells affected per animal in this group was significantly increased over the controls (Table 9), the mean number of aberrations per animal was comparable to the control group (Table 10).

For Group 3 (high level exposure), 26 animals out of 38 examined (68%) exhibited aberrant cells. Of the 950 cells examined in this group, 50 or 5.3% exhibited aberrations. Both the mean number of cells per animal (Table 9) and the mean number of aberrations per animal (Table 10) were significantly higher than the controls.

As indicated in Table 11, the proportion of animals showing aberrations and the proportions of examined cells showing aberrations in Groups 2 and 3 were significantly higher than the controls.

Approximately 100 metaphases were scanned on each slide for an estimate of polyploidy. The control group revealed only one animal with two cells that displayed polyploidy. Group 2 had two animals with one cell each that was polyploid. There was no evidence of any polyploidic cells in Group 3.

Other incidental findings included sporadic non-clonal hypodiploidy which was observed at comparable frequencies among all three groups.

E. Photographic Documentation

A minimum of five photographs of bone marrow cells was taken on each animal analyzed. The negatives and contact proofs as well as the vernier location of each photograph is on file at HLA.

Representative photographs from each group have been selected for inclusion in this report.

Group 1 - Page 17 .

Slide No.	Vernier Location	Observation
A - Slide B4 -	126.7 X 18.1	Normal
B - Slide B2 -	115.2 X 33.3	Normal
C - Slide B4 -	115.8 X 15.7	Normal
D - Slide B2 -	125.8 X 44.4	Gap

Group 2 - Page 18 .

Slide No.	Vernier Location	Observation
A - Slide B51 -	111.5 X 48.7	Normal
B - Slide B28 -	122.3 X 50.0	Exchange
C - Slide B56 -	121.1 X 42.4	Chromatid Break & Exchange
D - Slide B50 -	121.6 X 48.2	Normal

Group 3 - Page 19 .

Slide No.	Vernier Location	Observation
A - Slide B64 -	117.3 X 39.7	Multiple Aberrations
B - Slide B74 -	113.0 X 36.0	Exchange
C - Slide B90 -	110.2 X 38.3	Chromatid Break & Exchanges
D - Slide B41 -	119.7 X 37	Multiple Aberrations

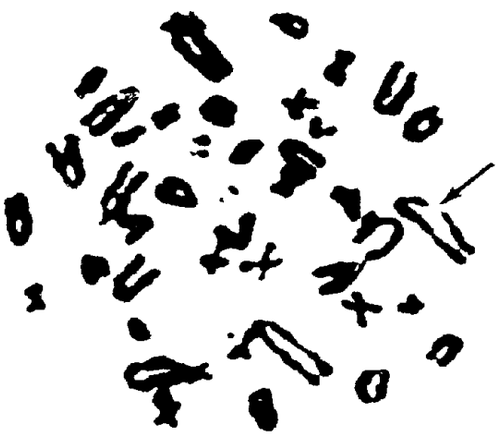
GROUP 1

A

B

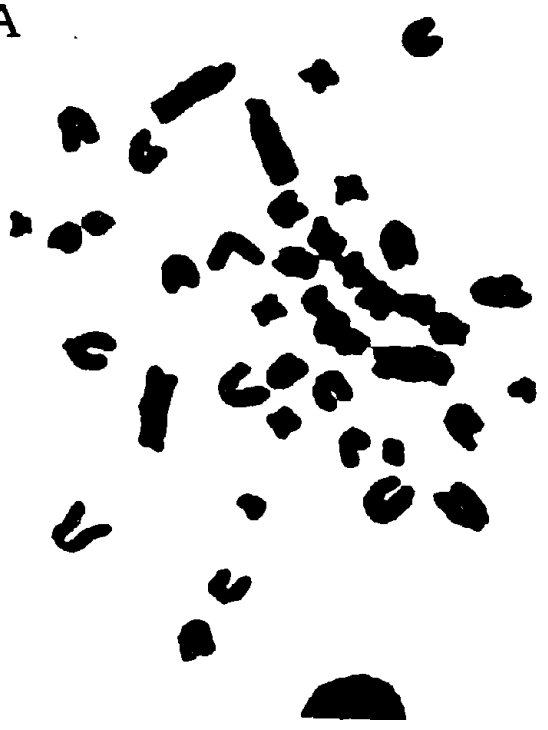
C

D

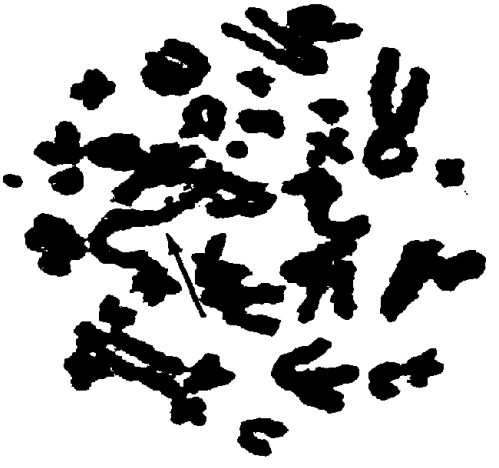


GROUP 2

A



B



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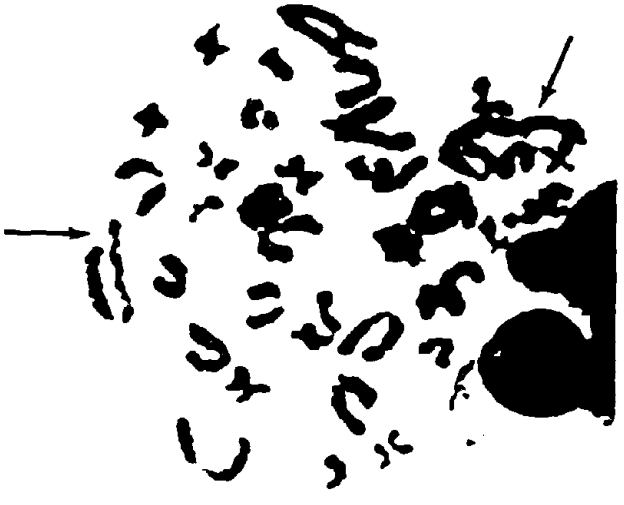
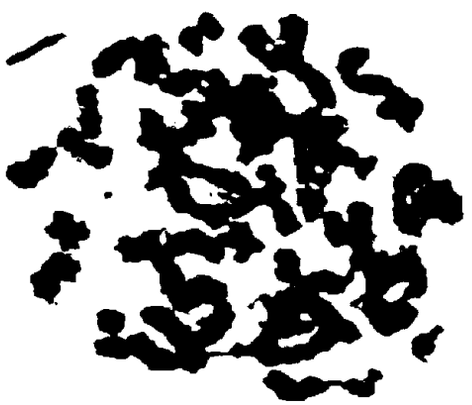


D

GROUP 3

A



B

C

D

IV. SUMMARY AND CONCLUSIONS

One hundred twenty sexually mature male albino rats were randomly assigned to three groups of 40 rats each. Two groups were each exposed by inhalation to a mixture of nitrous oxide (N_2O) and halothane vapor seven hours per day, five days a week, for 52 weeks. The remaining group was exposed to room air under the same conditions. Following the 52-week exposure period, the surviving animals were sacrificed and subsequent cytogenetic evaluations of bone marrow cells performed. Group designations for this study are listed below.

Group No.	No. of Male Rats	Treatment	Mean Analytical Concentration ppm ± S.D.
1	40	Air Control	---
2	40	N_2O + halothane	49.7 ± 5.1 1.1 ± 0.2
3	40	N_2O + halothane	498.6 ± 14.6 10.6 ± 1.8

Survival following the exposure period was 39/40, 37/40, and 38/40 for Groups 1, 2, and 3, respectively.

Mean terminal body weight data were similar among the control and treated groups.

The aberration most frequently found in the bone marrow cells of both treated groups was the chromatid gap. Gaps are not considered to be a valid measure of genetic damage; they may be artifacts of slide preparation. It is of interest to note, however, that the significant increase in the number of gaps was dose-related.

Chromatid breaks, which are considered to be mildly significant as a cytogenetic event, were the next most frequent aberrations observed. Chromatid breaks represent physical breakage of the chromatid arm by some agent which has disrupted the linear array of DNA within the structure. Since the breakage involves only one arm, it is believed to be a post-replication event which may be lethal to that cell and may not be passed on in subsequent cell divisions. Both treatment groups exhibited significant increases relative to the controls in the number of chromatid breaks without fragments and the high level group (Group 3) also showed a significant increase in the number of chromatid breaks with fragments.

Chromosomal breaks, which are pre-replication disruptions of linearity involving both chromatid arms, were comparable among all groups.

Chromosomal markers including exchange figures, rings, and miscellaneous marker chromosomes were significantly increased in Group 3, but not in Group 2. Exchange figures and marker chromosomes, which are considered to be the most significant aberrations with respect to cytogenetic evaluation, are the result of chromatid and/or chromosomal breakage where the broken ends reunite with other chromosomes incorrectly. This process results in complex chromosomal figures which can be identified under the light microscope because of their unique morphology.

The mean number of cells showing all types of aberrations (excluding gaps) was significantly higher for Groups 2 and 3, while only Group 3 showed a significant increase in the mean number of all aberrations (excluding gaps).

The incidence of polyploidy was essentially comparable among the control and treated groups.

Fifty-nine percent of the animals in Group 2 had aberrant cells. A quantitative, although statistically non-significant, increase in the mean number of markers and exchange figures was seen for this group. This non-significant increase could be due to the small sample size (25) of cells examined per animal and/or the possibility that the Group 2 exposure approximated the threshold level of induction of more serious genetic damage. Because of the lack of significant increases in the number of chromosomal markers and in the mean number of all aberrations but a significant increase in the number of cells showing all types of aberrations (excluding gaps), a conclusive conclusion regarding the mutagenic effect of the exposure to 50 ppm N_2O plus 1 ppm halothane cannot be made. However, chromatid-type damage was present in this group and could indicate a possible low-level effect on the bone marrow chromosomes.

Sixty-eight percent of the rats from Group 3 showed affected cells, thus ruling out a localized effect in only a few animals.

Group 3 contained significant increases in the number of chromosomal markers, in the mean number of all aberrations and in the mean number of cells showing all types of aberrations as well as significant increases in chromatid aberrations. Based on these data, it can be concluded that exposure to 500 ppm N_2O plus 10 ppm halothane can cause chromosomal damage to bone marrow cells in the rat.

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Report Preparation: Kapp and Krumm

Supervision: Hardy and Kapp

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