JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 12, Number 2, 1993
Mary Ann Liebert, Inc., Publishers

Uterine Changes in Female Mice Following Lifetime Inhalation of Wholly Vaporized Unleaded Gasoline: A Possible Relationship to Observed Liver Tumors?

JUDITH A. MACGREGOR,[1] WARD R. RICHTER,[2] and RENAE I. MAGAW[3]

ABSTRACT

An increased incidence of primary hepatocellular tumors was reported in female B6C3F$_1$ mice following lifetime exposure to high levels (2056 ppm) of wholly vaporized unleaded gasoline. This effect was not observed in male mice, nor in females exposed to either 67 or 292 ppm gasoline. No explanation for the sex and dose-specific effect was discussed in the initial report or has been subsequently published. At necropsy, a decreased incidence of enlarged/cystic uteri was also noted among high dose females; however, no correlating histopathologic changes were reported. Because the liver neoplastic response was limited to the females, and because spontaneous liver tumors are known to be influenced by the hormonal environment, we reexamined the uterine tissues microscopically. We observed a dramatic difference among treatment groups in the severity of cystic endometrial hyperplasia. The incidence of moderate cystic endometrial hyperplasia at the terminal sacrifice was 76% in the control animals whereas none of the animals at the highest exposure level had lesions that were graded moderate or severe. In addition, uterine atrophy was present in 35% of mice examined at the high exposure level and was absent in other groups. The changes noted are consistent with an altered hormonal influence on the uterus. The finding of a decrease in cystic endometrial hyperplasia and an increase in uterine atrophy in the same dose group in which liver tumors were found suggests that these effects may be interrelated.

INTRODUCTION

Gasoline is perhaps one of the most familiar substances to people in industrialized societies. Every year, 100 billion gallons of gasoline are produced in the United States alone. Thousands of workers at service stations and gasoline distribution terminals, and millions of consumers create a high potential for human exposure to gasoline.

One lifetime study on the health effects of gasoline has been conducted in the mouse. In this study, a compound-related increase in primary hepatocellular tumors was reported for female B6C3F$_1$ mice exposed to 2056 ppm wholly vaporized unleaded gasoline (IRDC, 1983; MacFarland et al. 1982, 1984a,b). Significant increases were not observed in female mice exposed to two lower treatment levels. Among all female mice at risk, liver tumor incidences were 10, 17, 15, and 37% for the 0, 67, 292, and 2056 ppm treatment groups, respectively. The majority of tumors appeared late in the study without other signs of hepatotoxicity. A compound-related increase in hepatocellular tumors was not present in male mice; incidence rates were 36, 26, 39, and 35% for the 0, 67, 292, and 2056 ppm treatment levels, respectively (Magaw et al., 1993). Other treatment-related effects were

[1]Chevron Research & Technology Co., P.O. Box 4054, Richmond, CA.
[2]Pharmaco-LSR, East Millstone, NJ.
[3]ICF Kaiser Engineers, 1800 Harrison Street, 7th Floor, Oakland, CA.

limited to a decrease in enlarged/cystic uteri observed at gross necropsy in females exposed to the high treatment level. No correlating histopathologic changes were reported for the gross observation and the effect was not thought to be of significance and was given little attention (IRDC, 1983).

No explanations for the increased incidence of liver tumors or the decreased incidence of enlarged/cystic uteri have been reported. It is unlikely that the carcinogenic response resulted from a direct mutagenic event. Unleaded gasoline has been extensively tested in several mutagenesis assays and the results of these studies support the general conclusion that unleaded gasoline is not mutagenic (Conaway et al., 1982; EPA, 1987; NESCAUM, 1989).

In addition, several aspects about the observed tumorigenic effect are unusual and also suggest that the tumors may not have been produced as a result of a direct action of wholly vaporized unleaded gasoline on hepatocytes. The finding of a hepatotumorigenic effect in female, but not male, $B6C3F_1$ mice is not common (Haseman and Huff, 1987; Haseman et al., 1987). The late appearance of the liver tumors without any other signs of hepatotoxicity and the fact that the final incidence observed in the high dose group was similar to that observed in control male mice in the same study are also noteworthy.

The development of hepatocellular tumors in the mouse is known to be hormonally dependent (Agnew and Gardner, 1952; Andervont, 1950; Warwick, 1971). Data and selected tissues from the original study were reviewed to determine whether there was any evidence of compound-related endocrine effects. Results of the review are presented in this paper. The possibility of a relationship between the observed effects on the uteri and the development of hepatocellular tumors is also explored.

METHODS

The final report of the lifetime inhalation study of wholly vaporized unleaded gasoline was thoroughly reviewed. The International Research and Development Corporation (IRDC, 1983) conducted a 2-year study in which groups of 100 male and 100 female $B6C3F_1$ mice were exposed to 0, 67, 292, and 2056 ppm gasoline by whole body inhalation. Interim sacrifices were conducted after approximately 3, 6, 12, and 18 months exposure. Terminal sacrifices were conducted after 103–107 weeks for male mice and after 113 weeks for female mice.

Selected tissues from the study were obtained and subjected to an independent histopathologic review. The original slides for livers, ovaries, and uteri of all female mice were requested from the laboratory. The laboratory could not locate the slides and, therefore, paraffin blocks containing tissues collected during the study were obtained instead. Blocks containing ovarian tissue were grossly examined and many were found to be too small to consistently obtain adequate slides. Therefore, effects on the ovaries could not be assessed. Blocks containing livers and uteri were recut, sections were stained with hematoxylin and eosin, and new slides were prepared. The liver slides were reviewed to confirm the original diagnoses. The uteri were evaluated histologically for the presence of any compound-related effect.

Careful attention was paid to the histopathologic observation of uterine endometrial hyperplasia, cystic endometrial hyperplasia, and atrophy because of the decrease in the incidence of enlarged/cystic uteri described as a possible treatment-related effect for female mice in the final study report. These lesions were carefully graded on a four-point scale of severity corresponding to subjective classifications of trace, mild, moderate, and severe. It was not possible to make precise measurements of thickness because the sections were recuts and because the original embedding was not done anticipating the need to make such measurements.

Hyperplasia and/or cystic hyperplasia were recorded when there was an increase in endometrial thickness beyond what was considered normal for an adult sexually mature female in anestrus. The grade of trace was used for the minimal amount of hyperplasia that could be subjectively differentiated from normal. A hyperplastic uterus with a grade of severe was approximately four times as large (in cross section) as a uterus with trace hyperplasia. The severe hyperplastic epithelium included multiple cystic glands, varying in size. The difference between a uterus with trace and severe hyperplasia was quite evident by examination of the slides with the naked eye. The grades of mild and moderate were equally spaced between the two extremes. Examples of uteri with trace and severe cystic hyperplasia are presented in Figures 1 and 2, respectively.

The diagnosis of atrophy was used when the uterus was smaller than a normal adult uterus. A four-point scale was also used to describe the severity of this lesion. The grade of trace represented the slightest decrease in

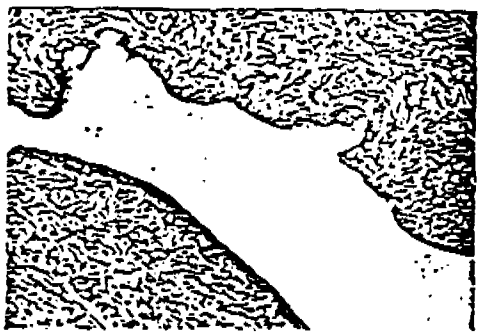


FIG. 1. Trace cystic endometrial hyperplasia of the uterus. Mouse exposed to 2056 ppm wholly vaporized unleaded gasoline and sacrificed at terminal sacrifice. Same magnification as Figure 2 with a 20× objective.

thickness of the endometrium and myometrium that could be detected. A uterus with moderate atrophy was approximately half normal size. Mild was intermediate between trace and moderate.

RESULTS

Table 1 indicates that cystic endometrial hyperplasia was present in the uterus of most of the female mice examined. Cystic hyperplasia was first observed in one animal that died after 9 months on study and the incidence increased by the 12-month interim sacrifice and remained high throughout the remainder of the study. Figure 3 presents low-power photomicrographs demonstrating each of the four severity grades. Overall, the incidence of the lesion was slightly decreased only at the highest treatment level (2056 ppm). However, there was a dramatic treatment-related change in severity of the lesion that was evident as early as the 12-month sacrifice time and that continued throughout the study.

Average severity grades were derived for each treatment group by multiplying the number of animals scored for each severity by the severity grade, summing the results for all animals in the treatment group, and dividing by the total number of animals in the group with cystic hyperplasia. Cystic hyperplasia was much more severe in the controls and at the low treatment level than at the mid and high treatment levels. The severity observed in the control and low exposure animals was representative of the spontaneous picture for "normal" animals of this age, but the altered pattern of severity observed at the two higher treatment levels was unusual and was considered to be treatment related.

When the severity of cystic hyperplasia was evaluated separately for liver tumor-bearing and non-tumor-bearing animals, there was a tendency toward the lesions to be milder in animals with tumors. Average severities were 1.8

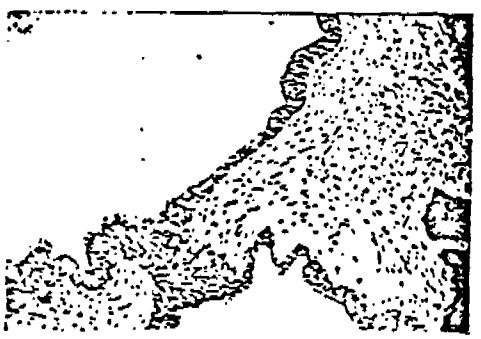


FIG. 2. Severe cystic endometrial hyperplasia of the uterus. Control mouse sacrificed at terminal sacrifice. Same magnification as Figure 1 with a 20× objective.

TABLE 1. INCIDENCE AND SEVERITY OF UTERINE CYSTIC HYPERPLASIA: ALL FEMALE MICE EXAMINED BY TIME OF DEATH

	Time of death															
	<12 months				*12–18 months*				*18-mo. to T-sac.*				*Terminal sacrifice*			
Treatment level[a]	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
No. examined	26	22	21	22	12	13	11	11	26	29	29	24	41	33	37	40
Cystic hyperplasia	0	1	0	0	9	8	11	5	17	23	18	11	40	32	37	34
Trace	0	0	0	0	0	0	0	1	3	2	2	1	0	3	5	16
Mild	0	0	0	0	2	1	4	2	3	6	10	6	6	9	26	18
Moderate	0	1	0	0	5	6	6	2	9	11	6	4	31	20	6	0
Severe	0	0	0	0	2	1	1	0	2	4	0	0	3	0	0	0
Average severity[b]	0	3.0	0	0	3.0	3.0	2.7	2.2	2.6	2.7	2.2	2.3	2.9	2.5	2.0	1.5

[a]Treatment levels: 1 = 0 ppm; 2 = 67 ppm; 3 = 292 ppm; 4 = 2056 ppm.

[b]Severity grades: trace = 1; mild = 2; moderate = 3; severe = 4.

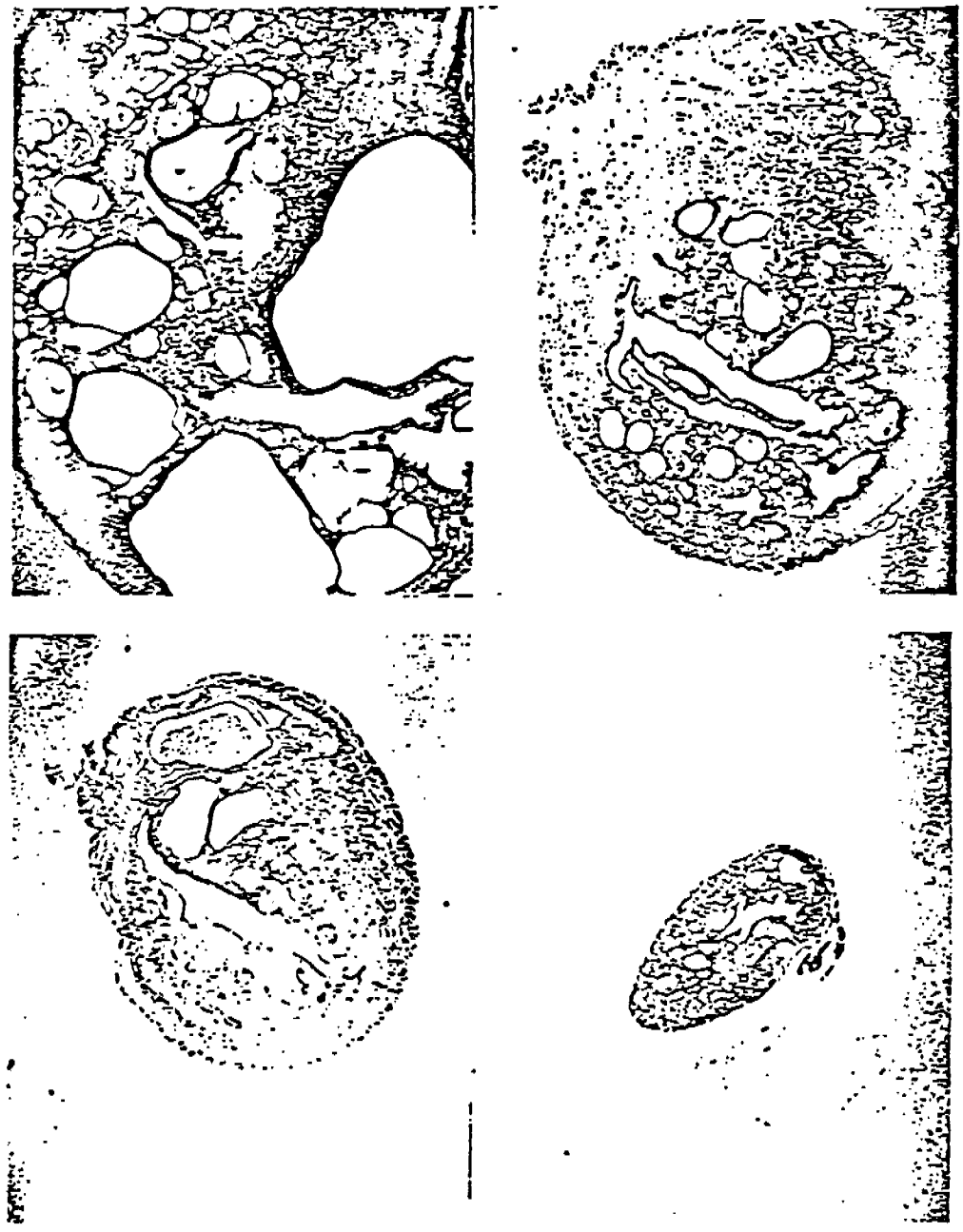


FIG. 3. Cross sections of uterine horns illustrating the four grades of severity. All at 20× magnification. Upper left, severe, group 1 animal; upper right, moderate, group 2 animal; lower left, mild, group 3 animal; lower right, trace, group 4 animal.

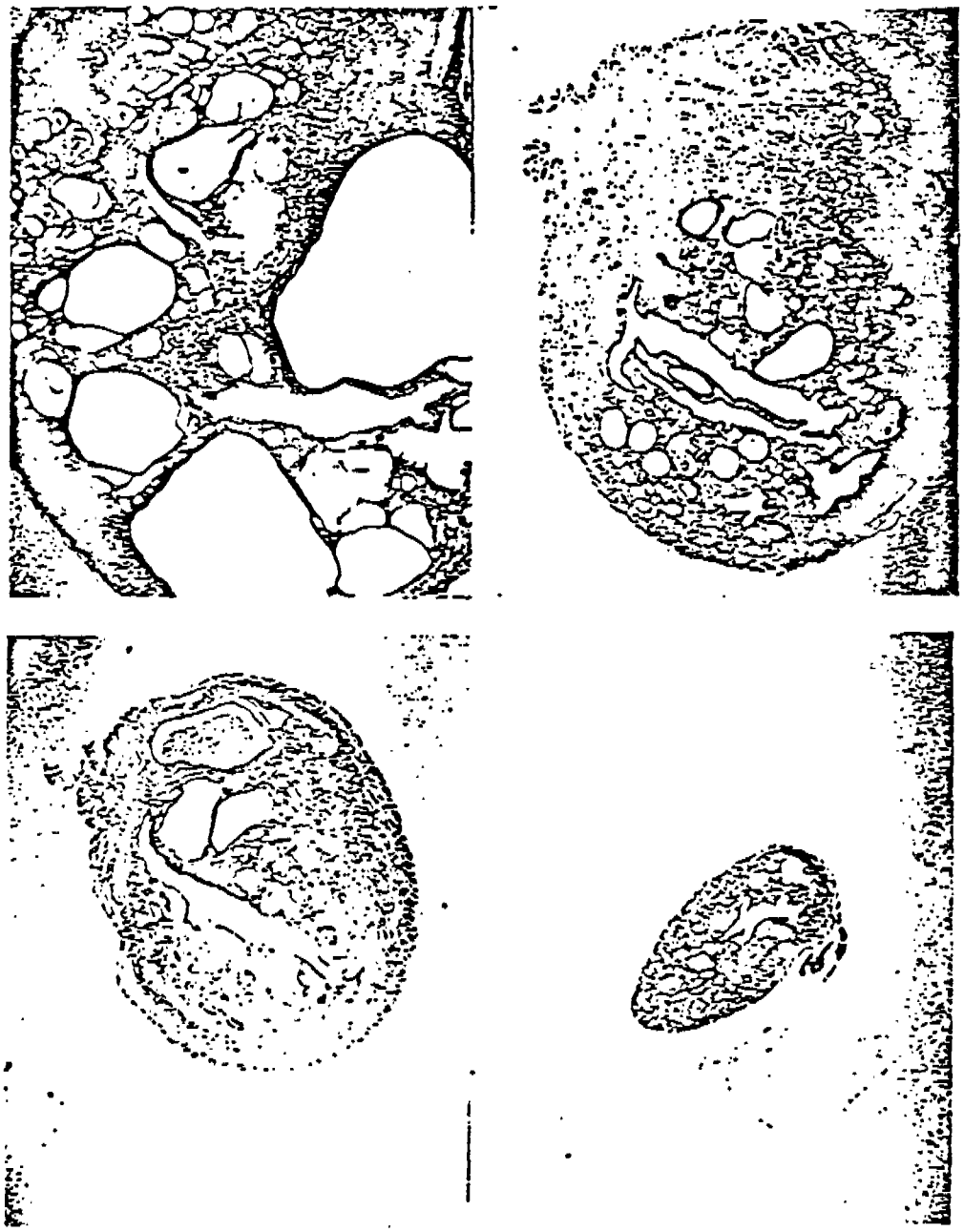


FIG. 3. Cross sections of uterine horns illustrating the four grades of severity. All at 20× magnification. Upper left, severe, group 1 animal; upper right, moderate, group 2 animal; lower left, mild, group 3 animal; lower right, trace, group 4 animal.

and 1.5 in tumor-bearing animals at the mid and high dose levels, respectively, and 2.3 and 1.9 in non-tumor bearing animals at the same dose levels. Because nearly all of the animals in the study developed cystic hyperplasti lesions, and the subjective nature of the severity scoring, it would be difficult to definitively conclude that difference between the tumor and non-tumor bearing groups exists.

Table 2 presents the incidence and severity of uterine atrophy. Atrophy was observed almost exclusively in mic exposed to 2056 ppm. The first lesion was observed in an animal exposed to 67 ppm that died between 12 and 1 months on study, but the majority of lesions were observed only at the end of the study. At terminal sacrifice, 14/4 mice exposed to 2056 ppm were diagnosed with uterine atrophy, while the lesion was absent from control animal and those exposed to 67 or 292 ppm.

DISCUSSION

Cystic endometrial hyperplasia is a common spontaneous condition in mice (Cosgrove et al., 1978, Malinin an Malinin, 1972, Sheldon and Greenman, 1979; Ward et al., 1979). It is generally believed to result from changes ir the relative levels of estrogen and progesterone (Chand and Chauhan, 1975, Lindahl and Willen, 1991; Tang et al. 1984). The incidence of this lesion in mice varies by strain and tends to increase with age. In many strains, nearly every female older than 18 months will have some degree of endometrial hyperplasia (Burek et al., 1982).

Exogenous estrogen can induce cystic hyperplasia (Tang et al., 1984). Several rodent diets have been shown t have estrogenic activity and this activity has been shown to increase the size and weight of the mouse uterus (Dran et al., 1981; Greenman et al., 1987; Thigpen et al., 1987a,b,c). Uterine changes that develop as an animal ages may merely be biological indicators of endocrine function and animals in which this aging effect is marked may be more sensitive indicators of differences in estrogen levels. Studies in which the uteri are affected to a marked extent may in effect, serve as biological test systems that allow any reduction in the normal development of cystic endometria hyperplasia to be easily observed.

The decreased severity and incidence of cystic endometrial hyperplasia observed after exposure to wholly vaporized unleaded gasoline are unusual, and indicate that the normal development of the lesions in exposed animals was altered. It is possible that a component of gasoline, or a group of compounds, interferes with norma hormonal influences on the uterus. Another plausible hypothesis involves modulation of the biotransformation o endogenous substances at high hydrocarbon exposure levels. Several halogenated hydrocarbons (e.g., chlordane, dieldrin, heptachlor, polybrominated biphenyls) are known to stimulate liver microsomal enzymes and have been shown to decrease the concentration of estrogen in rats and mice and to inhibit estrogen's uterotrophic activity in exposed animals (Bonhaus et al., 1981; Welch et al., 1979).

A review of the toxicologic studies on gasoline produced some supporting evidence for this possibility. Increased liver weights, a gross observation often associated with induction of liver enzymes, were observed in 90-day inhalation studies in rats (IIT, 1985) and monkeys (Kuna and Ulrich, 1984; MacFarland, 1984). In the IRDC lifetime inhalation study, liver weights were increased in the high dose female mice as early as the 3-month interim sacrifice, many months before effects were observed in the uteri.

TABLE 2. INCIDENCE AND SEVERITY OF UTERINE ATROPHY: ALL FEMALE MICE EXAMINED

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
Number examined	105	97	98	97
Atrophy	1	1	0	16
Trace	0	0	0	1
Mild	1	1	0	12
Moderate	0	0	0	1
Severe	0	0	0	2

Evidence for a possible alteration in the hormonal status of female mice exposed to high levels of unleaded gasoline is interesting because of the possibility of a connection between this effect and the occurrence of liver tumors in these animals. Mouse liver tumors are one of the most controversial endpoints in carcinogenesis testing. Mechanisms through which these tumors develop have not been fully worked out and remain a topic of considerable debate and research. Many factors can influence the spontaneous incidence or the induced incidence (ECETOC, 1982; Turusov and Takayama, 1979; Warwick, 1971) of mouse liver tumors and the incidence is known to vary from laboratory to laboratory and even from study to study within the same laboratory (Tarone et al., 1981).

The sex of the animal has been shown to have a marked effect. The spontaneous rate of hepatocellular tumors for B6C3F$_1$ and other strains of mice is known to be higher in males than females (Agnew and Gardner, 1952; Andervont, 1950; Haseman et al., 1985; Tarone et al., 1981; Ward et al., 1979). Haseman et al. (1985) reported 31% spontaneous incidence in B6C3F$_1$ males and 8% in females.

Reasons for this difference have been explored in many studies and the hormonal environment of the animals has been shown to play an important role. Experimentally, ovariectomy and the administration of testosterone have been shown to enhance spontaneous hepatocellular tumor incidences in females, while orchiectomy and estrogen administration reduce the incidence in males. Castrated males and females generally express similar spontaneous liver tumor rates (Agnew and Gardner, 1952; Andervont, 1950, Warwick, 1971).

In the lifetime inhalation study of wholly vaporized unleaded gasoline, the liver tumor incidences observed in all groups of female mice other than the high dose group were low and generally within the expected spontaneous range for female B6C3F$_1$ mice (Haseman et al., 1985; Tarone et al., 1981). The incidence in the high dose group was significantly greater than the other groups and was, in fact, similar to incidence rates observed in male mice in the same study (IRDC, 1983, MacFarland 1982, 1984; MacFarland et al., 1984; Magaw et al., 1993) and to expected spontaneous rates for male B6C3F$_1$ mice (Haseman et al., 1985; Tarone et al., 1981; Ward et al., 1979). These observations are consistent with the idea that uterine effects may serve as a marker for some endocrine disturbance that alters the incidence of hepatocellular tumors in female mice.

REFERENCES

AGNEW, L.R.C., and GARDNER, W.U. (1952). The incidence of spontaneous hepatomas in C3H, C3H (low milk factor), and CBA mice and the effect of estrogen and androgen on the occurrence of these tumors in C3H mice. Cancer Res. 12:757–761.

ANDERVONT, H.B. (1950). Studies on the occurrence of spontaneous hepatomas in mice of strains C3H and CBA. J. Natl. Cancer Inst. 11:581–592.

BONHAUS, D.W., McCORMACK, K.M., BRASELTON, W.E., and HOOK, J.B. (1981). Effect of polybrominated biphenyls on hepatic microsomal metabolism of estrogens and uterotrophic action of administered estrogen in rats. J. Toxicol. Environ. Health. 8:141–150.

BUREK, J.D., MOLELLO, J.A., and WARNER, S.D. (1982). Selected nonneoplastic diseases. in: *The Mouse in Biomedical Research Vol II Diseases* H.L. Foster, J.D. Small, and J.G. Fox (eds.). Academic Press: New York. pp. 425–440.

CHAND, S., and CHAUHAN, H.V.S. (1975). Cystic endometrial hyperplasia in sheep and goats. Indian J. Anim. Sci. 45:71–75.

CONAWAY, C.C., SCHREINER, C.A., and CRAGG, S.T. (1982). Mutagenicity evaluation of petroleum hydrocarbons, in: *The Toxicology of Petroleum Hydrocarbons. Proceedings of a Symposium... May, 1982.* H.N. MacFarland, C.E. Holdsworth, J.A. MacGregor, R.W. Call, and M.L. Kane (eds.): American Petroleum Institute. Washington, DC. pp. 128–138.

COSGROVE, G.E., SATTERFIELD, L.C., BOWLES, N.D., and KLIMA, W.C. (1978). Diseases of aging untreated virgin female RFM and BALB/c mice. J. Gerontol. 33:178–183.

DRANE, H.M., HEBERT, C.N., and SABA, N. (1981). Effect of diet on oestrogen bio-assay in mice. Fd. Cosmet. Toxicol. 19:93–96.

EUROPEAN CHEMICAL INDUSTRY ECOLOGY AND TOXICOLOGY CENTRE (ECETOC). (1982). *Hepatocarcinogenesis* in Laboratory Rodents: Relevance for Man. ECETOC Monograph No. 4, Brussels.

GREENMAN, D.L., FULLERTON, F.R., SUBER, R., and FARMER, J. (1987). Effects of purified (AIN-76A) and natural-ingredient (NIH-07) diets on responses of BALB/c and B6C3F$_1$ female mice to estradiol. J. Toxicol. Environ. Health. 22:351–362.

HASEMAN, J.K., and HUFF, J.E. (1987). Species correlation in long-term carcinogenicity studies. Cancer Lett. 37:125–132.

HASEMAN, J.K., HUFF, J.E., RAO, G.N., ARNOLD, J.E., BOORMAN, G.A., and McCONNELL, E.E. (1985). Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F1 (B6C3F$_1$) mice. J. Natl. Cancer Inst. 75:975–984.

HASEMAN, J K., HUFF, J E., ZEIGER, E., and McCONNELL, E.E (1987). Comparative results of 327 chemical carcinogenicity studies Environ Health Perspect 74:229–235.

IIT RESEARCH INSTITUTE. (1985). Thirteen-week inhalation toxicity study of a 0–145° gasoline distillate fraction in rats. Sponsored by the American Petroleum Institute. API Doc No 32-32405 February, 1985.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION (IRDC). (1983). Motor Fuel Chronic Inhalation Study. Vols 1–6 Sponsored by the American Petroleum Institute Sept. 15, 1983.

KUNA, R A., and ULRICH, C.E. (1984) Subchronic inhalation toxicity of two motor fuels J. Am Coll. Toxicol. 3:217–229.

LINDAHL, B., and WILLEN, R. (1991) Endometrial hyperplasia following estrogen treatment without the addition of gestagen. A follow-up study after withdrawal of estrogens Anticancer Res. 11:2071–2074.

MacFARLAND, H.N. (1982) Chronic gasoline toxicity, in. *The Toxicology of Petroleum Hydrocarbons. Proceedings of a Symposium May, 1982.* H.N. MacFarland, C.E. Holdsworth, J.A. MacGregor, R.W Call, and M.L. Kane (eds.). American Petroleum Institute Washington, DC. pp. 78–86.

MacFARLAND, H.N. (1984). Xenobiotic induced kidney lesions: Hydrocarbons. The 90-day and 2-year gasoline studies, in: *Advances in Modern Environmental Toxicology, Vol. 7. Renal Effects of Petroleum Hydrocarbons.* M.A. Mehlman, C.P. Hemstreet, J.J Thorpe, and N.K Weaver (eds.) Princeton Scientific Publishers, Inc.: Princeton, NJ. pp. 51–56.

MacFARLAND, H.N., ULRICH, C.R., HOLDSWORTH, C.E., KITCHEN, D.N., HALLIWELL, W.H., and BLUM, S.C. (1984) A chronic inhalation study with unleaded gasoline vapor. J Am Coll. Toxicol 3:231–248.

MACAW, R J., RICHTER, W.R., and MacGREGOR, J.A. (1993). A reevaluation of liver tumors in mice exposed to wholly vaporized unleaded gasoline J Am Coll Toxicol 12:195–199.

MALININ, G I., and MALININ, L.M. (1972). Age-related spontaneous uterine lesions in mice J Geronitol 27:193–196.

NORTHEAST STATES FOR COORDINATED AIR USE MANAGEMENT (NESCAUM) (1989) Evaluation of the health effects from exposure to gasoline and gasoline vapors. Air Toxics Committee.

SHELDON, W G., and GREENMAN, D L. (1979). Spontaneous lesions in control BALB/c female mice. J. Environ Pathol. Toxicol 3:155–167.

TANG, F.Y., BONFIGLIO, T A., and TANG, L K (1984) Effect of estrogen and progesterone on the development of endometrial hyperplasia in the Fischer rat Biol Reprod 31:399–413.

TARONE, R E., CHU, K C., and WARD, J M. (1981) Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL 6N x C3H HeN)F1 (B6C3F1) mice. J Natl Cancer Inst 66:1175–1181.

THIGPEN, J E LI, L.A., RICHTER, C B., LEBETKIN, E H., and JAMESON, C.W. (1987a) The mouse bioassay for the detection of estrogenic activity in rodent diets: I A standardized method for conducting the mouse bioassay Lab Animal Sci. 37:596–601.

THIGPEN, J E LI, L A., RICHTER, C B., LEBETKIN, E.H., and JAMESON, C.W (1987b) The mouse bioassay for the detection of estrogenic activity in rodent diets. II Comparative estrogenic activity of purified, certified and standard open and closed formula rodent diets Lab Animal Sci. 37:602–605.

THIGPEN, J E LEBETKIN E H., DAWES, M L., RICHTER, C.B., and CRAWFORD, D (1987c) The mouse bioassay for the detection of estrogenic activity in rodent diets III Stimulation of uterine weight by dextrose, sucrose, and corn starch Lab Animal Sci 37:606–609.

TURUSOV, V S., and TAKAYAMA, S (1979). Tumors of the liver, in *Pathology of Tumors of Laboratory Animals. Vol. II. Tumors of the Mouse* V S Turusov (ed.) International Agency for Research on Cancer, IARC Scientific Publication No 23. Lyon pp 193–211.

U S ENVIRONMENTAL PROTECTION AGENCY (EPA). (1987) Evaluation of the carcinogenicity of unleaded gasoline Office of Health and Environmental Assessment, Washington, D C., EPA 600 6-87-001.

WARD, J.M., GOODMAN, D G., SQUIRE, R A., CHU, K C., and LINHART, M S (1979) Neoplastic and nonneoplastic lesions in aging (C57BL 6N x C3H HeN)F1 (B6C3F1) mice. J Natl Cancer Inst 63:849–854.

WARWICK, G P (1971) Metabolism of liver carcinogens and other factors influencing liver cancer, in *Liver Cancer.* International Agency for Research on Cancer, IARC Scientific Publication No. 1, Lyon, pp 121–157.

WELCH, R M., LEVIN, W., KUNTZMAN, R., JACOBSEN, M., and CONNEY, A.H. (1971) Effect of halogenated hydrocarbon insecticides on the metabolism and uterotrophic action of estrogens in rats and mice Toxicol. Appl. Pharmacol 19:234–246.

Address reprint requests to:
Dr. Judith A. MacGregor
Chevron Research and Technology Co.
P.O. Box 4054
Richmond, CA 94804

JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 12, Number 2, 1993
Mary Ann Liebert, Inc., Publishers

A Reexamination of Liver Tumors in Mice Exposed to Wholly Vaporized Unleaded Gasoline

RENAE I. MAGAW,[1] WARD R. RICHTER,[2] and JUDITH A. MACGREGOR[3]

ABSTRACT

A single lifetime inhalation study of the effects of wholly vaporized unleaded gasoline has been conducted in the mouse. An elevation of hepatocellular tumors was reported only in females and only at the high treatment level (2056 ppm). Several reports of this study have appeared previously in the scientific literature, including a comprehensive summary published in this journal. Different incidence rates were reported for mouse liver tumors in these publications without explanations of how they were calculated of why they differed. To clarify the data, we recently examined the final report of the gasoline study, the individual animal data, and slides prepared from liver tissue collected during the study. The previously reported incidence rates did not take into consideration all animals at risk for tumor development or all animals with hepatocellular tumors. Revised incidence rates are presented for hepatocellular tumors in all mice on study. These rates are approximately 20–25% lower than previously reported.

INTRODUCTION

A lifetime inhalation study of the effects of wholly vaporized unleaded gasoline on mice was conducted by the International Research and Development Corporation (IRDC, 1983) and sponsored by the American Petroleum Institute. The results of the study indicated that wholly vaporized unleaded gasoline induced a statistically significant compound-related increase in hepatocellular tumors in female mice. No increase was observed in male mice.

The study was originally reported by MacFarland et al. in this journal (1984) and elsewhere (1982, 1984), and it was reviewed extensively by the U.S. Environmental Protection Agency (1987) and other organizations (HEI, 1985; NESCAUM, 1989). Slightly different incidence figures were presented for mouse liver tumors in the published reports. The differences could not be explained on the basis of information presented in the articles and raised questions about why the published rates differed. The data and the reports in question have been thoroughly reviewed and the actual liver tumor incidence observed in mice in this study was determined.

IRDC STUDY SUMMARY AND PUBLISHED INCIDENCE DATA

IRDC (1983) conducted a 2-year study in which groups of 100 male and 100 female B6C3F$_1$ mice were exposed to 0, 67, 292, and 2056 ppm of wholly vaporized unleaded gasoline by whole body inhalation. Interim sacrifices

[1]ICF Kaiser Engineers, 1500 Harrison Street, 7th Floor, Oakland, CA.
[2]Pharmaco-LSR, East Millstone, NJ.
[3]Chevron Research & Technology Co., P.O. Box 4054, Richmond, CA.

were conducted after approximately 3, 6, 12, and 18 months of exposure. Terminal sacrifices were conducted after 103–107 weeks for male mice and 113 weeks for female mice. The incidence rates for liver tumors have been reported several times, as shown in Table 1 (MacFarland, 1982; MacFarland et al., 1984; NESCAUM, 1989; EPA, 1987). Hepatocellular tumors were defined as adenomas and carcinomas in these publications.

LIVER TUMOR INCIDENCE REVIEW

In order to determine the true incidence of liver tumors observed in the study and the reasons why previously reported incidence rates vary, the IRDC study history and final report were carefully examined. In addition, an independent histopathological review of liver sections was conducted. Because the original slides could not be located by IRDC, paraffin blocks containing liver tissue were obtained from the laboratory. The blocks were recut, sections were stained with hematoxylin and eosin, and new slides were prepared.

The criteria utilized in the histopathological review for the diagnoses of hepatocellular adenoma and carcinoma were as summarized by Brooks and Roe (1985) and by Popp (1985) for adenomas and carcinomas, respectively. Briefly, adenomas were sharply demarcated and they compressed adjacent liver parenchyma. The cytology of the cells varied, with cytoplasm that was eosinophilic, basophilic, clear, or vacuolated. Carcinomas were characterized by some or all of the following: an increased degree of pleomorphism, greater nuclear/cytoplasmic ratio, trabecular plates several cells thick, and increased mitotic activity. Some were large with irregular or invasive growth patterns.

The review of the IRDC final report indicated that the previously reported incidence rates do not take into consideration *all mice at risk of tumor development or all animals with hepatocellular tumors*. The first hepatocellular tumor was observed at the 12-month interim sacrifice and thus all animals alive at that time should be considered at risk for tumor development and should be included in the incidence rates. The rates reported by MacFarland, NESCAUM, and those used by the EPA for estimating the carcinogenic potency of unleaded gasoline correspond to the incidence of hepatocellular tumors in mice that died or were killed during the 18-month to terminal sacrifice period. Animals that were killed or that died on study prior to 18 months were not included. The

TABLE 1. PRIMARY HEPATOCELLULAR NEOPLASMS INCIDENCE RATES REPORTED IN MICE

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
	Female mice			
EPA, pages 5–14[a]	8/57 (14)[b]	10/52 (19)	13/57 (23)	28/56 (50)
EPA, pages 3–15[c]	8/100 (8)	10/100 (10)	12/100 (12)	27/100 (27)
MacFarland[d]	8/57 (14)	10/52 (19)	12/57 (21)	27/56 (48)
NESCAUM[e]	8/57 (14)	10/52 (19)	12/57 (21)	27/56 (48)
Revised incidence[f]	8/79 (10)	13/76 (17)	12/79 (15)	29/78 (37)
	Male mice			
MacFarland[d]	23/51 (45)	15/42 (36)	20/44 (45)	24/54 (44)
NESCAUM[e]	23/51 (45)	15/42 (36)	20/44 (45)	24/54 (44)
Revised incidence[f]	27/75 (36)	18/70 (26)	28/71 (39)	27/77 (35)

[a] Incidence used to estimate carcinogenic potency in EPA (1987).

[b] Percentages are reported in parentheses.

[c] Incidence reported in summary section of EPA (1987).

[d] Incidences reported in MacFarland (1982, 1984) and MacFarland et al. (1984).

[e] Incidence reported in NESCAUM (1989).

[f] Corrected incidence determined from this review.

summary incidence figures reported for female mice by EPA differ. In this case, the numerators are based on mice that died during the 18-month to terminal sacrifice period, while the denominators represent the total number of mice initially started on test, including those that died prior to 12 months. These animals should not be considered at risk of tumor development and should not be included in the incidence rates.

The study history provides several possible explanations for why the data were reported incorrectly. The histopathologic evaluation of the study was carried out by several pathologists at different laboratories. Separate reports for the different sacrifice groups were prepared and reviewed at different times. The preliminary-draft terminal sacrifice report included data for animals that died on study after the 18-month interim sacrifice along with the data for animals sacrificed at the end of the experiment. It did not include or summarize any data for animals that died prior to these times. This report was reviewed, discussed, and summarized prior to completion of the final IRDC report. When the final report was issued for the entire study many months later, the various reviewers did not go back and incorporate data for animals that died previously.

In addition to problems in tabulating the data, there was a change in the terminology for proliferative liver lesions used during the course of the study that resulted in some mice with hepatocellular tumors being excluded from the reported incidence rates. The diagnosis of neoplastic nodule was used at the 12- and 18-month interim sacrifices, but not at other time periods. This term was proposed by Squire and Levitt (1975) to describe proliferative lesions only in the rat. It was proposed because of the difficulty in differentiating between hyperplastic and neoplastic lesions in the rat on morphologic grounds. The term neoplastic nodule is not typically used to describe mouse tumors, but was used for diagnosing lesions in this study for mice at these two interim sacrifices. Animals diagnosed with neoplastic nodules were not included in the incidence figures reported by MacFarland, NESCAUM, or EPA.

Lesions diagnosed as neoplastic nodules by the original pathologist were reevaluated as part of this review and their morphology was found to be consistent with the diagnosis of hepatocellular adenoma. The morphology of these lesions was also consistent with that of the lesions diagnosed as hepatocellular adenoma at later times in the study and animals with neoplastic nodules should be included in the tumor incidence figures.

REVISED TUMOR INCIDENCE

Tables 2 and 3 present the total number of animals with hepatocellular tumors by tumor type and for all tumor types combined for female and male mice, respectively. The overall revised incidence rates presented for all hepatocellular tumors include all animals considered to be at risk for tumor development (i.e., all animals alive at 12 months) and all animals with primary liver tumors, including those originally diagnosed as neoplastic nodules.

TABLE 2. PRIMARY HEPATOCELLULAR NEOPLASMS IN FEMALE MICE: NUMBERS OF ANIMALS WITH TUMORS AND REVISED INCIDENCE

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
Neoplastic nodule	0	3	0	1
Adenoma	1	4	4[a]	8[a]
Carcinoma	7	6	9	21
Total benign	1	7	4	9
Total benign and malignant				
With neoplastic nodules	8	13	12[b]	29[b]
Without neoplastic nodules	8	10	12[b]	28[b]
Revised incidence	8/79 (10%)	13/76 (17%)	12/79 (15%)	29/78 (37%)

[a]One mouse had an adenoma during the 18- to 24-month study period.

[b]One mouse had an adenoma and a carcinoma and is counted only for the most advanced lesion.

TABLE 3. PRIMARY HEPATOCELLULAR NEOPLASMS IN MALE MICE: NUMBERS OF ANIMALS WITH TUMORS AND REVISED INCIDENCE

	Treatment level			
	0 ppm	67 ppm	292 ppm	2056 ppm
Neoplastic nodule	4	3	5	2
Adenoma	12	4	5	5
Carcinoma	12[a]	14	19[b]	21[c]
Total benign	16	7	10	7
Total benign and malignant				
With neoplastic nobules	27[e]	18[d]	28[e]	27[e]
Without neoplastic nodules	24	15[d]	23[e]	25[e]
Revised incidence	27/75 (36%)	18/70 (26%)	28/71 (39%)	27/77 (35%)

[a] One mouse had a carcinoma at the 12 month sacrifice (0 ppm) or during the 12- to 18-month period (2056 ppm).

[b] Three mice had a carcinoma during the 12- to 18-month period.

[c] One mouse had a neoplastic nodule and a carcinoma and is counted only for the most advanced lesion.

[d] Three mice had an adenoma and a carcinoma and are counted only for the most advanced lesion.

[e] One mouse had an adenoma and a carcinoma and is counted only for the most advanced lesion.

The revised incidence rate observed in female mice exposed to 2056 ppm wholly vaporized unleaded gasoline is approximately 25% lower than previously reported. The revised rate is the result of correcting several inconsistencies in tabulating and analyzing data from this study. Inherent problems associated with multiple pathologists conducting the microscopic evaluations further complicated the analysis and should be avoided.

The incorrect incidence data have been used to characterize the potential risks associated with human exposure to gasoline by several regulatory groups (EPA, 1987; NESCAUM, 1989). These risks are based on a treatment-related effect observed only at the high dose level and, therefore, the incidence changes identified in this dose group could alter the estimated risks. This suggests that these risk assessments should be reevaluated.

REFERENCES

BROOKS, P.N., and ROE, F.J C. (1985). *Digestive System*. T.C. Jones, U. Mohr, and R.D. Hunt (eds.). Springer-Verlag: Berlin. pp. 47–51.

HEALTH EFFECTS INSTITUTE. (1985). Gasoline Vapor Exposure and Human Cancer. Evaluation of Existing Scientific Information and Recommendations for Future Research. Report of the HEI Health Review Committee.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION. (1983). Motor Fuel Chronic Inhalation Study. Vols. 1–6. Sponsored by the American Petroleum Institute. Sept. 15, 1983.

MacFARLAND, H.N. (1982). Chronic gasoline toxicity, in: *The Toxicology of Petroleum Hydrocarbons. Proceedings of a Symposium, May, 1982*. H N. MacFarland, C.E. Holdsworth, J.A. MacGregor, R.W. Call, and M.L. Kane (eds.): American Petroleum Institute: Washington, D C., pp. 78–86.

MacFARLAND, H N. (1984). Xenobiotic induced kidney lesions: Hydrocarbons. The 90-day and 2-year gasoline studies, in: *Advances in Modern Environmental Toxicology, Vol. 7, Renal Effects of Petroleum Hydrocarbons*. M.A. Mehlman, C.P. Hemstreet, J J. Thorpe, and N.K. Weaver (eds.): Princeton Scientific Publishers, Inc.: Princeton, NJ, pp. 51–56.

MacFARLAND, H.N., ULRICH, C R., HOLDSWORTH, C E., KITCHEN, D.N., HALLIWELL, W.H., and BLUM, S.C. (1984). A chronic inhalation study with unleaded gasoline vapor. *J. Am. Coll. Toxicol.* 3:231–248.

NORTHEAST STATES FOR COORDINATED AIR USE MANAGEMENT (NESCAUM) (1989). Evaluation of the health effects from exposure to gasoline and gasoline vapors. Air Toxics Committee.

POPP, J.A. (1985) *Digestive System*. T.C. Jones, U. Mohr, and R D. Hunt (eds): Springer-Verlag Berlin, pp. 39–45.

SQUIRE, R.A., and LEVITT, M.H. (1975). Report of a workshop in classification of specific hepatocellular lesions in rats. Cancer Res. 35:3214–3223.

U.S. ENVIRONMENTAL PROTECTION AGENCY. (1987). Evaluation of the carcinogenicity of unleaded gasoline. Office of Health and Environmental Assessment, Washington, D.C., EPA/600/6-87-001.

Address reprint requests to:
Dr. Judith A. MacGregor
Chevron Research and Technology Co.
P.O. Box 4054
Richmond, CA 94804

FUNDAMENTAL AND APPLIED TOXICOLOGY 9, 678–686 (1987)

A Two-Generation Reproduction Study with Monochlorobenzene Vapor in Rats[1]

R. S. NAIR,* J. A. BARTER,† R. E. SCHROEDER,‡ A. KNEZEVICH,‡ AND C. R. STACK§

*Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167; †PPG Industries, Inc., One PPG Place, Pittsburgh, Pennsylvania 15272; ‡Bio/dynamics, Inc., East Millstone, New Jersey 08873; and §Chemical Manufacturers Association, 2501 M Street, N.W., Washington, D.C. 20037.

A Two-Generation Reproduction Study with Monochlorobenzene Vapor in Rats. NAIR, R. S., BARTER, J. A., SCHROEDER, R. E., KNEZEVICH, A., AND STACK, C. R. (1987). *Fundam. Appl Toxicol.* 9, 678–686. Groups of 30 male and 30 female Sprague–Dawley CD rats, designated as the F_0 generation, were exposed to vapor of monochlorobenzene (MCB) at target concentrations of 0, 50, 150, or 450 ppm for 10 weeks prior to mating and during mating, gestation, and lactation. The progeny of the F_0 generation was designated as the F_1 generation and groups of 30 male and 30 female F_1 animals were exposed to the same concentrations of MCB as the F_0 parents. Exposure of F_1 animals was initiated 1 week postweaning and lasted 11 weeks prior to mating and through mating, gestation, and lactation. All F_2 pups were observed through weaning at which time they were killed. Observations made during the study included body weights, food consumption, mating and fertility indices, pup and litter survival indices, and histopathology of selected tissues. No mortality was observed during the course of this study. Body weights and food consumption for all treated groups were comparable to controls during the growth period. Maternal body weight data during gestation and lactation were also comparable between the control and treated groups. Mating and fertility indices for males and females for both generations appeared unaffected by treatment. Pup and litter survival indices for all treated groups were comparable to those of controls. Hepatocellular hypertrophy and renal changes (tubular dilation with eosinophilic material, interstitial nephritis, and foci of regenerative epithelium) were observed among F_0 and F_1 male rats exposed to 150 and 450 ppm MCB. The incidence of bilateral degeneration of the testicular germinal epithelium was increased among F_0 adults in the high-concentration group and this lesion was observed only unilaterally in the mid- and high-concentration group F_1 adults. The relationship of these testicular changes to exposure to MCB is unclear because there did not appear to be any increase in intensity and/or incidence of testicular lesions among F_1 adults that had longer exposure. In addition, overall mean mating and fertility indices for all groups were comparable for both generations. In summary, exposure of rats to MCB at levels of 50, 150, or 450 ppm did not have any adverse effects on reproductive performance or fertility of male and female rats.

Monochlorobenzene (MCB) is used as a solvent and an intermediate in the chemical industry. Under Section 4 of the Toxic Substances Control Act, in 1980 the EPA proposed a test rule for chlorobenzenes which included reproductive effects testing for MCB. In order to satisfy this need, the industry has voluntarily conducted a two-generation reproduction study with MCB. The results of this study are reported below. The reproduction study was designed to assess the long-term effects of MCB through two generations of rats and to determine whether the test substance produces abnormalities in parental activities from mating through lactation, during pregnancy, or during growth and

[1] This study was sponsored by the Chlorobenzenes Program Panel (CPP) under the auspices of the Chemical Manufacturers Association. Members of CPP include the Monsanto Co., PPG Industries, Inc., and Standard Chlorine Chemical Co., Inc.

0272-0590/87 $3.00

development of offspring from conception through maturity. The inhalation route was selected because it is anticipated to be one of the primary routes of potential human exposure.

The dose levels for this study were selected on the basis of the following previously reported toxicity studies with MCB. Mated Fischer 344 rats exposed to MCB vapor at 590 ppm daily for 6 hr per day on Days 6 through 15 of gestation exhibited a slight weight loss during the first 2 days of exposure and showed a significant increase in absolute and relative liver weights at study termination. No teratogenic effects were observed up to the highest level tested. 590 ppm (John *et al.*, 1984). In a subchronic inhalation study, rats, rabbits, and guinea pigs were exposed to 200, 475, or 1000 ppm MCB in the air, 7 hr/day, 5 days/week for a total of 32 exposures. Histopathological changes were observed in the liver and kidneys of all species at 475 and 1000 ppm (AIHA, 1964). Sullivan *et al.* (1983) investigated the excretion of monochlorobenzene following exposure to 100, 300, and 700 ppm of MCB for an 8-hr period. The total amount of MCB excreted (expired air and urine) did not increase disproportionately between 100 and 400 ppm, but the total excretion was increased more than twofold when concentration increased from 400 to 700 ppm. These data show that metabolic clearance of MCB from blood becomes saturated at exposure concentrations of 400 ppm for 8 hr. Sullivan's data also indicated a change in metabolic profile when exposure concentration increased from 400 to 700 ppm.

On the basis of the above data, a concentration of 450 ppm was selected as the high-exposure level because it was expected to produce liver and kidney changes. In addition, the metabolic profile at this concentration would be similar to that expected for the lower concentration generally experienced in the workplace. This concentration is also six times the current TLV (registered trademark of the American Conference of Governmental Industrial Hygienists). The middle and low concentrations selected were 150 and 50 ppm, respectively.

METHODS

Test material. MCB is a colorless liquid which has a boiling point of 132.1°C. The test material was supplied by Monsanto Company and gas–liquid chromatography analyses of all drums provided to the testing laboratory revealed a purity of 99.9%.

Animals and housing conditions. Male (129–233 g) and female (131–162 g) Sprague–Dawley-derived rats (CD, registered trademark of Charles River Breeding Laboratories, Portage, MI) were utilized for this study. Animals were 6 weeks of age on the first day of exposure and were acclimated to the housing conditions within the animal quarters (temperature 65–77°F, relative humidity 40–70%, 12-hr light/dark cycle) for at least 13 days prior to initiation of exposure to the test material. Rodent Laboratory Chow/5002 (registered trademark of Ralston Purina Company, St. Louis, MO) and water were available *ad libitum* during the nonexposure period. Water was also available in the exposure chamber during lactation. All animals were individually housed in suspended stainless steel cages with wire mesh floors except during lactation. On Day 20 of gestation, each female's cage was fitted with a stainless steel floor pan and bedding (hardwood shavings) was provided. The stainless steel floor pans were removed on Day 14 of lactation. Fresh bedding was provided on an as-needed basis.

Exposure chamber and operating conditions. For exposure to the test atmosphere of MCB, animal cages were placed in 6-m^3 stainless steel and glass inhalation chambers. The chambers were operated dynamically at an air flow rate of at least 2140 liters/min. This flow rate was calculated to provide one complete air change every 2.8 min. For generating an atmosphere of MCB vapor, the test material was fed into an atomizing nozzle via an FMI fluid metering pump. The vaporized test material was diluted with preconditioned air prior to entry into the exposure chamber. For determining analytical concentration of MCB, a MIRAN 1A organic vapor analyzer was used. At least four samples were drawn daily at hourly intervals from each exposure chamber. A daily nominal concentration was determined by dividing the difference in weight of the generation apparatus and test material before and after exposure by the total volume of air delivered during the day.

Experimental design. Four groups of 30 male and 30 female rats, designated as the F_0 generation, were exposed to MCB vapor (exposure chamber and operating conditions given above) for 6 hr/day, 7 days/week at target concentrations of 0, 50, 150, and 450 ppm MCB for a period of approximately 10 weeks prior to mating. For mating, one male rat was cohoused with one female rat within the same treatment group at night until evidence of mating was observed or until 10 nights of cohabitation elapsed. If mating had not occurred after 10 days, the

female was housed nightly with a different male within the same treatment group (random selection) for a second 10-day interval. The presence of copulatory plug and/or the presence of sperm in the vaginal smear was considered to be sufficient evidence that a particular female had mated. The day on which such evidence was observed was designated as Day 0 of gestation. Once the female had mated, she was housed individually for the remainder of the gestation period. Exposure of females and males continued through gestation and lactation. Dams were not exposed from Day 20 of gestation to Day 4 of lactation (day of parturition was considered Day 0 of lactation) in order to reduce the stress to dams at parturition and to pups resulting from physical removal of the mother for a period of 6 hr early in lactation. Except for this break, exposure of F_0 adults continued daily until termination.

The progeny of the F_0 generation were designated as the F_1 generation. After F_1 pups were weaned, all F_0 animals were killed. Some F_1 weanlings were used as parents for the succeeding generation. The selection procedure of F_1 pups to become the F_1 adult generation (30 animals/sex) was conducted to maximize representation from the number of litters available. The pups selected for the F_1 parental generation received the same concentrations of MCB as the F_0 parents. Their MCB exposure was initiated 1 week after weaning and was continued for at least 11 weeks prior to mating. Extreme care was taken during F_1 mating to avoid sibling matings. Exposure of the F_1 adults to the test material was continued through mating, gestation, and lactation phases with a break in treatment for the dams late in gestation and early lactation similar to that of the F_0 dams. F_1 adults were killed after the weaning of the F_2 pups. All F_2 pups were killed at weaning (Day 21 of lactation) and necropsied.

All adult and weanling animals were observed for mortality and clinical signs of toxicity twice daily. Detailed physical examinations were performed weekly. Male body weights were recorded weekly; female body weights were recorded weekly prior to mating, on Days 0, 4, 14, and 20 of gestation, on Days 0, 4, 7, 14, and 21 of lactation, and weekly again following lactation. Food consumption was measured weekly during the growth period. Litters were examined twice daily for general appearance of the pups and for dead pups. The number of pups in each litter and pup sex distribution were recorded at birth (Day 0) and Days 4, 7, 14, and 21 of lactation. On Day 4 of lactation all litters with greater than eight pups were culled to that number and the sex distribution *within litters* was equalized when possible. Individual pup weights were recorded on Days 0, 4, 7, 14, and 21 of lactation.

Complete gross postmortem examinations were conducted on all F_0 and F_1 parents, all F_1 weanlings not selected to become parents of the F_2 generation, and all F_2 weanlings. Liver and brain weights of F_0 and F_1 adults were recorded. Liver, kidneys, pituitary gland, and reproductive organs (males—testes, epididymides, seminal vesicle, and prostate; females—vagina, uterus, and ovaries) were examined microscopically for all F_0 and F_1 adult animals in the control and high-dose groups. Liver, kidneys, and testes of male rats in the low and mid-concentration groups were examined histologically, also. For histological evaluation, all tissues were fixed in 10% formalin. Following fixation, the tissues were dehydrated, defatted, and embedded in paraffin. A 6-μm section of each tissue (for paired organs one section from the left and right organ was taken) was stained with hematoxylin and eosin and evaluated by a pathologist on a scale of 1 to 5, where 1 is considered to be minimal (≤1% of tissue on slide affected), 2 is mild (2–10% of tissue on slide affected), 3 is moderate (11–30% of tissue on slide affected), 4 is moderately severe (31–60% of tissue on slide affected), and 5 is severe (>60% of tissue on slide affected).

The mating index for males (ratio of number of males for which mating was confirmed in at least one female to total number of males) and females (ratio of number of females showing evidence of mating to total number of females), pregnancy rate (ratio of number pregnant to number mated), and fertility index for males (ratio of number of males impregnating a female to number mating) were calculated for each of the two matings. Pup survival indices (ratio of number of pups alive at a given interval to number of pups alive at the previous interval) at various intervals during lactation were also calculated.

Statistical analyses Mean body weights, food consumption, organ weights, organ to body weight ratios, gestation lengths, and numbers of offspring were evaluated statistically using the following methods. A Bartlett's test (Snedecor and Cochran, 1972) was performed to determine if groups had equal variances. If variances were equal ($p > 0.01$), parametric procedures were used to test significance; if not ($p \leq 0.01$), nonparametric procedures were used. Parametric methods utilized included one-way analysis of variance (Snedecor and Cochran, 1972) which was followed by a Dunnett test (Dunnett, 1955, 1964) if a significant F-value was obtained. The Kruskal–Wallis test (Hollander and Wolfe, 1973) was the nonparametric method employed for determining equality of treatment effects, and if significant differences were obtained, Dunn's summed rank test (Hollander and Wolfe, 1973) was used to determine which treatments differed from controls. The nonparametric test for determining monotonic trend was the Jonckheere's test (Hollander and Wolfe, 1973) and the standard linear regression was utilized for the parametric case. Pup viability indices and pup survival indices were analyzed with the litter as the experimental unit and these data were transformed using the arc sine transformation (Snedecor and Cochran, 1972).

Incidence data, which included pregnancy rates, fertility indices, mating indices, and litter survival indices, were analyzed using contingency tables. First, a standard chi-square analysis (Snedecor and Cochran, 1972) was performed to determine if the proportion of incidences

differed between the groups tested. Next, each treatment group was compared to the control group using a 2 × 2 Fisher exact test (Bradley, 1968), and the significance level was corrected using the Bonferroni inequality (Miller, 1966). Also, an Armitage test (Armitage, 1955) for linear trend was performed.

RESULTS

Cumulative mean (± standard deviation) analytical concentrations for the low-, mid-, and high-concentration levels were 51 (±5), 151 (±8), and 451 (±25) ppm, respectively, for the F_0 generation and 49 (±4), 150 (±11), and 454 (±21) ppm, respectively, for the F_1 generation. These concentrations are comparable to the target concentration of 50, 150, and 450 ppm. The nominal concentration tended to be approximately 10% lower than the analytical concentration.

No mortality was observed in the treated and control groups during each of the F_0 and F_1 adult generations. Mean body weights during the growth period for males and females in the F_0 and F_1 generations were comparable for all groups. Food consumption for all groups was also comparable during the two generations.

Reproductive indices for the F_0 and F_1 adult generations are depicted in Table 1. Mating and fertility indices for males and females for both generations appeared unaffected by treatment. The mean (± standard deviation) number of days for each male and female to mate for the control, low-, mid-, and high-concentration groups in the F_0 generation was 4.2 ± 4.6, 3.1 ± 2.8, 2.8 ± 2.0, and 3.1 ± 2.9, respectively. In the F_1 generation, the mean (± standard deviation) number of days for mating to occur was 4.9 ± 4.2, 3.2 ± 2.3, 3.1 ± 2.4, and 4.6 ± 4.1 for the control, low-, mid-, and high-concentration groups, respectively.

Mean litter and pup survival indices for the F_1 and F_2 litters are shown in Table 2. In the F_1 litters, pup and litter survival for all treated groups was comparable to that of controls. In the F_2 litters, a slight decrease in pup survival index (Days 0–4) was observed in the high-concentration level only. The majority of the decrease in pup survival index was attributed to two dams; one dam lost 12 of 15 pups during lactation Days 0–4, and the other dam lost all 10 pups during the same time period. If this had been a compound-related response, more affected litters would have been expected. Therefore, pup survival indices for control and treated animals for both litters were considered comparable.

Table 3 shows the absolute and relative (to body weight) liver weights for the F_0 and F_1 generations. Significant increases in mean absolute or relative liver weight were observed in the F_0 and F_1 generations in the 150 and 450 ppm groups. The mean relative liver weight for males (F_1 generation) in the 50 ppm group was also significantly elevated when compared to that of the control group.

At necropsy, an increase in the incidence of small flaccid testes and dilated renal pelvis was observed in the high-dose group for both F_0 and F_1 adults. The incidence of small flaccid testes among F_0 adults was 0, 1, and 3 in the low-, mid-, and high-concentration groups, respectively, while in the F_1 adults the incidence for this observation was 0, 1, and 5 for the 50, 150, and 450 ppm, respectively. The incidence of dilated renal pelvis appeared to be elevated in F_0 high-dose males and all treated groups among F_1 adults when compared to that of controls (Table 4). Among F_1 adults, the increase in incidence was not dose related; therefore the relationship of this observation to treatment is considered to be equivocal.

Microscopic changes related to treatment were observed in the liver and kidney. Hepatocellular hypertrophy was observed in male rats. The incidence of this observation for F_0 adults was 0, 0, 5, and 14 in the control, low-, mid-, and high-concentration groups, respectively, while for F_1 adults the incidence was 2, 0, 3, and 7 in the 0, 50, 150, and 450 ppm groups, respectively. All affected male rats showed only minimal to mild hepatocellular hypertrophy. Only one high-concentration-

TABLE 1

A TWO-GENERATION REPRODUCTION SUDY IN RATS WITH MONOCHLOROBENZENE VAPOR: MATING, PREGNANCY, AND FERTILITY RATES

Group (ppm)	Mating: Females (No. mated[a]/total) No.	Mating: Females (No. mated[a]/total) Percentage	Mating: Males (No. mated[b]/total) No.	Mating: Males (No. mated[b]/total) Percentage	Pregnancy, females (No. pregnant/No. mated) No.	Pregnancy, females (No. pregnant/No. mated) Percentage	Fertility, males (No. impregnating[c]/No. mated) No.	Fertility, males (No. impregnating[c]/No. mated) Percentage
				F_0 mating (for F_1 litters)				
0	30/30	100.0	26/30	86.7	27/30	90.0	24/26	92.3
50	30/30	100.0	28/30	93.3	30/30	100.0	28/28	100.0
150	29/30	96.7	29/30	96.7	27/29	93.1	27/29	93.1
450	30/30	100.0	29/30	96.7	26/30	86.7	26/29	89.7
				F_1 mating (for F_2 litters)				
0	30/30	100.0	27/30	90.0	24/30	80.0	21/27	77.8
50	29/30	96.7	29/30	96.7	29/29[d]	100.0	29/29	100.0
150	29/30	96.7	29/30	96.7	23/29	79.3	23/29	79.3
450	28/30	93.3	25/30	83.3	25/28	89.3	22/25	88.0

[a] Number of females showing evidence of mating (plug and/or sperm and/or pregnancy).
[b] Number of males for which mating was confirmed in at least one female.
[c] Number of males mated with at least one female for which parturition was evident.
[d] Statistically significant at $p \leq 0.05$.

level female rat in the F_0 generation showed hepatocellular hypertrophy.

Renal degeneration and inflammatory lesions were limited to the male rats. The incidence of this observation is presented in Table 5. As depicted, the incidence and severity of renal changes were elevated in the mid- and high-concentration groups. These changes were considered to be related to exposure to MCB.

The incidence of unilateral or bilateral degeneration of the germinal epithelium of the testes was increased in the mid- and high-concentration groups in both the F_0 and F_1 generations (Table 6). However, there did not appear to be an increase in intensity and/or incidence of testicular lesions among F_1 animals that had longer exposure than F_0 animals as a result of their *in utero* exposure and potential exposure during lactation. Also, all animals in the high-concentration group in the F_0 generation showed bilateral lesions while all affected F_1 animals showed unilateral lesions only. In order to determine the effect of testicular lesions on reproductive performance, the reproductive performance of the animals that showed degeneration of the germinal epithelium was reviewed. These data revealed that the affected control animals in both the F_0 and F_1 generations were successful in siring litters, while in the mid-concentration group, all F_0 animals and two of three F_1 animals successfully sired litters. In the high-concentration group, three of six animals affected in each of the two generations were successful in siring litters.

DISCUSSION

This study was designed to evaluate the effects of monochlorobenzene vapor on the reproductive performance and fertility of rats through two consecutive generations. Target

TABLE 2

A TWO-GENERATION REPRODUCTION STUDY IN RATS WITH MONOCHLOROBENZENE VAPOR: PUP AND LITTER SURVIVAL INDICES

Group (ppm)	Pup viability index at birth (live/total born)[a]	Pup survival indices (days)		Litter survival index[d]	
		0–4[b]	4–21[c]	No.	Percentage
		F_1 litters			
0	96.9 ± 11.3[e]	92.4 ± 14.6	96.7 ± 10.2	27/27	100.0
50	96.1 ± 12.0	96.7 ± 8.3	95.0 ± 18.5	29/30	96.7
150	99.0 ± 2.4	94.1 ± 19.4	97.1 ± 10.2	26/27	96.3
450	96.4 ± 7.4	90.5 ± 20.5	93.5 ± 20.1	24/26	92.3
		F_2 litters			
0	98.1 ± 4.9	94.4 ± 10.9	88.4 ± 28.1	22/24	91.7
50	97.2 ± 5.6	93.2 ± 19.1	99.1 ± 3.3	28/29	96.6
150	97.9 ± 3.8	97.6 ± 5.7	89.1 ± 28.8	21/23	91.3
450	97.4 ± 4.9	87.7 ± 24.6	89.6 ± 28.7	22/25	88.0

[a] Total No. of live pups at Day 0/total No. of pups observed (live plus dead pups) at Day 0 for individual females.
[b] Total No. of live pups at Day 4 (precull)/total No. of live pups at Day 0 for individual females.
[c] Total No. of live pups at Day 21/total No. of live pups at Day 4 (postcull) for individual females.
[d] Total No. of litters with live pups at weaning (Day 21)/ total No. of litters with live pups at birth.
[e] Mean ± SD.
No statistically significant differences from control.

TABLE 3

MEAN LIVER WEIGHTS FOR F_0 AND F_1 GENERATIONS OF RATS EXPOSED TO MONOCHLOROBENZENE VAPOR

Dose level	Males		Females	
	Absolute (g)	Relative (g/100 g body wt)[a]	Absolute (g)	Relative (g/100 g body wt)[a]
		F_0 generation		
Control	19.25 ± 2.21[b]	3.61 ± 0.35	11.45 ± 1.29	3.77 ± 0.30
50 ppm	19.03 ± 3.12	3.60 ± 0.34	12.00 ± 1.26	3.89 ± 0.23
150 ppm	21.48 ± 2.33[c]	4.06 ± 0.30[c]	12.08 ± 1.13	3.95 ± 0.21[d]
450 ppm	21.84 ± 3.84[c]	4.12 ± 0.61[c]	13.27 ± 1.54[e]	4.41 ± 0.33[c]
		F_1 generation		
Control	18.31 ± 2.15	3.47 ± 0.32	12.36 ± 2.27	4.16 ± 0.60
50 ppm	19.46 ± 2.55	3.73 ± 0.36[d]	12.70 ± 1.58	4.17 ± 0.35
150 ppm	21.71 ± 3.49[c]	4.15 ± 0.46[e]	13.13 ± 1.55	4.36 ± 0.41
450 ppm	23.35 ± 4.09[c]	4.44 ± 0.40[e]	13.95 ± 1.99[e]	4.59 ± 0.37[e]

[a] Terminal body weight.
[b] Mean ± SD.
[c] Significantly different from control $p \leq 0.01$ using the nonparametric Dunn test.
[d] Significantly different from control $p \leq 0.05$ using the parametric Dunnett test.
[e] Significantly different from control $p \leq 0.01$ using the parametric Dunnett test.

TABLE 4

INCIDENCE OF DILATED RENAL PELVIS IN F_0 AND F_1 ADULTS

	Male (MCB, ppm)				Female (MCB, ppm)			
	0	50	150	450	0	50	150	450
F_0 adults	1	1	2	5	5	4	6	5
F_1 adults	1	4	6	4	0	1	2	2

exposure levels were 50, 150, and 450 ppm and the analytical measurements revealed that chamber concentrations were in agreement with the target exposure concentrations.

No adverse effect of treatment was evident on body weight, food consumption, physical observations, reproductive performance, or fertility in both F_0 and F_1 generations. Evaluation of pup delivery, nursing, and weaning data revealed no compound-related changes in either generation.

Absolute and/or relative liver weights were significantly elevated in the mid- and high-concentration groups for both F_0 and F_1 adults. In the low-concentration group, only the relative liver weights of the F_1 male rats were elevated. Histopathological examination of the liver revealed hepatocellular hypertrophy in the centrilobular region in males only in the mid- and high-concentration groups. Hepatocellular hypertrophy may be a reflection of the ability of monochlorobenzene to induce hepatic enzymes. In the absence of microscopic changes, the biological significance of changes in the liver weight of low-concentration-group males and mid- and high-concentration-group females is unclear. Exposure of rats to MCB vapor at 150 and 450 ppm also resulted in degenerative and inflammatory lesions in the kidneys. The incidence and severity of the microscopic changes in kidneys increased with dose and are considered to be treatment related. The observations of histological changes in the liver and kidney following exposure to MCB are not unexpected because these observations have been reported previously (Hol-

TABLE 5

INCIDENCE OF RENAL CHANGES AMONG MALE RATS INHALING MONOCHLOROBENZENE FOR TWO GENERATIONS[a]

	Group (ppm)							
	F_0 adults				F_1 adults			
	0	50	150	450	0	50	150	450
Total No. of animals examined	30	30	30	30	30	30	30	30
U/tubular dilation/eosinophilic material	0	3	2	3	4	4	6	6
B/tubular dilation/eosinophilic material	0	1	4	15	4	3	8	16
U/chronic interstitial nephritis	0	0	0	1	1	2	1	0
B/chronic interstitial nephritis	1	2	7	9	0	1	6	11
B/foci of regenerative epithelium	0	1	5	8	1	0	4	10
U/foci of regenerative epithelium	0	0	0	0	0	0	1	1

Note U/, unilateral B/, bilateral.

[a] These lesions were absent in female rats at the same concentrations.

TABLE 6

INCIDENCE AND SEVERITY OF UNILATERAL AND BILATERAL DEGENERATION OF GERMINAL EPITHELIUM AMONG MALE RATS EXPOSED TO VAPOR OF MONOCHLOROBENZENE

	Group (ppm)							
	F_0 adults				F_1 adults			
	0	50	150	450	0	50	150	450
Total examined	30[a]	30	30	30	30	30	30	30
Unilateral								
Minimal	1	—	—	—	—	—	—	1
Mild	—	—	1	—	—	—	1	—
Moderate	—	—	—	—	—	—	—	—
Moderately severe	—	—	—	—	—	—	1	5
Severe	—	—	1	—	—	—	1	—
Bilateral								
Minimal	—	—	—	3	1	—	—	—
Mild	—	—	—	—	—	—	—	—
Moderate	—	—	—	1	—	—	—	—
Moderately severe	—	—	—	—	—	—	—	—
Severe	—	—	—	2	—	—	—	—

[a] Two sections of testes evaluated histologically and severity of lesions graded from minimal to severe.

lingsworth *et al.*, 1956; AIHA, 1964; Dalich and Larson, 1985; NTP, 1985).

There was also an increase in the incidence of degenerative testicular changes (unilateral or bilateral) in high-dose males (F_0 and F_1 generations) and the F_1 mid-dose males. The relationship between testicular damage and exposure to MCB is unclear because: (i) As shown in Table 1, mean mating, pregnancy, and male fertility indices for both F_0 and F_1 generations were comparable for all groups. (ii) Overall, the number of males not siring litters in the control, low-, mid-, and high-concentration groups in the F_0 generation was 6, 2, 3, and 4, respectively, while in the F_1 generation the incidence was 9, 1, 7, and 8 for the control, low-, mid-, and high-concentration groups, respectively. Thus, the number of males not siring litters in the F_0 and F_1 generations was similar. However, if one examines the number of males not siring litters among animals with testicular lesions, three of six animals with testicular lesions in the high-dose group in each generation did not sire litters. (iii) The mean number of days for males and females to mate in each group was comparable for all groups in the two generations. While there was a dose-related increase in the incidence of degeneration of the germinal epithelium in both F_0 and F_1 adults, there was no increase in incidence and/or severity of lesions in the F_1 generation when compared to the F_0 generation. Since the length of exposure in the F_1 adult is longer as a result of the *in utero* exposure as well as the potential exposure during lactation, the F_1 adults should have been more severely affected. Moreover, previous subchronic studies in rats given MCB at equivalent or higher doses did not show any histologic testicular damage (Hollingsworth *et al.*, 1956; AIHA, 1964; NTP, 1985).

In summary, exposure to monochlorobenzene at levels of 50, 150, and 450 ppm did not have any adverse effects on reproductive performance or fertility of male and female rats through two consecutive generations.

ACKNOWLEDGMENTS

The technical support of Dr. Jim Terrill and of Cathy Houseman, Ellen Whiting, Gregory McGurr, and Joann B. Brink (all from Bio/dynamics, Inc.) is gratefully recog-

nized. We thank Pat Ploudre (Monsanto Company) for her efforts in typing this manuscript.

REFERENCES

American Industrial Hygiene Association (AIHA) (1964). Hygiene guide series: Chlorobenzene (monochlorobenzene, chlorobenzol, phenyl chloride). *Amer. Ind. Hyg. Assoc. J* **25**, 97–99.

ARMITAGE, P. (1955). Test for linear trends in proportions and frequencies. *Biometrics* **11**, 375–386.

BRADLEY, J. V. (1968). *Distribution-Free Statistical Tests*. pp. 195–203. Prentice-Hall, Englewood Cliffs, NJ.

DALICH, G. M., AND LARSON, R. E. (1985). Temporal and dose–response features of monochlorobenzene hepatotoxicity in rats. *Fundam. Appl. Toxicol.* **5**, 105–116.

DUNNETT, C. W. (1955). Multiple comparison procedure for comparing several treatments with a control. *J. Amer. Stat. Assoc.* **50**, 1096–1121.

DUNNETT, C. W. (1964). New tables for multiple comparisons with a control. *Biometrics* **20**, 482–491.

HOLLANDER, M., AND WOLFE, D. A. (1973). *Nonparametric Statistical Methods*, pp. 114–116 and 131. Wiley, New York.

HOLLINGSWORTH, R. L., ROWE, V. K., OYEN, F., MAJLE, H. R., AND SPENCER, H. C. (1956). *A. M. A. Arch. Ind. Health.* **14**, 138.

JOHN, J. A., HAYES, W. C., HANLEY, T. R., JR., JOHNSON, K. A., GUSHOW, T. S., AND RAO, K. S. (1984). Inhalation teratology study on monochlorobenzene in rats and rabbits. *Toxicol. Appl. Pharmacol.* **76**, 365–373.

MILLER, R. G., JR. (1966). *Simultaneous Statistical Inference*. p. 15. McGraw-Hill, New York.

National Toxicology Program (1985). *Toxicology and Carcinogenicity Studies of Chlorobenzene (CAS No. 108-90-7) in F344/N Rats and B6C3F1 Mice (Gavage Studies)*. Technical Report Series, No. 261.

SNEDECOR, G. W., AND COCHRAN, W. G. (1972). *Statistical Methods*, 6th ed., pp. 250–253, 277–279, and 296–298. Iowa State Univ. Press, Ames, IA.

SULLIVAN, T. M., BORN, G. S., CARLSON, G. P., AND KESSLER, W. V. (1983). The pharmacokinetics of inhaled chlorobenzene in the rat. *Toxicol. Appl. Pharmacol.* **71**, 194–203.

JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 11, Number 3, 1992
Mary Ann Liebert, Inc., Publishers

A Female Rat Fertility Study with Inhaled Benzene

ROBERT A. KUNA,[1] MARK J. NICOLICH,[2] RAYMOND E. SCHROEDER,[3] and GEORGE M. RUSCH[4]

ABSTRACT

Pure benzene was administered to female rats by inhalation at dose levels of 1, 10, 30, and 300 ppm for 6 h/day. The exposure took place during a 10-week premating period and during mating, gestation, and lactation periods. There was no effect on female reproductive performance. A trend toward-reduced body and organ weights was observed in the 21-day-old pups at the 30 and 300 ppm dose levels. Except for reduced body and liver weights in the female pups at 300 ppm, these differences were not statistically significant. Kidney to body weight ratios for female pups were statistically higher than control values in the 10, 30, and 300 ppm exposure levels. These organ weights and organ-to-body weight ratio changes appear to have been reflective of differences in body weights. No treatment-related effects were seen in pup survival during lactation or in the gross postmortem evaluation of these pups on Day 21 of lactation. Thus exposure of dams to levels as high as 300 ppm resulted in only minimal effects on the pups.

INTRODUCTION

THE POTENTIAL FOR REPRODUCTIVE risk due to benzene exposures is not a new concern of the occupational health professional. Hett and Maak (1938) noted gonadal abnormalities in both male and female rats following an undefined benzene exposure. This early research was later confirmed (Wolf et al., 1956) in males of two other species, rabbits and guinea pigs. Although much of the original reproductive data were equivocal, they did stimulate continued interest in and study of the potential effects of benzene on reproduction.

Kuna and Kapp (1981) noted fetotoxicity in the form of decreased body weights after exposure of dams to 50 ppm benzene during days 6 to 15 of gestation. Similar observations have also been recorded by Green et al. (1978), Hudak and Ungvary (1978), and Murray et al. (1979), however, they were all associated with much higher chamber concentrations of benzene.

The present occupational exposure limits for benzene are: American Council of Governmental Industrial Hygienists 10 ppm; and Occupational Safety and Health Administration 1 ppm with a short-term exposure limit of 5 ppm (*Guide to Occupational Exposure Limits*, 1991). The above data describing reproductive risk were derived from exposures which were markedly in excess of present exposure guidelines. Therefore, the current study was designed to evaluate exposure levels which are routinely encountered in many occupational settings as well as higher exposures which have previously been demonstrated as having a fetotoxic effect.

Study sponsored by U.S. manufacturers of benzene under the auspices of the Chemical Manufacturers Association and American Petroleum Institute (Ref. # BP 3.0).

[1]National Starch and Chemical Company, Bridgewater, NJ.
[2]Exxon Biomedical Sciences, Inc., West Amwell, NJ.
[3]Biodynamics, Inc., East Millstone, NJ.
[4]Allied Signal, Inc., Morristown, NJ.

MATERIALS AND METHODS

The benzene used was from American Petroleum Institute/Chemical Manufacturers' Association and was shown to be approximately 99.96% pure (ASTM D-1016).

Five groups of 26 female and 13 proven fertile male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used. Animals were housed two per cage during the first week and individually during week 2. In the premating treatment period they were individually caged during exposure and nonexposure periods. For mating, one male and two females were housed per cage. Females were housed individually during gestation, parturition, and with litter during lactation. All cages were stainless-steel wire mesh. Food (Purina Laboratory Chow) and water were provided *ad libitum*, except for exposure periods when females were without food. Water analyses were performed during the premating treatment period and near termination of the study. Assays were performed for benzene, pesticides, metals, and PCBs. All analyses were done according to proposed or approved Environmental Protection Agency methodologies (Federal Register 44:69464). Animal rooms were maintained on a 12-h light/dark cycle, and temperature and humidity were monitored. The two latter parameters were also recorded in exposure chambers.

Benzene vapor concentrations (0, 1, 10, 30, and 300 ppm) were generated in 1 m^3 stainless steel and glass chambers using a flash-evaporation generating system. In this system the benzene was pumped into a heated flask (50°C for 1, 10, and 30 ppm exposures, and 80°C for the 300 ppm exposure), flash-evaporated, and the vapor mixed with the chamber air supply. The three-neck flask was heated using a Glas-Col heating mantle and temperature was measured using a Glas-Col laboratory pyrometer. Benzene delivery was accomplished using a Sage instrument Model 220 and 341 syringe pump. Modifications in chamber concentration were made by adjusting pump flow rates or chamber air flow. Air flow was determined by measuring the change in pressure across a one-inch orifice plate using a Dwyer magnehelic pressure gauge. Air flows were maintained between 150 and 250 L/min for the duration of the exposures.

Chamber atmospheric concentrations were monitored using a Wilks Instrument Co., Miran Long Pathlength Infrared Model 1A, or an on-line Tracor Gas Chromatograph Model 550. The exposure levels were determined by comparing the observed absorbance of these samples to a previously determined standard curve generated using the test material under the same instrumental settings. The gas chromatograph was used because of interference in the infrared monitoring procedure (pathlength 21.75 m) for the 10 and 1 ppm exposure groups.

Chamber exposures for all study group females were conducted 6 h/day, 5 days/week during a 10-week premating and mating period, and daily from days 0 to 20 of gestation and days 5 to 20 of lactation.

To determine if estrous was affected by treatment, daily vaginal smears were made and evaluated for each female beginning 2 weeks prior to initiation of mating.

During the mating period, two females were caged with the same male nightly. Females were then examined each morning for evidence of mating (vaginal plug and/or sperm in the vaginal smear). The day on which such evidence was observed was defined as Day 0 of gestation. The male was initially caged for 8 days with the females or until both were mated. Once mated, females were housed individually for the duration of gestation. If a female did not mate, she was placed with a different male for an additional 7-day interval which was followed, if necessary, with yet another 7-day interval with a third male.

Observations of females for mortality and gross clinical signs were made twice daily. Detailed physical examinations were performed weekly throughout the study. Body weights were recorded once weekly through completion of the mating period. Mated females were weighed on Days 0, 7, 14, and 21 of gestation and on Days 0, 4, 14, and 21 of lactation.

Pups were counted, weighed, and sexed on Days 0, 4, 14, and 21 of lactation. Litters were observed twice daily. On Day 4 of lactation, litters of more than 10 pups were randomly culled to 10 with an equal number per gender where possible. Pups that died were weighed and sexed by internal examination.

All dams were given a gross postmortem examination. Method of sacrifice was by overdose of ether. Abnormal tissues noted during these evaluations were saved in 10% neutral buffered formalin. Uteri were examined for the presence and number of implantation sites, and along with ovaries, were saved in 10% neutral buffered formalin solution.

Gross postmortem examinations, including internal gender determinations were performed on all pups sacrificed by ether overdose on Day 21 of lactation and pups found dead during lactation. The latter were also checked for the presence or absence of milk in the stomach. Liver, kidney, and in males, testes weights were recorded for each pup.

Thirty-three organs and tissues along with any abnormal lesions were saved from two pups per sex per litter in 10% buffered formalin for possible future histopathological examination. Pups found dead prior to Day 4 of lactation were preserved in 70% ethanol.

Statistical evaluation of dam body weights and body weight changes during the treatment period, as well as gestation length and the number of pups per litter was by a standard one-way analysis of variance. Data were first tested with Bartlett's test for equality of variance.

The pregnancy percentage, viability index, and lactation index were tested for equality using a Chi-square test. If this test indicated statistical differences, each treated group was compared with the control group using Fisher's Exact test. This was done only after correcting the alpha (Type I probability) level for multiple tests via the Bonferrone correction (Miller 1966).

Pup body weights at Days 0, 4, 14, and 21, organ weights and organ-body weight ratios at Day 21 were tested using a nested design. Pups are taken as nested within dams, and dams nested within treatment groups. The significance of the difference between groups is determined by the ratio of dose group mean square error divided by the dam within group mean square error. If the ratio indicated a statistically significant difference in means, individual group mean differences were determined by the least significant difference (two sample *t*-test with the pooled variance) technique (Snedecor and Cochran, 1967).

RESULTS

Mean chamber benzene concentrations and ranges, as determined analytically, are presented in Table 1. Body weights of dams were comparable between the control and treated groups during the treatment period. Likewise, mean body weight gain during this same interval was comparable between these same groups, and no inequality of variance was indicated (Table 2). The type and incidence of in vivo observations noted during weekly physical evaluations were within the limits of that normally observed in the strain of animal used in this investigation.

Parturition and lactation data are presented in Table 3. These data reflect a statistically significant decrease in the lactation index at the 10 ppm level, which was not seen in either the 30 or 300 ppm groups and was considered not to be biologically significant.

Mean pup body weights on Days 0, 4, and 14 of lactation did not show a significant difference from corresponding control values for either gender. Pup body and organ weight values are presented in Tables 4 and 5. The only changes of statistical significance in these data were the decreases observed at Day 21 in female body and liver weights associated with the 300 ppm exposure level. Mean organ to body weight ratios (Table 6) showed nonsignificant decreases in male and female pup liver weights, and a significant increase in female pup kidney weight at the 10, 30, and 300 ppm exposure levels.

Gross necropsy observations of benzene-exposed dams and pups as compared with control dams and pups did not reveal any treatment-related effects. Likewise, evaluation of gross pathology findings in dead pups recovered during the study or pups culled from litters at Day 4 did not reveal effects attributed to treatment.

DISCUSSION

Exposure of adult female Sprague-Dawley rats to benzene in a one-generation reproduction study produced no evidence of toxicity, body weight, and/or altered reproductive performance. However, Kuna and Kapp (1981),

TABLE 1. MEAN LEVELS AND RANGES OF BENZENE IN TEST CHAMBER ATMOSPHERE

Reproductive Phase	*Exposure Levels (ppm)*			
	1.0	*10*	*30*	*300*
Premating	1.0 (0.3–3.5)	9.8 (7.4–12.7)	30.3 (26.3–35.4)	299 (273–334)
Mating	0.7 (0.0–1.4)	9.8 (8.0–12.1)	30.9 (29.6–33.0)	300 (287–319)
Gestation	0.8 (0.2–1.4)	10.9 (8.0–14.1)	30.9 (29.6–33.0)	296 (260–319)
Lactation	1.2 (0.2–1.4)	12.7 (9.5–15.2)	30.7 (29.0–32.4)	295 (260–337)

TABLE 2. MEAN MATERNAL BODY WEIGHTS AND BODY WEIGHT GAIN (g) DURING GESTATION AND LACTATION

Group/ ppm	Gestation (Days) 0	7	14	21	Change in Body Weight 0–21	Lactation (Days) 0	4	14	21	Change in Body Weight 0–21
I/O	253 ± 20[a]	273 ± 22	300 ± 23	375 ± 32	122 ± 23	284 ± 25	289 ± 23	311 ± 24	322 ± 23	39 ± 13
N	22	22	22	22	22	21[b]	21[b]	21	21	21
II/1	255 ± 21	273 ± 24	301 ± 26	376 ± 32	121 ± 20	282 ± 24	291 ± 22	319 ± 23	320 ± 22	38 ± 15
N	22	22	22	22	22	22	22	22	22	22
III/10	261 ± 19	282 ± 20	311 ± 23	388 ± 30	126 ± 21	288 ± 24	298 ± 26	325 ± 23	325 ± 24	37 ± 19
N	24	24	24	24	24	24	24	24	24	24
IV/30	254 ± 21	273 ± 23	301 ± 25	384 ± 30	130 ± 18	289 ± 23	294 ± 25	318 ± 26	328 ± 25	39 ± 12
N	19	19	19	19	19	19	19	19	19	19
V/300	254 ± 28	273 ± 28	301 ± 29	377 ± 39	126 ± 22	289 ± 34	296 ± 31	316 ± 28	330 ± 35	41 ± 18
N	23	23	23	23	23	23	22	22	22	22

[a]Mean ± standard deviation.

[b]One dam initiated delivery, pup in vaginal canal; however no litter found next day. No data presented for this animal.

N = number of observations.

TABLE 3. PARTURITION AND LACTATION DATA

Observation	Group I	II	III	IV	V
Percentage pregnant	88.5	96.2	92.3	84.0	88.5
No. pregnant[a]/no. mated	23/26	25/26	24/26	21/25	23/26
No. of litters	21	22	24	19	23
Mean gestation length (days)	21.7 ± 0.1	21.8 ± 0.1	21.9 ± 0.1	21.7 ± 0.1	21.6 ± 0.1
Mean no. pups/litter	11.7 ± 0.6	11.8 ± 0.6	12.6 ± 0.6	11.8 ± 0.7	12.0 ± 0.6
Gender ratio (M/F)	123/120	137/117	148/145	101/122	144/117
Mean no. UFC[b]	1.3	1.0	0.8	1.1	1.5
Viability index (%)	97.1	98.8	99.0	99.5[c]	96.9
No. pups alive at 4 days post-cull/No. pups born alive	236/243	251/254	290/293	217/218	253/261
Lactation index (%)	99.0	100.0	94.3[c]	99.4	98.0
No. pups alive at 21 days/No. pups alive at 4 days-postcull	195/197	206/206	216/229	177/178	198/202

[a] Number pregnant = number of females with either litters, uterine implants, or scars.

[b] Unaccounted-for-concepti, derived by subtracting total number of pups at birth from number of implant scars.

[c] Significantly different from control, $p < 0.05$.

Green et al. (1977), and Hudak and Ungvary (1978) have all observed decreases in dam body weights after comparable exposures to benzene during the period of gestation. One explanation for this difference is that the above body weight decreases were seen at doses in excess of what was reported in this paper. When using a chronic exposure of 300 ppm, Snyder et al. (1978) only observed rat body weight decreases after 30 weeks. Therefore, the benzene exposure concentrations used in this study were neither high enough nor of long enough duration to cause a response of maternal weight loss.

Investigators (Green et al., 1978, Hudak and Ungvary, 1978, Kuna and Kapp, 1981; Murray et al., 1979) studying the teratogenicity of benzene have all observed decreased fetal body weights at Day 20 of gestation with exposure levels to 2200 ppm. In this study, a similar fetal body weight response was observed in new born birth weights, 300 ppm group. Although male and female pup weights in the 300 ppm group were lower than control pup weights at all observation times, statistical significance could only be established for 21-day-old female pups. Male and female pups in the 30 ppm exposure group tended to have lower body weights at Days 14 and 21 of lactation. The above pup body weight changes from this investigation and historical references were observed at different time intervals. This is most probably due to the difference in benzene exposure schedules to the dams, but does not diminish the similarity and/or consistency of biological response. The data indicates that poor pup weight gain in male and female rat pups appears to be an effect of benzene exposure to adult rats.

In conjunction with the reported body weight decreases in this study there was also a statistically significant reduction in female pup liver weights in the 300 ppm group at Day 21 of lactation. Nonsignificant decreases in liver weights and liver to body weight ratios were seen in male pups in the 30 and 300 ppm exposure levels. Kidney to body weight ratios were statistically higher than control values in the 10, 30, and 300 ppm exposure levels for female pups and non-statistically higher in male pups. Although no other long-term benzene reproduction studies are available for comparison of the data, these differences in liver and kidney to body weight ratios may simply reflect differences in body weights rather than a direct response to benzene exposure.

In conclusion, exposure of female rats to 1, 10, 30, and 300 ppm benzene from premating through lactation did not produce any treatment-related effects in the dams. However, pup body and liver weight decreases were noted at the termination of the study in the 30 and 300 ppm groups along with increased kidney to body weight ratios in the 10, 30, and 300 ppm exposure groups for male (not statistically significant) and female pups. The significance of these changes is unknown at this time.

TABLE 4. MALE PUP BODY WEIGHTS (g) AND ORGAN WEIGHT (g) DATA

Group/ ppm	Day 0 Body Wt.	Day 4 Postcull Body Wt.	Day 14 Body Wt.	Day 21 Body Wt.	Organ weights		
					Testes	Liver	Kidneys
I/0	6.27 ± 0.66*	9.69 ± 1.49	23.29 ± 3.26	37.56 ± 5.73	0.16 ± 0.04	1.71 ± 0.39	0.44 ± 0.08
N	123	102	101	101	101	101	101
II/1	6.40 ± 0.55	10.03 ± 1.31	23.60 ± 3.15	38.24 ± 7.20	0.18 ± 0.05	1.71 ± 0.44	0.47 ± 0.10
N	137	111	111	111	111	110	111
III/10	6.36 ± 0.63	9.99 ± 1.74	23.32 ± 4.09	37.92 ± 7.68	0.17 ± 0.04	1.69 ± 0.47	0.47 ± 0.10
N	148	112	110	108	108	108	108
IV/30	6.24 ± 0.64	9.80 ± 1.65	21.82 ± 3.68	33.92 ± 6.22	0.15 ± 0.04	1.51 ± 0.41	0.42 ± 0.09
N	101	80	79	79	79	79	78
V/300	6.05 ± 0.56	9.49 ± 1.49	21.94 ± 3.01	34.14 ± 5.75	0.15 ± 0.03	1.45 ± 0.35	0.42 ± 0.09
N	144	102	101	101	101	101	101

*Mean ± standard deviation.

N = number of observations.

TABLE 5. FEMALE PUP BODY WEIGHTS (g) AND ORGAN WEIGHT (g) DATA

Group/ ppm	Day 0 Body Wt.	Day 4 Postcull Body Wt.	Day 14 Body Wt.	Day 21 Body Wt.	Organ Weights	
					Liver	Kidney
I/0	5.99 ± 0.54[a]	9.20 ± 1.28	22.20 ± 3.03	36.3 ± 5.20	1.69 ± 0.36	0.44 ± 0.08
N	120	95	94	94	94	94
II/1	5.95 ± 0.48	9.25 ± 1.34	21.94 ± 2.87	35.45 ± 5.89	1.59 ± 0.41	0.44 ± 0.08
N	117	95	95	95	95	94
III/10	6.11 ± 0.61	9.73 ± 1.58	22.80 ± 3.97	37.71 ± 7.14	1.78 ± 0.43	0.49 ± 0.10
N	145	117	112	108	108	108
IV/30	6.01 ± 0.62	9.43 ± 1.50	21.77 ± 3.42	33.68 ± 5.40	1.56 ± 0.33	0.45 ± 0.08
N	122	98	98	98	98	98
V 300	5.79 ± 0.51	9.18 ± 1.47	20.85 ± 2.95	32.59 ± 5.05[a]	1.46 ± 0.34[b]	0.44 ± 0.08
N	117	99	98	97	97	96

[a]Mean ± standard deviation.

[b]Significantly different from control group, $p < 0.05$.

N = number of observations.

TABLE 6. MEAN ORGAN/DAY 21 BODY WEIGHT RATIOS × 10-2

Group/ ppm	Males			Females	
	Testes	Liver	Kidney	Liver	Kidney
I/0	0.426 ± 0.068[a]	4.493 ± 0.513	1.177 ± 0.108	4.626 ± 0.546	1.223 ± 0.103
N	101	101	101	94	94
II/1	0.470 ± 0.145	4.438 ± 0.479	1.226 ± 0.145	4.438 ± 0.674	1.256 ± 0.095
N	111	110	111	95	94
III/10	0.443 ± 0.059	4.435 ± 0.665	1.241 ± 0.125	4.700 ± 0.492	1.295 ± 0.103[b]
N	108	108	108	108	108
IV/30	0.442 ± 0.078	4.380 ± 0.536	1.239 ± 0.093	4.601 ± 0.507	1.330 ± 0.115[b]
N	79	79	78	98	98
V/300	0.446 ± 0.060	4.216 ± 0.470	1.229 ± 0.142	4.442 ± 0.469	1.333 ± 0.136[b]
N	101	101	101	97	96

[a]Mean ± standard deviation.

[b]Significantly different from control, $p < 0.01$.

N = number of observations.

REFERENCES

GREEN, J. D., LEONG, B. K., and LASKIN, S. (1978) Fetotoxicity of inhaled benzene in rats. Toxicol. Appl. Pharmacol. 46:9–18.

Guide to Occupational Exposure Values—1991. 2nd ed. American Conference of Government and Industrial Hygienists.

HETT, J. and MAAK, H. (1938) Benzol and Keimdrusen. Klinische Wochenschr. 17:1376.

HUDAK, A. and UNGVARY, G. (1978). Embryotoxic effects of benzene and its methyl derivatives: toluene, xylene. Toxicology 11:55–63.

KUNA, R. A. and KAPP, R.W. (1981). The embryotoxic/teratogenic potential of benzene vapor in rats. Toxicol. Appl. Pharmacol. 57:1–7.

MILLER, R. J. (1966) *Simultaneous Statistical Inference*. McGraw Hill. New York.

MURRAY, F.J., JOHN, J.A., RAMPY, L.W., KUNA, R.A., and SCHWETZ, B.A. (1979). Embryo toxicity of inhaled benzene in mice and rabbits. Am Ind. Hygiene Assoc. J. 40:993–998.

SNEDECOR, G.W and COCHRAN, W.G. (1967). *Statistical Methods*, 6th Ed. Iowa State University Press. Ames IA.

SNYDER, C.A., GOLDSTEIN, B.D., SELLAKUMAN, A., WOLMAN, S., BROMBERG, I., ERLICKMAN, M.N., AND LASKIN, S. (1978). Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm. J. Toxicol. Environ Health 4:605–618.

WOLF, M.A., ROWE, V.K., MCCOLLISTER, D.D., HOLLINGSWORTH, R.L., and OYEN, F. (1956). Toxicological studies of certain alkylated benzenes and benzene. Arch. Ind. Health 14:387–398.

Address reprint requests to:
R. A. Kuna
National Starch & Chem Co.
Finderne Ave.
Bridgewater, NJ 08807

TERATOLOGY EVALUATION OF METHYL TERTIARY BUTYL ETHER IN RATS AND MICE

C. Clifford Conaway

Texaco Inc., Beacon Research Center, Beacon, New York

Raymond E. Schroeder

Bio/dynamics Inc., East Millstone, New Jersey

Neil K. Snyder

American Petroleum Institute, Washington, D.C.

Mated CD Sprague-Dawley rats and CD-1 mice were exposed during the period of organogenesis to target concentrations of 0, 250, 1000, and 2500 ppm methyl t-butyl ether (MTBE). None of the control or test-group animals died during the treatment or posttreatment periods. Females were sacrificed on d 20 (rats) or d 18 (mice). No adverse effects of treatment were reflected in maternal parameters of body weight, water consumption, or liver weight or in physical examination data for either species. Food consumption fell in the groups of treated rats during d 9–12; similar but nonsignificant effects were observed for mice during d 12–15.

In rats, no treatment-related changes were recorded in the uterine implantation data, fetal size parameters, or fetal sex distribution data. Examination of fetuses for external abnormalities, skeletal malformations, or ossification variations did not reveal any changes caused by MTBE exposure.

A slight increase in fetal resorptions was observed in the groups of mice exposed to low and high concentrations. This increase was attributed to two females in each group that had an unusually high number of resorptions, rather than to the treatment itself. No significant effects were observed in any groups of treated mice on external and soft-tissue examination or evaluation of skeletal abnormalities or ossification variations. The incidence of fused sternebrae in the high-concentration group increased slightly, which might be attributed to fetotoxicity.

This study was conducted under the auspices of the American Petroleum Institute for the following sponsor companies: Arco Chemical Company, Exxon Corporation, Chemische Werke Huls AG, Petrolex Chemical Corporation, Phillips Petroleum Company, Shell Oil Company, and Texaco Chemical Company.

The authors acknowledge the technical assistance of Maureen Yourneff, Kathryn Sloan, and Karen Van Skiver (Reproduction Teratology Section) and of Roger Ben-Dyke (Inhalation Toxicology Section) of Bio/dynamics, Inc.

Present address for C. Clifford Conaway is Post Office Box 12842, Arlington, Virginia 22209.

Present address for Neil K. Snyder is Atlantic Richfield Co., Los Angeles, California 90051.

Requests for reprints should be sent to C. E. Holdsworth, American Petroleum Institute, 1220 L Street, N.W., Washington, D.C. 20005.

Journal of Toxicology and Environmental Health, 16:797–809, 1985

INTRODUCTION

Methyl *t*-butyl ether (MTBE) has been approved by the U.S. Environmental Protection Agency as an octane booster in motor fuel at concentrations as high as 11% (*Federal Register*, 1979, 1981). Human exposure may occur during the processes of manufacture, distribution, and blending of MTBE; the greatest potential for exposure, however, is from the inhalation of gasoline vapor containing MTBE at service stations.

MTBE has a characteristic odor but a low degree of acute toxicity. It is slightly irritating to the eyes and mucous membranes and minimally irritating to intact skin (Reynolds et al., 1974). The 4-h LC50 for MTBE in Sprague-Dawley rats is approximately 35,000 ppm. When 3000 ppm MTBE was administered 6 h/d, 5 d/wk for 9 d, signs of anesthesia—i.e., a lack of response to stimuli—were occasionally observed. In addition, nasal irritation and liver enlargement were observed when the animals were sacrificed (American Petroleum Institute, 1984). Irritation, congestion, and localized inflammation of the nasal cavity and respiratory tract were also reported when Sprague-Dawley rats were exposed to 1000 ppm MTBE for 13 wk; signs of light anesthesia occurred during the periods of exposure (Huls, 1980). MTBE has been tested for toxic effects at concentrations of 10 or 15% (v/v) in high-octane premium gasoline. Addition of MTBE did not increase acute toxicity of motor gasoline, but it did lengthen the barbiturate-induced sleep time, reduce spontaneous motor activity, and cause slight disturbances in motor coordination (Snamprogette S.P.A., 1972).

Methyl *t*-butyl ether was not mutagenic using the Ames *Salmonella* microsome assay; it did not induce significant sister chromatid exchanges or chromosomal aberrations in Chinese hamster ovary cells in vitro. Although MTBE was not mutagenic in the L5178Y mouse lymphoma assay without microsomal activation, it did induce significant mutations in the presence of a microsomal S9 fraction. It was surmised that a metabolite, possibly formaldehyde, was the mutagenic agent, rather than MTBE itself (Arco Chemical Company, 1980).

The present study was undertaken to determine the potential embryotoxic or teratogenic effects of MTBE in Sprague-Dawley rats and CD-1 mice.

METHODS

Mated Sprague-Dawley-derived rats (CD), Charles River Breeding Laboratories, Inc., Kingston, N.Y.), 57 d old and weighing approximately 203 g at d 0 of pregnancy, were randomly distributed into 4 groups of 25 each. Pregnancy was confirmed by the presence of sperm in a vaginal smear or a copulatory plug. Each mated female was identified with a metal ear tag and housed individually in a stainless-steel,

suspended wire mesh cage, with tap water provided. Certified Purina laboratory chow (5002) was provided ad libitum, except during chamber exposures when the food and water were removed. Artificial light was cycled 12 h on, 12 h off. The temperature was maintained at 65–75°F, with relative humidity ranging between 43 and 71%. Male rats used for breeding were selected from an in-house colony. Mating was effected by overnight cohabitation with two females per male.

Mated female strain CD-1 mice, 72 d old at the initiation of mating and weighing approximately 26 g, were randomly distributed into 4 groups of 30. The mating and husbandry procedures were identical to those used with the rats, except that the temperature fluctuated between 67 and 76°F, with a relative humidity of 35–72%. Pregnancy was confirmed by a copulatory plug.

The methyl *t*-butyl ether (lots 0-28496 and AN-1993) was provided by the American Petroleum Institute. The purity of the MTBE was determined to be 95.03–98.93% using gas chromatography, with less than 0.80% *t*-butyl alcohol, 0.43% methanol, and 0.25% diisobutylene as contaminants.

The MTBE was transported via Teflon tubes to FMI fluid metering pumps (model PP6-20), which delivered the test material directly to air atomizing nozzles (¼ in., JSS Spraying Systems) mounted on the inlet portals of 10-m^3 stainless-steel and glass chambers. The chambers were operated at an airflow rate of 3000 l/min, with 1 complete air change every 3.3 min (t_{99} = 15.3 min). Exposures were conducted 6 h/d during d 6–15 of gestation for both species, with target concentrations of 0 (group I), 250 (group II), 1000 (group III), and 2500 (group IV) ppm. Exposure concentrations of MTBE were monitored every 30 min using a Miran 1-A infrared analyzer and a calibration curve. Nominal concentrations for each exposure chamber were calculated daily. The aerosol content was determined at least once per week using a Royco portable particle monitor (model 218).

Maternal body weights were recorded for both species on d 0, 6, 12, 15, and 18 and on d 20 for rats. Detailed physical examinations for signs of toxicity were performed at the same time that weights were recorded, after exposures, during d 6–15. Food and water consumption were recorded for the following intervals of gestation: d 6–9, d 9–12, d 12–15, and d 15–18 for both rats and mice, and d 18–20 for rats. Dams were sacrificed on d 20 (rats) or d 18 (mice) by CO_2 inhalation. Laparotomies were performed, and dams and pups were examined carefully for gross abnormalities. Each fetus was weighed, the crown–rump distance was recorded, and the sex of each rat fetus was determined from the anogenital distance. Mice were sexed internally at evisceration or during the soft-tissue examination. Late and early resorptions were scored. When no uterine implantation sites were observed, the uterus was stained with a 10% solution of ammonium sulfide (Salewski, 1964) to visualize the foci of implantation. One-third of the fe-

tuses in each litter were preserved intact in Bouin's solution and processed for the evaluation of soft tissue using Wilson's (1965) free-hand razor-blade sectioning technique. The remaining two-thirds of the fetuses were eviscerated, fixed, cleared, and stained with Alizarin red S for skeletal examination (Crary, 1962).

The equality of means was evaluated statistically using a one-way analysis of variance (ANOVA) technique, followed by a multiple-comparison procedure if necessary. Bartlett's test (Snedecor and Cochran, 1967) was performed to determine if groups had equal degrees of variance. If the variances were equal, parametric procedures were used; if not, nonparametric procedures were utilized. A one-way ANOVA, using the F distribution to assess the significance level (Gill, 1978), was followed by Dunnett's (1955) test if significant differences among the means were indicated. If nonparametric methods were required, the Kruskal–Wallis test was used. If significant differences were indicated, Dunn's summed rank test was performed to determine which treatment groups differed from the control group (Hollander and Wolfe, 1973). Methods for analyzing trends were also utilized, i.e., regression analysis or Jonckheere's test for monotonic trend (Hollander and Wolfe, 1973).

Incidence data for treatment versus control groups—i.e., pregnancy rates, the percentage of fetuses with malformations, the percentage of litters containing malformed fetuses, and ossification variations—were analyzed using standard chi-square analysis (Snedecor and Cochran, 1967). This analysis was followed by a 2 × 2 Fisher exact test (Bradley, 1968), using the Bonferroni inequality correction (Miller, 1966) to insure the stated significance level. Armitage's (1955) test was performed to determine if there was a linear dosage–response relationship.

RESULTS

Experimental Exposures: Rats and Mice

The cumulative mean chamber concentrations ± SD for rat exposures as determined by infrared (Miran) were 0, 250 ± 5, 1000 ± 12, and 2430 ± 91 ppm, for group I (0 ppm), group II (250 ppm), group III (1000 ppm), and group IV (2500 ppm), respectively. Corresponding mean nominal concentrations were 0, 260 ± 10, 1100 ± 41, and 3000 ± 127 ppm. For mice, the Miran cumulative mean concentrations were 0, 280 ± 26, 1110 ± 119, and 2710 ± 255 ppm, respectively; corresponding cumulative mean nominal concentrations were 0, 250 ± 19, 1000 ± 94, and 3500 ± 153 ppm, respectively, for group I, group II, group III, and group IV.

A leak was discovered in the sampling manifold late in the study and may have caused an underestimation of the chamber concentrations from Miran data by as much as 10%. The possibility of error was further indicated by higher nominal concentration data. No correction factor, however, was applied to the Miran data to account for the leak. A substantial amount of aerosol was measured in the mid- and high-concentration chambers; the particle diameter was determined to be less than 1–2 μm for a substantial proportion of the aerosol, which is in the respirable range. The presence of aerosol would also explain some of the discrepancies observed between the mean analytical and mean nominal concentrations.

Experimental Results—Rats

Maternal changes. All dams survived the treatment and observation period. The mean body weight and the mean weight gain during the pretreatment (d 0–6), treatment (d 6–15), and posttreatment (d 15–20) intervals were comparable in the control and treatment groups. The mean food consumption data were unremarkable, except that during the d 9–12 interval there was a significant reduction in mean food consumption in all three treatment groups. Although there was considerable variability in water consumption and a possible dose-related increase during d 18–20, no concentration-related changes in water consumption were observed during the period of MTBE exposure.

No treatment-related effects were noted during the evaluations of the live test animals. Upon sacrifice, the mean liver weights, both absolute and relative to the d 20 body weight, were not significantly affected. No gross postmortem effects attributed to the exposure were recorded. The pregnancy rates for the control and treatment groups were comparable.

The mean numbers of corpora lutea, uterine implantations, resorptions, and live fetuses (Table 1) were not significantly different in the treatment and control groups. Complete resorption of litters occurred in 1 low-concentration-group female and 1 mid-concentration-group female, while 1 control-group female had an in utero litter consisting of 12 resorption sites and a single live fetus. Only 1 dead fetus was recovered during the d 20 sacrifices; it was from the litter of a low-concentration-group female.

Fetal morphologic changes. The mean fetal weights and crown–rump distances were statistically comparable in all groups. A preponderance of male fetuses (58%) in the mid-concentration group was attributed to biological variability rather than to a treatment effect. External malformations noted in the control and treated fetuses are presented in Table 2. The incidence of external malformations was

TABLE 1. Corpora Lutea and Uterine Implantation, Rats[a]

Treatment group (ppm)	Number of pregnant females	Mean per pregnant females ± SD		Resorptions		Live fetuses	
		Corpora lutea	Implants	Mean number ± SD	Mean percent of implants ± SD	Mean number ± SD	Mean percent of implants ± SD
I (0)	25	14.8 ± 1.3	13.8 ± 1.3	1.4 ± 2.5	10.6 ± 19.6	12.4 ± 3.0	89.4 ± 19.6
II (250)	23	15.1 ± 2.1	13.9 ± 1.5	1.5 ± 2.5	11.3 ± 20.5	12.4 ± 3.2	88.4 ± 20.4
III (1000)	25	14.0 ± 2.4	12.7 ± 1.8	0.9 ± 2.6	7.2 ± 19.8	11.8 ± 3.1	92.8 ± 19.8
IV (2500)	24	14.3 ± 2.2	13.0 ± 2.1	1.2 ± 1.6	10.0 ± 14.4	11.8 ± 3.1	90.0 ± 14.4

[a] No statistically significant differences from the control group.

comparable in the control and treatment groups. Pale fetuses (3.6% in the control group, 0.7% in the low-concentration group, 0.3% in the mid-concentration group, and 1.4% in the high-concentration group) were not regarded as biologically significant.

The incidence of soft-tissue malformations, on both a per fetus and

TABLE 2. Summary of Fetal External Malformations, Rats[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I (0)	Absence of cranium and multiple cranio/facial defects	1/309	0.3	1/25	4.0
	Total	1/309	0.3	1/25	4.0
II (250)	No malformations	0/256	—	0/22	—
III (1000)	Exencephaly with open eye, protuding tongue, and fleshy outgrowth above snout	1/295	0.3	1/24	4.2
	Total	1/295	0.3	1/24	4.2
IV (2500)	Darkened area on tail	1/284	0.4	1/24	4.2
	Edematous with domed cranium and eye defects	1/284	0.4	1/24	4.2
	Total	2/284	0.7	2/24	8.3

[a] No statistically significant differences from the control group.
[b] Number of fetuses with the malformation/number of fetuses examined per group.
[c] Number of litters containing fetuses with a malformation/number of litters evaluated.

a per litter basis, was not statistically different in the control and treatment groups (Table 3).

Fetal skeletal abnormalities. The incidence of skeletal malformations, on both a per fetus and a per litter basis, were statistically comparable in the control and treatment groups (Table 4). A cervical rib and reduced cranial ossification were found in the one fetus with a cranial/facial malformation. In the mid-concentration group, reduced cranial ossification was recorded for the fetus with exencephaly, and facial malformations were noted upon external examination. In the high-concentration group, fused ribs and fused cervical vertebral transverse processes were observed in the edematous fetus.

Experimental Results—Mice

Maternal changes. A slight increase in the incidence of lacrimation was seen among females during the treatment period. The food consumption data were not remarkable except for a slight nonsignificant, dose-related decrease in mean food consumption during d 12–15. A slight, dose-related decrease in mean water consumption, nonsignificant, was observed during the d 9–12 gestation interval. No mortality occurred among the treated females that were exposed to MTBE. One low-concentration-group female died on d 0 of gestation, and an addi-

TABLE 3. Fetal Soft-Tissue Malformations, Rats[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I	Renal pelvis distended, ureter				
(0)	distended, or both	4/102	4.0	3/25	2.0
	Edematous (subcutaneous)—thoracic and abdominal region	1/102	1.0	1/25	4.0
	Total	5/102	4.9	4/25	16.0
II					
(250)	Ureter distended	1/95	1.1	1/22	4.5
	Portion of right cerebral hemisphere outside cranial cavity (between cranium and skin layer)	1/95	1.1	1/22	4.5
	Total	2/95	2.1	2/22	9.1
III	Ureter distended	1/96	1.0	1/24	4.2
(1000)	Total	1/96	1.0	1/24	4.2
IV	Renal pelvis and ureter distended, or				
(2500)	both	3/93	3.2	2/24	8.3
	Total	3/93	3.2	2/24	8.3

[a] No statistically significant differences from the control group.
[b] Number of fetuses with the malformation/number of fetuses examined per group.
[c] Number of litters containing fetuses with the malformation/number of litters evaluated.

TABLE 4. Fetal Skeletal Malformations, Rats[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I	Cervical rib and reduced cranial				
(0)	ossification:	1/207	0.5	1/25	4.0
	Total	1/207	0.5	1/25	4.0
II	Wavy ribs (bilateral)	1/191	0.5	1/22	4.5
(250)	Total	1/191	0.5	1/22	4.5
III	Reduced cranial ossifications	1/199	0.5	1/24	4.2
(1000)	Total	1/199	0.5	1/24	4.2
IV					
(2500)	Angulated rib (unilateral)	1/191	0.5	1/24	4.2
	Fused ribs (bilateral) and fused cervical vertebral transverse process	1/191	0.5	1/24	4.2
	Total	2/191	1.0	2/24	8.3

[a] No statistically significant differences from the control group.
[b] Number of fetuses with the malformation/number of fetuses examined per group.
[c] Number of litters containing fetuses with a malformation/number of litters evaluated.

tional mated female was added to the group to provide a total of 30 mated females.

The mean body weight and the mean body weight gain for d 0–6, d 6–15, and d 15–18 were comparable in the control and treatment groups. Mean liver weight and liver weight as a proportion of the corrected d-18 body weight were not affected by MTBE exposures. No treatment-related effects were recorded during the gross postmortem examination of the mice.

The implantation data for the control and treatment groups (Table 5) were not significantly different. The mean number of resorption sites and the mean percentage of resorptions/implants were slightly higher in the low- and high-concentration groups than in the control group, a difference that could be attributed to a high number of resorptions in two litters in each of these treatment groups. In the low-concentration group, 2 females had 10/11 (90.9%) and 7/9 (77.8%) resorptions/implants, respectively. In the high-concentration group, two females had litters comprised entirely of resorptions. Excluding the data for females in the low- and high-concentration groups with a large number of resorption sites, resorption data for these groups were similar to those for the control group. The mean number of live fetuses per litter was comparable in the control and treatment groups.

Fetal morphologic changes. There were no significant differences in the mean fetal weights and crown–rump distances of either sex be-

TABLE 5. Uterine Implantation Data, Day 18 Gestation Sacrifices, Mice[a]

Treatment group (ppm)	Number of pregnant females[b]	Mean number of implantations ± SD	Resorptions		Live Fetuses		Total number dead fetuses
			Mean number ± SD	Percentage of implants ± SD	Mean number ± SD	Percentage of implants ± SD	
I (0)	26	11.5 ± 1.8	1.0 ± 1.2	9.0 ± 10.0	10.1 ± 2.0	90.4 ± 9.7	2
II (250)	28[c]	12.1 ± 1.7	1.9 ± 2.3	16.6 ± 21.9	10.2 ± 3.2	83.1 ± 22.0	1
III (1000)	24	11.2 ± 2.0	1.3 ± 1.1	11.0 ± 9.1	9.9 ± 2.0	88.2 ± 9.6	2
IV (2500)	29	11.0 ± 1.8	1.0 ± 2.1	17.1 ± 25.6	9.9 ± 3.3	82.1 ± 25.4	2

[a] No statistically significant differences from the control group.

[b] Excludes females that delivered prematurely (one control and one group III female).

[c] Includes two females whose uteri were stained with ammonium sulfide and in which foci were identified but that had no fetuses. Data for these females were not included in the calculations of mean uterine implantation data for this group.

TABLE 6. Summary of Fetal External Examination Data, Mice[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I	Cleft palate	1/281	0.4	1/27	3.7
(0)	Total	1/281	0.4	1/27	3.7
II	Spina bifida, filamentous				
(250)	tail, imperforate anus	1/266	0.4	1/26	3.8
	Total	1/266	0.4	1/26	3.8
III	Kinked tail	1/251	0.4	1/25	4.0
(1000)	Portion of tail dark purple	2/251	0.8	2/25	8.0
	Total	3/251	1.2	3/25	12.0
IV	Kinked tail	1/290	0.3	1/27	3.7
(2500)	Hindlimb flexure	1/290	0.3	1/27	3.7
	Portion of tail dark purple	1/290	0.3	1/27	3.7
	Total	3/290	1.0	3/27	11.1

[a] No statistically significant differences from the control group.

[b] Number of fetuses (delivered pups) with the malformation number of fetuses (delivered pups) examined per group.

[c] Number of litters containing fetuses (delivered pups) with a malformation number of litters evaluated.

tween the control and treatment groups. No treatment-related effects on the fetal sex distribution were reported. The incidence of malformations observed upon external examination (Table 6) was comparable in the control and treatment groups. Malformations recorded during the soft-tissue examinations and the number of fetuses and litters affected were also comparable in the control, low-, mid-, and high-concentration groups (Table 7).

Fetal skeletal abnormalities. The incidence of skeletal malformations in the mouse, on both a per fetus and a per litter basis, was comparable in the control, low-, and mid-concentration groups (Table 8). In the high-concentration group, the incidence of fetuses with skeletal malformations and of litters containing such fetuses increased, but the increase was not significant. Cleft palates, not noted during the external evaluations and suggested by irregularities in the ossification pattern of the palatine/maxillary bone, were observed in one control-group fetus and two high-concentration-group fetuses. Thus, the total incidence of cleft palate observed in the external, soft-tissue, and skeletal evaluations in this study was 0.7% (2/281), 0% (0/265), 0.4% (1/251), and 0.7% (2/290) for the control, low-, mid-, and high-concentration groups, respectively. A low incidence of sternebral abnormalities (i.e., fused sternebrae) was observed in the treatment groups, while none were observed in the control group.

TABLE 7. Fetal Soft-Tissue Malformations, Mice[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I					
(0)	Renal pelvis distended	2/91	2.2	2/27	7.4
	Brain tissue (cerebral hemisphere—left side) present between cranium and overlying skin layer	1/91	1.1	1/27	3.7
	Total	3/91	3.3	3/27	11.1
II					
(250)	No malformations	0/86	—	0/27	—
III	Eye—collection of blood between				
(1000)	lens and eyelid	1/83	1.2	1/25	4.0
	Cleft palate[d]	1/83	1.2	1/25	4.0
	Total	2/83	2.4	2/25	8.0
IV	Heart defect—aortic and				
(2500)	pulmonary arches exist as a common vessel	1/95	1.1	1/27	3.7
	Cerebral hemisphere—lateral ventricles distended	1/95	1.1	1/27	3.7
	Cleft palate[d]	1/95	1.1	1/27	3.7
	Total	3/95	3.2	3/27	11.1

[a] No statistically significant differences from the control group.

[b] Number of fetuses (delivered pups) with the malformation/number of fetuses (delivered pups) examined per group.

[c] Number of litters containing fetuses (delivered pups) with the malformation/number of litters evaluated.

[d] Not noted during the fetal external examination.

DISCUSSION

Although there is some indication that food consumption declined in both rats and mice during portions of the gestation period, this did not have any significant effect on the terminal maternal body weight, maternal weight gain, or fetal body weight in either species.

The mean number of resorptions per litter in the control group of Sprague-Dawley rats was 1.4, somewhat elevated from the laboratory historical mean of 0.7 but within the range of 0.3–1.8 recorded for 24 studies. The incidence of resorptions in control mice was within the normal range; mice typically resorb 10–30% of fetuses, but the percentage depends on the strain of mouse and the laboratory. The incidence of soft-tissue malformations and major skeletal abnormalities in rats and mice exposed to MTBE did not increase significantly. A slight dose-related increase in minor skeletal abnormalities in mice (Table 8) was not statistically significant. It was concluded that MTBE adminis-

TABLE 8. Fetal Skeletal Malformations, Mice[a]

Treatment group (ppm)	Malformation	Fetuses		Litters	
		Number[b]	%	Number[c]	%
I	Cleft palate[d]	2/190	1.1	1/27	3.7
(0)	Fused ribs (unilateral) and misaligned thoracic centra	1/190	0.5	1/27	3.7
	Total	3/190	1.6	2/27	7.4
II	Fused sternebrae	1/180	0.6	1/26	3.8
(250)	Scrambled sternebrae	1/180	0.6	1/26	3.8
	Fused ribs (unilateral) and vertebral defects:	1/180	0.6	1/26	3.8
	Total	3/180	1.7	3/26	11.5
III	Fused sternebrae	2/168	1.2	2/25	8.0
(1000)	Fused ribs (unilateral)	1/168	0.6	1/25	4.0
	Angulated ribs (unilateral)	1/168	0.6	1/25	4.0
	Total	4/168	2.4	4/25	16.0
IV	Cleft palate[e]	2/195	1.0	2/27	7.4
(2500)	Fused sternebrae	4/195	2.1	4/27	14.8
	Total	6/195	3.1	6/27	22.2

[a] No statistically significant differences from the control group.

[b] Number of fetuses (delivered pups) with the malformation/number of fetuses (delivered pups) examined per group.

[c] Number of litters containing fetuses with the malformation/number of litters evaluated.

[d] Observations of the palatine/maxillary ossifications suggest a cleft-palate malformation; one of these fetuses was noted during the external examination to have a cleft palate. The second fetus with malformations of the palatine/maxillary ossifications was not noted to have a cleft palate.

[e] Observation of the palatine/maxillary ossifications suggest a cleft-palate malformation; however, a palatal defect was not noted during the fetal external examination of these same fetuses.

tered by inhalation at the concentrations tested was not maternally toxic, embryotoxic, or teratogenic.

REFERENCES

American Petroleum Institute. 1984. A nine day inhalation study of MTBE in the rat. Submitted by Biodynamics Inc., East Millstone, N.J., to the American Petroleum Institute.

Arco Chemical Company. 1980. Methyl tertiary butyl ether: Acute toxicological studies. Arco Chemical Co., Newton Square, PA 19073.

Armitage, P. 1955. Tests for linear trends in proportions and frequencies. *Biometrics* 11:375–386.

Bradley, J. V. 1968. Fisher Exact Test. In *Distribution Free Statistical Tests*. pp. 195–203. Englewood Cliffs, N.J., Prentice-Hall.

Chemische Werke Huls AG (Huls). 1980. Methyl tertiary butyl ether (Inveron): Three month inhalation toxicity in rats. Submitted by Inveresk Research International, Edinburgh, Scotland to Chemische Werke Huls AG.

Crary, D. D. 1962. Modified benzyl alcohol clearing of alizarin-stained specimens without loss of flexibility. *Stain Technol.* 37:124–125.

Dunnett, C. W. 1955. A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Soc.* 50:1096–1121.

Federal Register. 1979. 44:12242–12249 (March 6).
Federal Register. 1981. 46:38582–38586 (July 28).
Gill, J. L. 1978. *Design and Analysis of Experiments in the Animal and Medical Sciences*. Ames: Iowa State University Press.
Hollander, M., and Wolfe, D. A. 1973. Chapter 6 In *Nonparametric Statistical Methods*. New York: Wiley.
Miller, R. G., Jr. 1966. *Simultaneous Statistical Inference*. New York: McGraw-Hill.
Reynolds, R. W., Smith, J. S., and Steinmetz, I. 1974. Methyl ethers as motor fuel components. Presented at 168th National Meeting of Division of Petroleum Chemistry, American Chemical Society, Atlantic City, N.J.
Salewski, E. 1964. Farbemethode zum makroskopischen nachweis von implantations stellen am uterus der ratte. *Arch. Pathol. Exp. Pharmakol.* 247:367
Snamprogette S. P. A. 1972. Research on "solvent SB" (CISB), alone or as a component of various mixtures. Report submitted by the Instituto di Farmacologia della Facolta di Medicina e Chirurgia dell Universitita Cattolica del Sacro Cuore di Roma to Snamprogette S.P.A.
Snedecor, G. W., and Cochran, W. G. 1967. One Way Classifications. In *Statistical Methods*, 6th ed., Chapter 10. Ames: Iowa State University Press.
Wilson, J. G. 1965. Methods for administering agents and detecting malformations in experimental animals. In *Teratology: Principles and Techniques*, eds. J. G. Wilson and J. Workany, pp. 262–277. Chicago: University of Chicago Press.

Received January 28, 1985
Accepted March 14, 1985

METHYL TERTIARY BUTYL ETHER INHALATION IN RATS: A SINGLE GENERATION REPRODUCTION STUDY

ROBERT W. BILES,* RAYMOND E. SCHROEDER† AND CHARLES E. HOLDSWORTH‡

***Exxon Biomedical Sciences, Incorporated**
Exxon Research & Engineering Company
East Millstone, New Jersey

†Bio/dynamics Inc.
East Millstone, New Jersey

‡American Petroleum Institute
Washington, D.C.

Male rats exposed to target concentrations of methyl tertiary butyl ether (MtBE) at 300, 1300 and 3400 ppm for 6 hours/day, 5 days/week for 12 weeks were mated to female rats exposed to the same concentrations for a 3-week period. Exposures continued through the mating period and the females continued exposures during gestation and from days 5-21 lactation of the litters (F_{1a}) (no exposures days 0-4 lactation). A second litter (F_{1b}) was produced under the same mating and post mating exposure regimen. No adverse effect of treatment was observed with the adult animals (Fo) throughout the in-life portion of the study. The only remarkable finding was an increased incidence of dilated renal pelves in the low- and high-dose females (Fo). All gonad weights, male accessory reproductive organ weights, organ-to-body weight ratios and reproductive organ histopathology were unremarkable upon comparison of treated animals with air sham controls. The mating indices and fertility indices in exposed animals for both mating intervals (F_{1a} and F_{1b}) were not significantly different from controls. Pregnancy rates were comparable between treated and control females for the first litter interval (F_{1a}) but were slightly lower (not statistically significant) than control on the second litter inter-

1. Address all technical comments questions to Robert W. Biles, Exxon Biomedical Sciences, Inc., Exxon Research & Engineering Co., P.O. Box 235, East Millstone, NJ 08873. Address reprint requests to Charles E. Holdsworth, American Petroleum Institute, 1220 L Street, NW, Washington, DC 20005.

2. Key words: inhalation, methyl tertiary butyl ether, oxygenated hydrocarbons, reproduction study, single generation.

3. Abbreviations: CHO, Chinese hamster ovary, MtBE, methyl tertiary butyl ether

val (F_{1b}). Treated animal mean gestation length and the mean number of pups at birth were not statistically different from controls. The pup viability indices at birth were comparable for control and treated groups for the F_{1a} generation, but the mid- and high-dose groups displayed a slight statistically significant decrease in the F_{1b} generation; the decrease was not considered to be biologically significant and perhaps not treatment-related. Litter survival indices were comparable between control and treated groups for both litter intervals. Pups of mid- and high-dose females had slightly lower (not statistically significant) mean weights at days 14 and 21 of lactation but this was not considered treatment-related. The most frequent post-mortem observation for pups sacrificed at day 21 of lactation was dilated renal pelves. This did not appear to be related to treatment. It is concluded that MtBE inhalation in rats results in little adverse reproductive toxicity as shown in a two litter, one generation reproduction assay in rats.

INTRODUCTION

In the late 1970s, considerable attention was placed on the development and use of oxygenated hydrocarbons as either high octane motor fuels or as octane improvers. This research effort was primarily driven by the anticipation of an EPA-mandated decrease in lead antiknock usage (Reynolds et al., 1974). Methyl tertiary butyl ether (MtBE) emerged as one of the leading oxygenated compounds for use in gasoline blending. Currently, MtBE is used as an octane improver for motor gasoline, and the use has increased as leaded gasoline phasedown continues as promulgated by the EPA (Ember, 1984).

The primary route of exposure to workers is through inhalation which occurs during the production and transportation processes, as well as when MtBE is blended into gasoline. The primary source of potential exposure of the general public is from gasoline vapors from those gasolines blended using varying amounts of MtBE (Reynolds et al., 1974).

Animal studies have been used to assess the acute and subchronic toxicities of MtBE. As well, studies have been completed to assess the genotoxic and teratogenic potential of MtBE. The acute inhalation LC_{50} to rats has been reported to be $\approx$ 35,000 ppm-4 hour (Arco Chemical Company, 1980) and the acute rat oral LD_{50} has been reported to be $\approx$ 4 g/kg (API, 1980).

One subchronic inhalation study (Huls, 1980) conducted in rats for 6 hours/day, 5 days/week for 13 weeks produced minimal to no toxic effects through the dose range tested (250, 500 and 1000 ppm). Another subchronic inhalation study (API, 1984) (6

hours/day for 9 days) in rats, conducted essentially as a probe for the present study, produced a reduced response to auditory stimuli, increased absolute and relative liver weights and some minor deviations in clinical parameters at the highest exposure level of 3000 ppm. These findings of relatively low toxicity suggested the selection of the maximum exposure concentration for the current study in order to assure some toxicity in the parental generation.

Other toxicity findings relevant to the present study have been derived from short-term mutagenicity assays and a teratology study in rats and mice. MtBE was not mutagenic when tested in *Salmonella* and *Saccharomyces* or in tests for sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary (CHO) cells *in vitro* (API, 1980). MtBE was mutagenic in the L5178Y mouse lymphoma assay only in the presence of an S-9 metabolic activation system (API, 1980). An inhalation teratology study (Conaway et al., 1985) in rats and mice exposed to MtBE at nominal concentrations of 260, 1100 and 3300 ppm produced no treatment-related effects in the maternal rats or their offspring and only slight increases in fetal resorptions and fused sternebrae in mice.

The work reported here was conducted to evaluate whether exposure to MtBE would (1) affect the fertility, reproductive systems and performance of male and/or female rats, and (2) be detrimental to the development of the resulting offspring.

METHODS

Test Material. MtBE was provided through the American Petroleum Institute and was shown by GC analysis to be 95-99% pure. Other prominent materials identified in these analyses were (1) secondary-butyl methyl ether, $< 0.45\%$, (2) methanol, $< 0.43\%$, (3) tertiary-butyl alcohol, $< 0.79\%$ and (4) diisobutylene, $< 0.22\%$.

Inhalation Chamber Design, Vapor Generation and Analyses. The 10 cubic meter stainless steel and glass exposure chambers were operated dynamically at an airflow rate of 3000 liters per minute. The liquid was metered from reservoirs via Teflon tubing directly to air atomizing nozzles. The MtBE was atomized into the chamber air stream which, in turn, vaporized the test material. Target concentrations were 0 (control), 250, 1000 and 2500 parts per million MtBE (ppm v/v in air). The concentration of MtBE was determined in each chamber (including control) every 30 minutes during the exposure period with a MIRAN® I-A General Organic Vapor Analyzer. Backup analyses included daily checks of chamber concentrations and weekly verification of the MIRAN® using on-line gas chromatography.

Animals. Male and female CD® (Sprague-Dawley derived) rats, approximately four weeks old at receipt, were obtained from Charles River, Kingston, NY, and acclimated for 30 days. Animals were fed Purina Rat Chow (certified diet), and water was available *ad libitum*; however, neither were available during exposure periods. Hard-

wood shavings were placed in each female's cage on day 20 of gestation and fresh litter was provided as needed through day 14 of lactation. All animals (male and female) were sorted into treatment groups with a computerized program which also sorted among the groups to equalize mean body weights between the groups. The animals were housed individually in stainless steel mesh cages except during the mating period and during lactation. Temperatures were maintained between 62-72°F; relative humidity was kept between 30-70%; and there was a 12/12 hour light/dark cycle.

Experimental Design. Four exposure groups were established, each with 15 males and 30 females: Group I - 0 ppm (sham control), Group II - 250 ppm, Group III - 1000 ppm and Group IV - 2500 ppm. Exposures were conducted for 6 hours/day, 5 days/week during the premating interval, which was 12 weeks for males and 3 weeks for females. Two mating intervals were conducted, and in each interval two females were caged nightly with one male from the same treatment group. Once mated, females were removed to litter. After a five-day interval, all unmated females were randomly reassigned to a different mating unit and exposed to a male from the same treatment group for five additional days. Females unmated after 10 days of mating were randomly assigned to a different mating unit for a third five-day interval.

Males continued to be exposed 6 hours/day, 5 days/week during mating intervals and mating rest intervals, while mated females were exposed daily 7 days/week, 6 hours/day from day 0-20 of gestation. Females were not treated from day 21 of gestation to day 4 of lactation. On day 5 of lactation, daily exposure for the females was resumed for 6 hours/day until day 20 of lactation. However, the litters (F_{1a}) were not exposed.

The F_{1a} litters were weaned, and the Fo males and Fo females underwent a two-week mating rest period, after which a second mating period began in order to produce a second litter (F_{1b}) of animals. (Fo) males were sacrificed at the end of this mating period while (Fo) females underwent the same exposure regimen described for the first mating period. Fo females were sacrificed at the end of the (F_{1b}) weaning.

During the first and second mating periods, females that did not mate were treated 7 days/week, 6 hours/day until all F_{1a} (F_{1b}) litters were weaned. Females that mated but did not deliver a litter resumed daily treatment at completion of a 26-day postmating interval.

In-life observations for Fo males and females were made for mortality and gross signs of toxicologic and pharmacologic effects. Body weights were obtained initially and weekly thereafter for males, while females were weighed weekly until mated and then again on days 0, 7, 13 and 20 of gestation and days 0, 7, 14 and 21 of lactation.

At sacrifice, each Fo animal was examined grossly with particular attention given to the reproductive organs. The testes and epididymides from each male and ovaries from each female were evaluated microscopically.

The newborn pups from both litters were inspected and counted shortly after birth and weighed on days 0, 7, 14 and 21 of lactation. External sexing was confirmed by internal inspection of gonads at sacrifice. On day 4 of lactation, each litter with greater than 10 pups was culled to that number using a randomization technique. Pups found dead at parturition and during lactation were grossly examined both internally and externally for malformations. Pups were sacrificed on day 21 of lactation and given a gross postmortem examination. The gonads and abnormal tissue were saved for histopathological evaluation.

Statistical procedures employed for parametric data included Bartlett's test for equal variance and standard one-way ANOVA with F distribution (Snedecor and Cochran, 1967). If significant differences among means occurred, then Dunnett's test (Dunnett, 1955) was used to determine which treatment means were different from control. For nonparametric data, the Kruskal-Wallis test (Gill, 1978) for equality of means was used. If differences among means occurred, then Dunn's summed rank test (Hollander and Wolff, 1973) was used to determine which treatment means were different from the control. All statistical tests, except Bartlett's test, were conducted at the 0.05% and 0.01% two-sided risk level.

RESULTS

Fo-Adults. General. No control or treated animals died during the treatment or mating periods. Mean weekly body weights and mean weight gain for males during the pretreatment period, mating and post mating periods were comparable between all groups (treatment and control); no adverse effect of treatment was evident. Likewise, no treatment-related effect was evident in body weight data for the female animals during pretreatment and post mating periods. Gestation and lactation body weights were also comparable between control and treated groups for both litter intervals. The types and incidences of in-life observations were similar between

TABLE 1
Analysis of MtBE Exposure Concentrations

Chamber Concentration	Animals	Group I	II	III	IV
Target (ppm)	Male & Female	0	250	1000	2500
Actual[a] Exposure[a] (ppm)	Male &	0	290 ± 22	1180 ± 107	2860 ± 254
	Female	0	300 ± 7	1240 ± 37	2980 ± 132

[a]Cumulative mean ± SD.
[b]MIRAN®: a miran leak was discovered and corrected during male exposures. Actual final analytical exposure values are shown in above table.

control and treated animals; no adverse effect of treatment was noted. The most common types of in-life observations noted were nasal discharge, rales, alopecia and lacrimation.

Necropsy, Organ Weights and Histopathology. The only finding of note from the gross post-mortem examinations was an increased incidence of dilated renal pelves in females from Groups II (250 ppm) and IV (2500 ppm). The incidence of dilated renal pelves for females in the control and treated groups II, III and IV were 1/30, 4/30, 0/30 and 5/30.

Fo-Adults. General. No control or treated animals died during the treatment or mating periods. Mean weekly body weights and mean weight gain for males during the pretreatment period, mating and post mating periods were comparable between all groups (treatment and control); no adverse effect of treatment was evident. Likewise, no treatment-related effect was evident in body weight data for the female animals during pretreatment and post mating periods. Gestation and lactation body weights were also comparable between control and treated groups for both litter intervals. The types and incidences of in-life observations were similar between control and treated animals; no adverse effect of treatment was noted. The most common types of in-life observations noted were nasal discharge, rales, alopecia and lacrimation.

Necropsy, Organ Weights and Histopathology. The only finding of note from the gross post-mortem examinations was an increased incidence of dilated renal pelvis in females from Groups II (250 ppm) and IV (2500 ppm). The incidence of dilated renal pelves for females in the control and treated groups II, III and IV were 1/30, 4/30, 0/30 and 5/30.

No treatment-related effects were evident in adult gonad weight data. Organ to body weight ratio data for gonads from both adult male and females were not different between treated groups and controls. Mean ovary weight and mean ovary to body weight ratio for the Fo females were comparable between the control and treated groups (data not shown). In the Fo males, mean weight data (absolute and relative to terminal body weight) for the testes, epididymides, seminal vesicles and prostate were comparable between the control and treated groups (data not shown). Microscopic evaluations of the gonads of both male and female animals revealed no treatment-related effects.

Mating, Fertility and Pregnancy Indices. For both mating intervals (F_{1a} and F_{1b}), the mating indices for males and females were not statistically different between the control and treated groups (Table 2). The male fertility indices were also comparable between the control and treated groups for both litter intervals (Table 3). No adverse effect of treatment was evident in male fertility data. During the first litter interval (F_{1a}), pregnancy rates were comparable between the control, low- and high-dose groups and slightly lower (not statistically significant) than controls in the mid-dose (Group III) group (Table 3). In the second litter interval, pregnancy rates were slightly

TABLE 2
Mating Indices for Male and Female Rats Exposed to MtBE for Two Mating Intervals

Group [Target ( )]	F_{1a} Litter Interval				F_{1b} Litter Interval			
	Males[a]		Females[b]		Males		Females	
	No.[c]	%	No.[d]	%	No.	%	No.	%
I (0 ppm)	12	80.0	27	90.0	12	80.0	25	83.3
II (250 ppm)	14	93.3	30	100.0	14	93.3	29	96.7
III (1000 ppm)	14	93.3	27	90.0	14	93.3	29	96.7
IV (2500 ppm)	15	100.0	29	96.7	12	80.0	25	83.3

[a]N = 15.
[b]N = 30.
[c]Number of males mating with a female.
[d]Number of females mating.

TABLE 3
Male Fertility Indices and Pregnancy Rates for Rats Exposed to MtBe for Two Mating Intervals

Group [Target ( )]	F_{1a} Litter Interval				F_{1b} Litter Interval			
	Male Fertility Indices		Pregnancy Rate		Male Fertility Indices		Pregnancy Rate	
	No.[a]	%	No.[b]	%	No.	%	No.	%
I (0 ppm)	11/12	91.7	25/27	92.6	11/12	91.7	22/25	88.0
II (250 ppm)	14/14	100.0	28/30	93.3	13/14	92.9	23/29	79.3
III (1000 ppm)	13/14	92.9	20/27	74.1	13/14	92.9	23/29	79.3
IV (2500 ppm)	14/15	93.3	24/29	82.8	11/12	91.7	19/25	76.0

[a]Number of males impregnating a female/number of males mating a female.
[b]Number of pregnant females/number of mated females.

lower (not statistically significant) in each treated group. No clear dose relationship was evident in either litter interval. In the control group, two females did not mate with a male during either mating interval; all treated females mated at least once during these same intervals. During both mating periods, 28 control (93.3%), 28 low-dose (93.3%), 25 mid-dose (83.3%) and 26 high-dose females (86.7%) delivered at least one litter.

Gestation Length, Parturition Data and Litter Survival Indices. Mean gestation length data (range 22.1 to 22.3 days) were comparable between the control and treated groups for both litter intervals. The mean number of pups at birth (live, dead and total) was also comparable between control and treated groups during both litter intervals (Table 4). Pup viability indices at birth were comparable for control and treated groups for the first litter interval, but in the second litter interval, the mid- and high-dose groups displayed a slight, but significant, decrease in the pup variability index (Table 5). The pup viability indices seen in the mid- and high-dose groups during the second litter interval (F_{1b}) were similar to the pup viability index observed for the control group during the first litter interval (F_{1a}). Litter survival indices (ratio of litters weaned to litters with live pups at birth) were comparable between control and treated groups for both litter intervals (Table 5).

Pups. Mean pup body weights (distinguished by sex) throughout the lactation period for both litter intervals were comparable in the control and low-dose groups. The mean pup weights for the mid-and high-dose groups were comparable to controls at days 0 and 7 of lactation, but on days 14 and 21 of lactation they were slightly (but not statistically significant) lower than controls (Table 6). Pup survival indices during the day 0-4 and 4-21 lactation intervals are summarized for both litter intervals in Table 7.

During the first litter interval, pup survival indices for the day 0-4 period were comparable between the control and high-dose group and significantly lower than control in the low- and mid-dose groups. Pup survival indices for the day 4-21 period were comparable between the control and treated groups.

In the mid-dose group, the decrease in pup survival index during the day 0-4 interval was, in part, attributed to the loss of one entire litter containing 12 pups at birth; excluding data for this litter, the day 0-4 pup survival index for the mid-dose would be 94.0%.

In the second litter interval, pup survival indices during the day 0-4 interval were comparable between the control and each of the treated groups. Likewise, during the day 4-21 interval, pup survival indices were comparable between the control and treated groups (Table 7).

Pup sex distribution indices at day 0 (pre-cull) and day 21 (post-cull) were similar between the control and treated groups for both litter intervals (data not shown).

The most frequent post-mortem observation for pups sacrificed at day 21 of lactation was dilated renal pelves. The incidence of this observation was comparable in the

TABLE 4
Number of Litters and Parturition Data for Rats Exposed to MtBE for Two Mating Intervals

Group [Target ( )]	# of Litters	F_{1a} Litter Interval			# of Litters	F_{1b} Litter Interval		
		Mean # of Pups at Birth[a]				Mean # of Pups at Birth		
		Live	Dead	Total		Live	Dead	Total
I (0 ppm)	25	13.2	0.3	13.5	22	13.3	0.1	13.4
II (250 ppm)	28	12.4	0.3	12.7	23	12.8	0.3	13.1
III (1000 ppm)	20	11.5	0.7	12.2	23	12.1	0.6	12.7
IV (2500 ppm)	24	13.3	0.4	13.7	19	12.3	0.6	12.9

[a]Data collected at the interval litter examination.

TABLE 5
Pup Viability Indices and Litter Survival Indices for Two Litter Intervals

Group [Target ( )]	F_{1a} Litter Interval				F_{1b} Litter Interval			
	Pup Viability Indices		Litter Survival Indices		Pup Viability Indices		Litter Survival Indices	
	No.[a]	%	No.[b]	%	No.	%	No.	%
I (0 ppm)	330/338	97.6	25/25	100.0	292/295	99.0	22/22	100.0
II (250 ppm)	347/354	98.0	27/27	100.0	295/302	97.7	22/23	95.7
III (1000 ppm)	230/243	94.7	18/19	94.7	278/291*	95.5	23/23	100.0
IV (2500 ppm)	320/329	97.3	24/24	100.0	234/245*	95.5	19/19	100.0

[a]Total number of live pups at birth/total number of pups (live and dead) at birth.
[b]Number of females that wean litters (day 21)/number of females with live pups at birth (day 0).
*$P < .05$.

TABLE 6
Mean Pup Body Weight Data During Lactation—Both Litter Intervals[1]

Group [Target ( )]	Mean Pup Weight[a] (grams)							
	Lactation Days							
	0		7		14		21	
	Male	Female	Male	Female	Male	Female	Male	Female
	F_{1a} Litter Interval							
I (0 ppm)	6.2	5.9	13.2	13.0	23.6	23.0	36.2	35.1
II (250 ppm)	6.0	5.6	13.1	12.4	24.1	22.8	36.9	34.7
III (1000 ppm)	5.8	5.5	12.5	12.2	21.6	21.0	32.9	32.5
IV (2500 ppm)	6.0	5.6	12.8	12.6	22.4	21.9	33.8	33.1
	F_{1b} Litter Interval							
I (0 ppm)	6.0	5.7	13.2	12.5	23.8	22.7	36.9	35.1
II (250 ppm)	6.1	5.7	13.3	12.6	23.6	22.6	36.8	34.4
III (1000 ppm)	6.0	5.6	12.8	12.2	22.7	21.7	34.7	33.1
IV (2500 ppm)	6.1	5.7	13.1	12.5	22.8	22.1	34.9	33.6

[a]Litter as the experimetnal unit.

TABLE 7
Pup Survival Indices for Both Litter Intervals

Group [Target ( )]	Pup Survival Index			
	Lactation Days			
	0-4 (pre-cull)		4-21 (post-cull)	
	No.[a]	%	No.[b]	%
	F_{1a} Litter Interval			
I (0 ppm)	324/330	98.2	247/250	98.8
II (250 ppm)	317/347**	91.4	246/254	96.9
III (1000 ppm)	205/230**	89.1	162/165	98.2
IV (2500 ppm)	305/320	95.3	235/236	99.6
	F_{1b} Litter Interval			
I (0 ppm)	282/292	96.6	211/218	96.8
II (250 ppm)	275/295	93.2	210/213	98.6
III (1000 ppm)	273/278	98.2	214/215	99.5
IV (2500 ppm)	231/234	98.7	182/182**	100.0

[a]Number of pups on day 4 (pre-cull)/ number of live pups on day 0.
[b]Number of pups on day 21/ number of pups on day 4 (post-cull).
**$P < .01$.

control and high-dose groups for both litter intervals, but was slightly (not statistically significant) elevated in the low- and mid-dose groups for both litter intervals (data not shown).

Other observations noted for the weanling day 21 pups during the gross post-mortem examination occurred infrequently and did not appear to indicate a relationship with treatment.

DISCUSSION

The present study was designed as an evaluation of the potential reproductive system toxicity of methyl-t-butyl ether. No adverse effects of treatment were seen in either male or female adult rats exposed to MtBE for approximately 28 weeks (males) and 16 weeks (females) at the approximate concentrations of 300, 1200 and 3000 ppm. Although a 9 day probe study at 3000 ppm indicated some toxic potential (irritability, possible CNS effects) the rats at all doses in the present study gained weight commensurate with control animals and displayed no unusual toxicological signs. The type and incidence of in-life signs such as rales, lacrimation, nasal discharge and alopecia

were also similar to the controls. In gross post-mortem, the only finding of note was a nonsignificant increase in dilated renal pelves in females of the low- and high-dose groups. The effect was not considered to be treatment-related since it was apparently not dose-related.

Gonad weights and gross and histopathological observations of the gonads from both male and female rats exposed to MtBE were uniformaly consistent with control animals; no treatment-related effects were evident. Also, weights and histopathology results of organs of the male reproductive tract (testes, epididymides, seminal vesicles and prostate) were indistinguishable between control and treated animals. Consequently, MtBE displays little to no structural effect on the reproductive system of the adult rat.

With respect to reproductive function, no adverse effects of treatment were seen in male and female mating indices, male fertility indices, litter survival indices, litter size, gestation length, or sex ratio of offspring. No treatment-related effects with respect to pregnancy rates were evident during the first litter interval; however, pregnancy rates from the second litter interval were slightly, but not statistically significantly lower than the controls and no clear dose-response relationship was evident. No adverse effect on female fertility was indicated from this study. In summary, no significant effects on reproductive performance were observed in this study at concentrations of MtBE as high as 3000 ppm.

The effects of parental MtBE exposures were minimal to the offspring. The significant decrease in pup viability in mid and high dose groups of the F_{1b} generation is borderline with respect to biological significance. None of the pup viability indices of either generation are below approximately 95%. The outstanding viability (99%) of the F_{1b} control group contributes to the statistical significance seen in the F_{1b} mid and high dose groups. Pup survival indices during the day 0-4 lactation period were significantly ($P < .05$) lower than controls in the low- and mid-dose groups during the first litter interval. However, the F_{1a} high-dose group displayed no reduction in pup survival when compared to controls, and also no reduction in pup survival was seen in any treated group during the second litter interval. Moreover, pup survival indices for the day 4-21 lactation period (post-cull) were comparable between the control and treated groups for both litter intervals. Consequently, the reduced pup survival in the F_{1a} low- and mid-dose groups is not believed to be a treatment-related effect. Pups nursing to mid- and high-dose females had slightly lower body weights at days 14 and 21 of lactation for both litter intervals. The reduction in mean pup weights did not demonstrate a clear dose relationship. Moreover, the differences from controls were not statistically significant, and the mid- and high-dose groups weights were comparable on days 0 and 7 of lactation. Finally, gross external and internal examination of pups delivered and subsequently weaned to MtBE treated females did not reveal any adverse effects on any organ system due to treatment.

The current scientific literature is unexpectedly silent with respect to one or more

generation reproduction studies with aliphatic, aromatic or oxygenated aliphatic/aromatic hydrocarbons. Pesticides, drugs, and food additives, and certain chlorinated hydrocarbons make up the majority of these studies. One multi-generation dietary study of 1-3 butanediol in rats has been published which did report a decrease in pregnancy rates of F_{1a} rats and some effects in fetal growth; however no terata, dominant lethal or chromosomal aberrations were noted (Hess et al., 1981).

Finally, with respect to reproductive outcome, a report of a two species (rat and mouse) inhalation teratology study on MtBE at doses similar to the present study concluded that MtBE is not maternally toxic, embryotoxic or teratogenic (Conaway et al., 1985). This present study indicates that an oxygenated hydrocarbon, MtBE, possesses little adverse reproductive toxicity potential as defined in a two litter, one generation reproduction assay in rats.

ACKNOWLEDGEMENT

The authors would like to acknowledge the invaluable assistance of the MtBE Ad Hoc Task Force Participants including C. C. Conaway, K. Hoover, C. Kirwin, S. Ridlon, R. N. Roth, G. S. Simon, N. K. Snyder, L. Stewart and F. B. Thomas.

REFERENCES

API (1980). Rationale and Program for Human and Environmental Health Effects of the Gasoline Additive Methyl-t-butyl Ether.

API (1984). A Nine Day Inhalation Study of MtBE in the Rat. Submitted by Bio dynamics Inc., East Millstone, NJ to the American Petroleum Institute.

ARCO CHEMICAL COMPANY. (1980). Methyl Tertiary Butyl/Ether: Acute Toxicological Studies.

CONAWAY, C.C., SCHROEDER, R.E. and SNYDER, N.K. (1985). Teratology Evaluation of Methyl Tertiary Butyl Ether in Rats and Mice. J. Tox. and Environ. Hlth. 16:797-809.

DUNNETT, C.W. (1955). A Multiple Comparison Procedure for Comparing Several Treatments with a Control. J. Amer. Statist. Soc. 50:1096-1121.

EMBER, L. (1984). EPA Study Backs Cut in Lead Use in Gas. Chemical and Engineering News, April 9.

GILL, J.L. (1978). Design and Analysis of Experiments in the Animal and Medical Sciences, Iowa State University Press.

HESS, F.G., JR., COX, G.E., BAILEY, D.E., PARENT, R.A. and BECCI, P.J. (1981). Reproduction and Teratology Study of 1,3-butanediol in Rats. Journal of Applied Toxicology 1:202-209.

HOLLANDER, M. and WOLFF, D.A. (1973). Nonparametric Statistical Methods, Ch. 6. New York: John Wiley and Sons.

HULS (1980). Methyl Tertiary Butyl Ether (Driveron): Three Month Inhalation Toxicity in Rats. Submitted by Ineresk Research International, Edinburgh, Scotland to Chemische Werke Huls AG.

REYNOLDS, R.W., SMITH, J.S. and STEINMETZ, I. (1974). Methyl Ethers as Motor Fuel Components. Presented at 168 National Meeting of Division of Petroleum Chemistry, American Chemical Society, Atlantic City, NJ.

SNEDECOR, G.W. and COCHRAN, W.G. (1967). Chapter 10, One Way Classifications in Statistical Methods, 6th Edition. Ames: Iowa State University Press.

Received June 12, 1987
Accepted August 4, 1987

SUBCHRONIC INHALATION TOXICITY AND REPRODUCTIVE ASSESSMENT IN RATS OF THREE CHLORINATED PROPENES

F. R. Johannsen, G. J. Levinskas

Monsanto Company, St. Louis, Missouri

G. M. Rusch, R. E. Schroeder

Bio/dynamics, Incorporated, East Millstone, New Jersey

Groups of 15 male and 15 female Sprague-Dawley rats were exposed to 1 of 3 chloropropene (2,3-Di = DCP; 1,2,3-Tri = TRCP; and 1,1,2,3-Tetra = TECP) vapors to provide information on repeated exposures and the potential for reproductive impairment by the most likely route of occupational exposure. Target exposure concentrations were 0, 1, 5, and 15 ppm, 6 h/d, 5 d/wk for 13 wk. The following parameters were evaluated: pharmacotoxic signs, survival, body weights, hematology, clinical blood chemistry, urine analysis, gross and histopathology (over 40 tissues/rat), organ weights, and selected weight ratios. Signs of nasal irritation were noted in rats exposed to 15 ppm of either DCP or TRCP but not TECP. Small decreases in overall body weight were observed in female rats exposed to 15 ppm TCP. An increase (~15%) in spleen weight, with no corresponding histopathological or clinical findings, was observed in 15 ppm DCP-treated male rats. No other effects considered related to treatment were observed following exposure to any of the three chlorinated propenes.

Additional groups of 10 male and 20 female Sprague-Dawley rats were exposed to DCP, TRCP, or TECP vapors at target concentrations of 0, 1, or 5 ppm for 6 h/d, 5 d/wk for a 10-wk premating period, a mating period, and the first 14 d (females only) of gestation. Females were allowed to deliver litters and the offspring were evaluated during a 21-d lactation period. Mating, pregnancy, and fertility indices were generally comparable among all test groups, although female mating and pregnancy indices of both DCP-treated females were lower than expected in the regular and postrecovery reproduction phase. No effects were seen on pup survival, sex distribution, body weights, organ weights, and ratios. A modest reduction in pup body weights was observed following TECP exposure but was attributed to large litter size. No treatment-related effects were seen following necropsy of adults or weanlings, nor were such effects noted following microscopic evaluation of gonads from parental animals.

Presented in part at the 68th Annual Meeting of the Federation of American Societies for Experimental Biology, St. Louis, Missouri, April 1-6, 1984.

The authors wish to express their gratitude to Mrs. Diana Jones, Monsanto Co., for assistance in preparation of this manuscript.

Present address for G. M. Rusch is Allied-Signal, Inc., Morristown, NJ.

Requests for reprints should be sent to Dr. Frederick R. Johannsen, Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

Journal of Toxicology and Environmental Health, 33:291–302, 1991

INTRODUCTION

Chlorinated propenes are important chemical intermediates used in industrial and agricultural chemical processes. Because of the volatility of this chemical class, inhalation of vapors in the workplace is considered to be one of the principal potential occupational exposure routes. A paucity of toxicity data by the inhalation route exists in the literature for chloropropene isomers other than the 1,3-dichloropropene isomer. A threshold limit value (TLV) of 1 ppm has been established for dichloropropene (isomers undefined) (ACGIH, 1986).

Inhalation toxicity data for 1,3-dichloropropene include single 4-h LC50s of 729–900 ppm for Fischer 344 rats (Parker et al., 1982; Stott et al., 1988) and subchronic inhalation studies both with 1,3-dichloropropene (Torkelson and Oyen, 1977; Torkelson and Rowe, 1981; Stott et al., 1988; Hanley, 1987) and with a mixture of 1,3-dichloropropene and 1,2-dichloropropane (Parker et al., 1982; Linnett et al., 1988). Lomax et al. (1989) also have reported on the chronic inhalation toxicity of technical-grade 1,3-dichloropropene in rats and mice.

The present study was designed to provide a comparative toxicologic profile of three chloropropene isomers not previously evaluated toxicologically, including the isomer of 2,3-dichloropropene (DCP), 1,2,3-trichloropropene (TRCP), and 1,1,2,3-tetrachloropropene (TECP). For comparative purposes, each chlorinated propene was tested under a similar exposure regimen following subchronic inhalation exposure. Additional information was also derived on the ability of these chlorinated propenes to affect reproduction/fertility in the rat at exposure levels that were anticipated to produce no more than minimal irritation.

In preparation for this study, a series of range-finding studies were used for subsequent dose level selection. In 1-mo inhalation pilot studies, groups of 5 male and 5 female Sprague-Dawley rats were exposed 6 h/d, 5 d/wk to vapors of either 2,3-DCP, 1,2,3-TRCP, or 1,1,2,3,-TECP (Monsanto Company, unpublished observations, 1976). Each chlorinated propene was evaluated at target levels of 0, 5, 20, or 100 ppm. Deaths related to treatment resulted in groups of rats exposed to the high dose (100 ppm) of both TECP (1M, 2F) and TRCP (5M, 5F). Reduced mean body weights were observed primarily in female rats, in treated groups exposed to mid and high levels of DCP and TRCP and all levels of TECP. Signs of irritation (droopy eyelids, alopecia) generally correlated to those test levels with observed weight loss. Exposure to TRCP produced the most significant irritation (droopy lids, lacrimation, red nasal discharge). Only gross pathologic, not histopathologic, examinations were performed. Noteworthy findings included red nasal and anal discharge on 2 high-dose (100 ppm) TRCP-treated males and the failure of lungs to collapse, likely due to pulmonary edema, in those rats that died on test after exposure to 100 ppm TECP.

METHODS

Test Material and Atmosphere Generation

Each of the chlorinated propenes used in these studies were analyzed by gas chromatography to verify purity, as follows: DCP, >99%; TRCP, 95%; TECP, >99%.

Each test article was placed in a 30-ml midget impinger while nitrogen gas was passed through the vessel. The resultant vapor was directed into a 760-l dynamic airflow inhalation chamber system and diluted with room air to produce the desired exposure concentrations. Chambers were operated at average flow rates of 130 l/min for DCP and TECP exposures and at 142 l/min for TRCP. This provided the theoretical 99% equilibration times of 27 and 25 min, respectively (Silver, 1946). After 2 wk of exposure the delivery system for TRCP was modified to one using a metering pump to assure uniform generation of the cis and trans isomers. In this system, the test article was placed in a glass syringe and metered into the side tube of a 250-ml filtering flask using a Sage model 341 syringe pump. This flask was warmed on a heating mantel with an autotransformer. Nitrogen gas was then passed through the flask and the resultant vapor was directed into the inhalation chamber.

In developing the infrared chamber monitoring conditions, scans were made on each material, and then a wavelength most sensitive for each test agent was selected. The cell pathlength was determined as the one giving the best response over the range of exposure levels. The conditions selected are given in Table 1.

Chamber concentrations were determined intermittently at least three times daily by direct analysis of chamber samples using a Miran model IIA infrared (IR) analyzer (Wilks Instrument Co., Darien, Conn.). Exposure levels were determined by comparison of the observed absorbance of these samples to a previously determined calibration curve generated using the same test material under the similar instrumental settings. Estimation of the consistency of the IR was obtained by periodic comparison of daily nominal concentrations throughout the course of each study. No significant differences were observed that indicated a change in the characteristics of the IR monitor.

The IR measurements for the low-dose (1 ppm) TRCP concentrations appeared to be subject to water-vapor interference resulting in an over-

TABLE 1. Exposure Conditions

Compound	Wavelength (μm)	Pathlength (dial reading)
2,3-DCP	11.2	13.0
1,2,3-TRCP	12.6	14.0
1,1,2,3-TECP	10.9	9.0

estimation of actual exposure concentrations. A colorimetric technique using 4-*p*-nitrobenzyl pyridine reagent (Rusch et al., 1976) was used to independently determine chamber concentrations at this test level. From these results it was concluded that some water-vapor interference occurred and that actual concentrations were even closer to target levels than indicated by IR. No such problems were encountered at other TRCP levels.

Animals

Charles River Sprague-Dawley-derived rats were acclimatized and assigned to study according to a random-number table procedure. Rats for the subchronic phase were 7 wk of age at study inception, while those used in the reproduction phase were 62 d old. Rats were pair-housed in stainless steel wire caging during exposure and singly housed at all other times, except during mating periods. Water and rodent feed (Purina Laboratory Chow, St. Louis, Mo.) were available ad libitum during nonexposure periods.

Experimental Evaluation of Animals from 13-Week Toxicity Studies

Groups of 15 male and 15 female rats were exposed to target concentrations of 0 (concomitant control), 1, 5, or 15 ppm of the respective chloropropene for 6 h/d, 5 d/wk for 13 wk.

All animals were observed for survival twice daily and given a detailed physical examination on a weekly basis throughout the study period. Individual body weights were recorded weekly from 10 d prior to exposure through termination.

Clinical parameters were determined for all rats in the control and the highest dose groups (15 ppm) exposed to each chloropropene.

Measurements were taken at the study midpoint (d 44–45) and for all surviving animals in all test groups just prior (d 85–88) to term. These parameters included hematology (hemoglobin, hematocrit, erythrocytes, clotting time, and total and differential leukocytes) and serum chemistry (serum glutamic pyruvic transaminase, alkaline phosphatase, urea nitrogen, and glucose). Urinalysis (gross appearance, specific gravity, pH, protein, bilirubin, ketones, glucose, occult blood, and sediment) was performed on all animals in each of the 15-ppm chloropropene groups and the control group on study d 47–48 and again on d 89. Complete necropsies were performed on all animals sacrificed at study term. Organ weights and organ/body weight ratios were recorded/calculated for brain, gonads, heart, kidneys, liver, lungs, pituitary and spleen. Microscopic examination of the following tissues were performed for all control and high-dose (15 ppm DCP, TCP and TECP) animals: adrenals, bone marrow, brain, eye, gonads, heart, colon, duodenum, ileum, kidneys,

liver, lung, lymph nodes, mammary gland, pancreas, pituitary, salivary gland, skeletal muscle, skin, spinal cord, spleen, stomach, thyroid, urinary bladder, uterus, prostate, and any gross lesions or tissue masses possibly attributable to treatment.

Experimental Evaluation of Animals from Reproduction Study

Groups of 10 male and 20 female rats were exposed to nominal concentrations of 0 (concomitant control), 1, or 5 ppm of either DCP, TRCP, or TECP. Test concentrations were selected such that no more than minimal irritation would result, inasmuch as this toxicological parameter would, in itself, significantly limit the potential level of repeated exposure occupationally. Exposures occurred in the same chambers with rats in the subchronic phase. Rats in the reproduction phase, however, were exposed for a 10-wk premating period, a mating period (not in excess of 30 d), and from d 0 through d 14 (day exposure stopped) of gestation (females only). All exposures were 6 h/d, 5 d/wk.

During the mating period, male and female rats from the same treatment group were cohoused nightly, initially one male per two females. Males were cohoused with females up to 10 consecutive days. If mating had not occurred following this interval, females were paired with a different male for a second or if necessary even a third 10-d interval. Females were examined each morning for evidence of mating (vaginal plug or sperm). Gestation d 1 was defined as the day mating was confirmed. Mated females were housed individually for the duration of gestation.

On d 19 of gestation, dams were provided nesting material and were examined twice daily for signs of parturition. The day all pups were delivered was defined as day 0 of lactation. All litters were weaned on d 21.

Both DCP-treated groups contained a higher frequency of female rats that had not mated or did not bear litters after the full 30-d mating cycle. These rats (6 per group) were held for a 60-d period during which they incurred no exposures to DCP. Then each female was caged with a proven male rat of the same strain from an in-house breeding colony. Each female was caged with the same male nightly for 10 consecutive days. Females unmated after this interval were caged with a different male nightly for an additional 10-consecutive-day period. Mating and pregnancy were determined, and if pregnant the animal entered the study phase as outlined previously.

All adult rats were observed twice daily for overt signs of toxicity and survival. Detailed physical examinations were made weekly. Individual body weights for both adult male and female rats were recorded weekly throughout the study, beginning at initiation of the 10-wk premating period. Mated females were weighed on d 0, 6, 15, and 20 of gestation and on d 0, 4, 14, and 21 of lactation.

Litters were observed daily for the presence of dead pups, which

were recorded. Surviving pups of each sex were weighed on d 0, 1, 4, 14, and 21 of lactation.

All adult male rats on study were sacrificed after completion of the 30-d mating period; chloropropene exposures were terminated after mating. Mated females were sacrificed at weaning or 26 d postmating if pregnancy was not observed. Female rats from the DCP-treated groups were retained for a posttreatment period described earlier. Complete necropsy was performed on each adult animal with particular attention given to the reproductive tract. The following organ weights were recorded and body-weight ratios calculated: brain, gonads, epididymides, spleen, heart, lungs, kidneys, and liver. Microscopic evaluations of the ovaries, testes, and/or epididymides were made for each adult animal. Offspring were killed at weaning (d 21) and a thorough gross necropsy was performed. Sex was determined by internal inspection of the gonads.

Statistical Methods

Body weights, organ weights, and weight ratios from compound-treated groups were compared to controls using Dunnett's multiple comparison test (Dunnett, 1964). Hematology, clinical chemistry parameters, and gestation length, offspring weight, and number of offspring from treated groups were compared to controls using appropriate tests of variance (*F*-test), and when significant, the Student's *t*-test was employed as modified by Cochran (Snedecor and Cochran, 1967). Survival indices (offspring, litter, mortality), mating indices, pregnancy rates, and fertility indices were compared using the chi-square method of analysis (Snedecor and Cochran, 1967).

RESULTS

Analytical Concentrations

Chamber sample analyses indicated that rats in the 1, 5, and 15 ppm test groups were actually exposed to the following cumulative mean analytical concentrations (±SD):

DCP: 1.3 (±0.4), 4.9 (±0.7), and 15.0 (±1.4) ppm
TRCP: 2.8 (±0.9), 5.5 (±1.8), and 14.9 (±2.4) ppm
TECP: 1.0 (±0.3), 5.0 (±1.4), and 14.3 (±2.3) ppm

The vast majority of daily analytical values were within ±5%, and no overlap between test levels was observed.

Subchronic Study Phase

Daily Observations and Mortality With the exception of a single accidental death occurring in the TECP high-dose group during blood drawing on test d 45, all other test animals survived the duration of the study. No abnormal behavioral reactions were noted in any of the treated animals. Red nasal discharge and yellow staining of the anogenital fur were noted with increased frequency in the 15 ppm DCP and TRCP groups, and both the 5 and 15 ppm TECP test groups. A 9% decrease in body weight gain for female rats in the 15 ppm TRCP group was observed at the conclusion of the study. No other effects on animal body weight gain related to chloropropene treatment were seen.

Hematology, Serum Chemistry, Urinalysis A few scattered observations of statistically significant differences between control and individual exposed groups were observed in hematology, serum chemistry, and urinalysis parameters measured at study term. However, none appeared to be outside of normal biological limits, nor did any appear to be exposure related.

Organ Weights and Weight Ratios Elevated mean, absolute (12%) and relative (15%), spleen weights were observed in terminally killed male rats exposed to 15 ppm DCP. Similar findings were not evident in groups of female rats at this exposure level, nor were similar findings observed in any lower DCP-treated groups. The lack of any significant changes in the hematological profile and the lack of corresponding histopathological changes in these 15 ppm DCP-treated male rats suggest these findings are unrelated to treatment. Analysis of remaining absolute and relative organ weights from other DCP-, TRCP-, and TECP-treated groups resulted in only sporadic, statistically significant differences between various treated and control groups, with no apparent dose- or treatment-related trend evident.

Gross and Microscopic Pathology No differences were observed in gross pathology findings between control rats and those treated with DCP, TRCP, or TECP. Microscopic examination of animals of both chloropropene-treated and control groups revealed no changes that could be correlated to 13-wk exposure to DCP, TRCP, or TECP.

Reproduction Phase

Parental Generation Observations and Mating Indices A single male rat in the 1 ppm DCP group was killed in a moribund state prior to scheduled sacrifice. No treatment-related differences were observed in group body weights between chloropropene-treated (DCP, TRCP, and TECP) and control animals during the premating phase or in females during gestation and lactation.

Small differences in mating indices (male and female) were observed in both DCP-treated groups when compared to control values (Table 2).

TABLE 2. Mating and Fertility Indices of Rats Exposed to 2,3-Dichloropropene (DCP), 1,2,3-Trichloropropene (TRCP), or 1,1,2,3-Tetrachloropropene (TECP) Vapors

		Mating		Fertility	
Vapor	ppm	Female[a]	Male[a]	Female[b]	Male[c]
Control	0	20/20	9/10	19/20	9/9
DCP	1	17/20	6/10	14/17	6/6
	5	18/19[d]	8/10	14/18	8/8
TRCP	1	19/20	8/10	17/19	8/8
	5	20/20	8/10	20/20	8/8
TECP	1	20/20	9/10	17/20	9/9
	5	20/20	9/10	20/20	9/9

Note. No significant differences between treated and control groups.
[a]Number mated vs. group total.
[b]Number pregnant vs. number mated.
[c]Number of males impregnating vs. number mated.
[d]Excludes consideration of one hermaphrodite.

However, none of these differences were statistically significant. No apparent effects in similar indices were seen in TRCP- or TECP-treated rats. With the exception of the DCP-treated groups, pregnancy and impregnation indices appeared normal in all treated groups (Table 2).

Posttreatment Recovery Group Six female rats, all from the 1 and 5 ppm DCP groups, that either did not mate or conceive during the 30-d mating period were retained for a 60-d posttreatment recovery period. Several of these individuals mated, became pregnant, and bore normal litters. However, both the mating index and the pregnancy rate for each group remained somewhat lower than expected (Table 3). No clear evidence of a dose response was seen, nor were there any physical findings at necropsy that were indicative of a treatment-related effect.

Litter Data Gestational length, fetal viability at birth, litter size (Table 4), and litter survival (Table 5) did not appear adversely affected by DCP, TRCP, or TECP exposures.

TABLE 3. Mating and Pregnancy Indices of 2,3-Dichloropropene (DCP) Treated Rats Cohabitated with Proven Males in the Postrecovery Mating Phase

	Number of females		
DCP (ppm)	Cohabitated	Mated	Pregnant
1	6	4	2
5	5[a]	3	1

[a]Excludes hermaphrodite.

TABLE 4. Gestation Length, Fetal Viability at Birth, and Litter Size of Offspring from Rats Exposed to 2,3-Dichloropropene (DCP), 1,2,3-Trichloropropene (TRCP), or 1,1,2,3-Tetrachloropropene (TECP) Vapors

Vapor	ppm	Gestation length (d)	Fetal viability	
			Alive	Dead
Control	0	22.2	10.2	0.3
DCP	1	21.8	10.9	0.2
	5	22.1	10.6	0.4
TRCP	1	22.1	10.8	0.1
	5	22.1	10.9	0.2
TECP	1	21.9	11.1	0.4
	5	21.9	13.8[a]	0.2

[a]Significantly ($p \leq .01$) different from control.

Necropsy and Organ Weights The only remarkable gross lesion observed after necropsy of control and chloropropene-treated adult rats was that of a hermaphrodite in the 5 ppm DCP-treated female group. This animal had a fully formed uterus with primordial epididymis, testes, and seminal vesicles. Gross lesions were confirmed microscopically. This observation is considered representative of a congenital malformation and not treatment related. This animal did not mate throughout the course of both the full and postrecovery portion of the study.

Histopathology Microscopic examination of the gonads of DCP-, TRCP-, and TECP-treated rats was generally unremarkable. Sporadic, subtle microscopic findings (mild focal testicular atrophy) were found in the testes of two males from the low-dose 2,3-DCP group in the reproduction phase. No such findings were observed at the next higher (5 ppm) test level in this study phase or even at 15 ppm in the subchronic phase. Thus, no dose-response relationship was established. This type of lesion

TABLE 5. Survival and Growth Indices of Offspring from Rats Exposed to 1,1,2,3-Tetrachloropropene (TECP) Vapors

TECP (ppm)	Mean number (± SD) of pups alive at		Mean pup weight (g ± SD) at d			
	Birth	d 21	0	4	14	21
0	10.2 ± 3.4	10.2 ± 2.1	6.5 ± 0.7	10.7 ± 1.5	27.7 ± 3.7	45.6 ± 7.4
1	11.1 ± 3.1	10.6 ± 2.9	6.3 ± 0.5	10.3 ± 1.5	26.8 ± 4.9	43.2 ± 7.7
5	13.8 ± 2.1[a]	12.6 ± 2.5[a]	6.2 ± 0.7	9.4 ± 1.2[a]	24.4 ± 4.1[b]	39.7 ± 6.2[b]

[a]Significantly different from control, $p < .01$.
[b]Significantly different from control, $p < .05$.

occurs spontaneously with increasing frequency in rats in an age-related fashion (Heywood and James, 1985). Due to the protocol design, these males were of an age (~6 mo) before termination when such lesions begin to manifest themselves. An evaluation of several studies of >6 mo duration at this laboratory confirmed the age change of this lesion in the CD rat. Thus, this lesion is considered to be spontaneous in nature and unrelated to compound administration. No such effects were noted in other TRCP- or TECP-treated groups.

Weanling Generation

Offspring Data and Necropsy Mean pup survival of chloropropene-treated groups was comparable to control levels, with one exception. Pup survival from dams administered 5 ppm DCP was significantly lower than control values after 1 and 4 d of lactation. These differences were attributable to the loss of a single litter before d 1 of lactation in a dam for which d 0 of gestation had not been confirmed. This single incident was judged to be unrelated to treatment.

Significant reductions in group mean body weights were observed on lactation d 4, 14, and 21 from pups whose dams were exposed to 5 ppm TECP (Table 5). This study group also exhibited a significantly ($p < .01$) increased number of pups delivered at d 0 and remaining alive at weaning (lactation d 21). Inasmuch as litters were not culled, it is possible that reduced pup weights at the 5 ppm TECP level were reflective of a reduced nutritional status due to the increased survivorship in this study group. Mean pup weights have been shown to decrease with increasing litter size. Khera et al. (1989) reported a difference of approximately 5 g between mean litter weights of dams nursing 10 pups per litter versus 14 pups per litter. This 5-g disparity would account for the entire weight differential observed in the present study observed through the 21-d lactation period. No other effects on pup weight or weight gain were observed in other chloropropene-treated groups. Sex distribution observed in this study was judged to have exhibited normal distribution. Necropsy of pups found dead during lactation and killed on d 21 of lactation revealed no treatment-related effects.

DISCUSSION

Lethal concentrations of the 1,3-DCP isomer in laboratory animals can produce lung, liver, and kidney injury (Torkelson and Oyen, 1977). However, multiple exposures to 1,3-DCP or a DCP-containing mixture at lower exposure levels have resulted only in slight toxicological effects in several mammalian species (Highman and Heppel, 1946; Torkelson and Oyen, 1977; Torkelson and Rowe, 1981). Rats, guinea pigs, rabbits, and dogs exposed to up to 3 ppm 1,3-DCP for 6 mo exhibited no histopatho-

DUE TO CLERICAL ERROR

IN THE BATE LABELING

PROCESS. THIS BLANK PAGE

HAS BEEN INSERTED TO MAINTAIN

BATES PAGE ORDER.

THIS PAGE IS

NOT

A

DOCUMENT.

logic effects, except for slight, apparently reversible changes in the kidneys of male rats at the highest test level (Torkelson and Oyen, 1977). Subsequent data from the same laboratory (Torkelson and Rowe, 1981) reported no gross or microscopic changes in either the kidney or liver (or any other tissue or organ) in mice or rats exposed to 1,3-DCP concentrations of 12–93 ppm for 13 wk. More recent data from that facility, using Fischer 344 rats and B6C3F1 mice, have shown depressed growth rates and degenerative change of the respiratory tract in animals exposed to 1,3-DCP levels at and above 90 ppm for 13 wk. Hyperplasia of the urinary bladder epithelium was also observed in female mice.

The results observed in the present comparative study with three chloropropene derivatives are consistent with subchronic inhalation toxicity results previously reported for 1,3-DCP. In the present study, no specific target organ toxicity was observed with 2,3-DCP, TRCP, or TECP when administered at atmospheric concentrations up to 15 ppm for 3 mo. Specifically, no changes in organ weights, gross or microscopic pathology, or clinical chemistry findings indicative of a toxicologic effect were observed in any of the tissues or organs for which toxicologic lesions had been reported after high-level acute exposure with 1,3-DCP. The slight splenic weight increase seen with 2,3-DCP in the present study is of questionable significance since it was not accompanied by corresponding hematologic or pathologic changes. The fact that this organ has not been reported as a target site in previous studies or with other chlorinated propenes in this study supports a contention that this finding is unrelated to exposure.

Findings in the reproductive phase of this study have shown no reproductive and target tissue (testes) effects similar to that of a halogenated alkane, dibromochloropropane (DBCP). In the case of DBCP, effects were noted in male germinal tissue that affected male fertility (Rao et al., 1983). This lack of reproductive target tissue toxicity is consistent with previous subchronic reports for 1,3-DCP (Torkelson and Oyen, 1977; Stott et al., 1988). The lack of effects on male fertility observed after treatment with DCP, TRCP, or TECP is also consistent with results of a two-generation inhalation reproduction study in rats with the 1,3-DCP isomer (Breslin et al., 1989). The lower-than-expected mating frequency seen with DCP-treated females, while not significantly different from controls, is of possible relationship to treatment in light of the reduced mating/pregnancy frequencies also seen during the postrecovery period. Still, no apparent physical aberrations related to treatment were observed at necropsy to resolve this issue. No such effects were apparent following either TRCP or TECP exposure at either exposure level evaluated.

The results of the present study extend our knowledge regarding the subchronic toxicologic effects of chlorinated propenes. The lack of significant toxicity observed at levels greater than those expected to be

tolerated because of irritation from occupational exposure thus supports the present dichloropropene TLV and suggests that this occupational standard would possess an acceptable margin of safety for TECP and TRCP as well.

REFERENCES

American Conference of Governmental Industrial Hygienists. 1986. *Documentation of the Threshold Limit Values and Biological Exposure Indices*, 5th ed. Washington, D.C.: ACGIH.

Breslin, W. J., Kirk, H. D., Streeter, C. M., Quast, J. F., and Szabo, J. R. 1989. 1,3-Dichloropropene: Two generation, inhalation reproduction study in Fischer 344 rats. *Fundam. Appl. Toxicol.* 12:129–143.

Dunnett, C. W. 1964. New tables for multiple comparisons with a control. *Biometrics* 20:482–491.

Hanley, T. R., Jr., John-Green, J. A., Young, J. T., Calhoun, L. L., and Rao, K. S. 1987. Evaluation of the effects of inhalation exposure to 1,3-dichloropropene on fetal development in rats and rabbits. *Fundam. Appl. Toxicol.* 8:562–570.

Heywood, R., and James, R. W. 1985. Current laboratory approaches for assessing male reproductive toxicity: Testicular toxicity in laboratory animals. In *Reproductive Toxicology*, ed. R. L. Dixon, pp. 147–160. New York: Raven Press.

Highman, B., and Heppel, L. A. 1946. Toxicology of 1,2-dichloropropane (propylene dichloride). III. Pathologic changes produced by a short series of daily exposures. *Arch. Pathol.* 42:525–534.

Khera, K. S., Grice, H. C., and Clegg, D. J., eds. 1989. Chapter IV. Multigeneration studies. In *Current Issues in Toxicology*, pp. 21–40. New York: Springer-Verlag.

Linnett, S. L., Clark, D. G., Blair, D., and Cassidy, S. L. 1988. Effects of subchronic inhalation of D-D (1,3-dichloropropene/1,2-dichloropropane) on reproduction in male and female rats. *Fundam. Appl. Toxicol.* 10:214–223.

Lomax, L. G., Stott, W. T., Johnson, K. A., Calhoun, L. L., Yano, B. L., and Quast, J. F. 1989. The chronic toxicity and oncogenicity of inhaled technical grade 1,3-dichloropropene in rats and mice. *Fundam. Appl. Toxicol.* 12:418–431.

Parker, C. M., Coate, W. B., and Voelker, R. W. 1982. Subchronic inhalation toxicity of 1,3-dichloropropene/1,2-dichloropropane (D-D) in mice and rats. *J. Toxicol. Environ. Health* 9:899–910.

Rao, K. S., Burek, J. D., Murray, F. J., John, J. A., Schwetz, B. A., Bell, T. J., Potts, W. J., and Parker, C. M. 1983. Toxicologic and reproductive effects of inhaled 1,2-dibromo-3-chloropropane in rats. *Fundam. Appl. Toxicol.* 3:104–110.

Rusch, G. M., La Mendola, S. L., Katz, G. V., and Laskin, S. 1976. Determination of low levels of dimethylcarbamoyl chloride in air. *Anal. Chem.* 48:2259–2261.

Silver, S. D. 1946. Constant flow gassing chambers: Principles influencing design and operation. *J. Lab. Clin. Med.* 31:1153–1161.

Snedecor, G. W., and Cochran, W. G. 1967. *Statistical Methods*, 6th ed. Ames: Iowa State University Press.

Stott, W. T., Young, J. T., Calhoun, L. L., and Battjes, J. E. 1988. Subchronic toxicity of inhaled technical grade 1,3-dichloropropene in rats and mice. *Fundam. Appl. Toxicol.* 11:207–220.

Torkelson, T. R., and Oyen, F. 1977. The toxicity of 1,3-dichloropropene as determined by repeated exposure of laboratory animals. *Am. Ind. Hyg. Assoc. J.* 38:217–223.

Torkelson, T. R., and Rowe, V. K. 1981. Halogenated aliphatic hydrocarbons containing chlorine, bromine and iodine. In *Patty's Industrial Hygiene and Toxicology*, eds. G. D. Clayton and F. E. Clayton, Vol. IIB, pp. 3433–3601. New York: Wiley.

Received June 14, 1990
Accepted February 11, 1991

JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 12, Number 2, 1993
Mary Ann Liebert, Inc., Publishers

A Reexamination of Liver Tumors in Mice Exposed to Wholly Vaporized Unleaded Gasoline

RENAE I. MAGAW,[1] WARD R. RICHTER,[2] and JUDITH A. MACGREGOR[3]

ABSTRACT

A single lifetime inhalation study of the effects of wholly vaporized unleaded gasoline has been conducted in the mouse. An elevation of hepatocellular tumors was reported only in females and only at the high treatment level (2056 ppm). Several reports of this study have appeared previously in the scientific literature, including a comprehensive summary published in this journal. Different incidence rates were reported for mouse liver tumors in these publications without explanations of how they were calculated of why they differed. To clarify the data, we recently examined the final report of the gasoline study, the individual animal data, and slides prepared from liver tissue collected during the study. The previously reported incidence rates did not take into consideration all animals at risk for tumor development or all animals with hepatocellular tumors. Revised incidence rates are presented for hepatocellular tumors in all mice on study. These rates are approximately 20–25% lower than previously reported.

INTRODUCTION

A lifetime inhalation study of the effects of wholly vaporized unleaded gasoline on mice was conducted by the International Research and Development Corporation (IRDC, 1983) and sponsored by the American Petroleum Institute. The results of the study indicated that wholly vaporized unleaded gasoline induced a statistically significant compound-related increase in hepatocellular tumors in female mice. No increase was observed in male mice.

The study was originally reported by MacFarland et al. in this journal (1984) and elsewhere (1982, 1984), and it was reviewed extensively by the U.S. Environmental Protection Agency (1987) and other organizations (HEI, 1985; NESCAUM, 1989). Slightly different incidence figures were presented for mouse liver tumors in the published reports. The differences could not be explained on the basis of information presented in the articles and raised questions about why the published rates differed. The data and the reports in question have been thoroughly reviewed and the actual liver tumor incidence observed in mice in this study was determined.

IRDC STUDY SUMMARY AND PUBLISHED INCIDENCE DATA

IRDC (1983) conducted a 2-year study in which groups of 100 male and 100 female B6C3F$_1$ mice were exposed to 0, 67, 292, and 2056 ppm of wholly vaporized unleaded gasoline by whole body inhalation. Interim sacrifices

[1]ICF Kaiser Engineers, 1800 Harrison Street, 7th Floor, Oakland, CA.
[2]Pharmaco-LSR, East Millstone, NJ
[3]Chevron Research & Technology Co., P O Box 4054, Richmond, CA

were conducted after approximately 3, 6, 12, and 18 months of exposure. Terminal sacrifices were conducted after 103–107 weeks for male mice and 113 weeks for female mice. The incidence rates for liver tumors have been reported several times, as shown in Table 1 (MacFarland, 1982; MacFarland et al., 1984; NESCAUM, 1989; EPA, 1987). Hepatocellular tumors were defined as adenomas and carcinomas in these publications.

LIVER TUMOR INCIDENCE REVIEW

In order to determine the true incidence of liver tumors observed in the study and the reasons why previously reported incidence rates vary, the IRDC study history and final report were carefully examined. In addition, an independent histopathological review of liver sections was conducted. Because the original slides could not be located by IRDC, paraffin blocks containing liver tissue were obtained from the laboratory. The blocks were recut, sections were stained with hematoxylin and eosin, and new slides were prepared.

The criteria utilized in the histopathological review for the diagnoses of hepatocellular adenoma and carcinoma were as summarized by Brooks and Roe (1985) and by Popp (1985) for adenomas and carcinomas, respectively. Briefly, adenomas were sharply demarcated and they compressed adjacent liver parenchyma. The cytology of the cells varied, with cytoplasm that was eosinophilic, basophilic, clear, or vacuolated. Carcinomas were characterized by some or all of the following: an increased degree of pleomorphism, greater nuclear/cytoplasmic ratio, trabecular plates several cells thick, and increased mitotic activity. Some were large with irregular or invasive growth patterns.

The review of the IRDC final report indicated that the previously reported incidence rates do not take into consideration all mice at risk of tumor development or all animals with hepatocellular tumors. The first hepatocellular tumor was observed at the 12-month interim sacrifice and thus all animals alive at that time should be considered at risk for tumor development and should be included in the incidence rates. The rates reported by MacFarland, NESCAUM, and those used by the EPA for estimating the carcinogenic potency of unleaded gasoline correspond to the incidence of hepatocellular tumors in mice that died or were killed during the 18-month to terminal sacrifice period. Animals that were killed or that died on study prior to 18 months were not included. The

TABLE 1. PRIMARY HEPATOCELLULAR NEOPLASMS INCIDENCE RATES REPORTED IN MICE

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
	Female mice			
EPA, pages 5–14[a]	8/57 (14)[b]	10/52 (19)	13/57 (23)	28/56 (50)
EPA, pages 3–15[c]	8/100 (8)	10/100 (10)	12/100 (12)	27/100 (27)
MacFarland[d]	8/57 (14)	10/52 (19)	12/57 (21)	27/56 (48)
NESCAUM[e]	8/57 (14)	10/52 (19)	12/57 (21)	27/56 (48)
Revised incidence[f]	8/79 (10)	13/76 (17)	12/79 (15)	29/78 (37)
	Male mice			
MacFarland[d]	23/51 (45)	15/42 (36)	20/44 (45)	24/54 (44)
NESCAUM[e]	23/51 (45)	15/42 (36)	20/44 (45)	24/54 (44)
Revised incidence[f]	27/75 (36)	18/70 (26)	28/71 (39)	27/77 (35)

[a] Incidence used to estimate carcinogenic potency in EPA (1987).

[b] Percentages are reported in parentheses.

[c] Incidence reported in summary section of EPA (1987).

[d] Incidences reported in MacFarland (1982, 1984) and MacFarland et al. (1984).

[e] Incidence reported in NESCAUM (1989)

[f] Corrected incidence determined from this review.

summary incidence figures reported for female mice by EPA differ. In this case, the numerators are based on mice that died during the 18-month to terminal sacrifice period, while the denominators represent the total number of mice initially started on test, including those that died prior to 12 months. These animals should not be considered at risk of tumor development and should not be included in the incidence rates.

The study history provides several possible explanations for why the data were reported incorrectly. The histopathologic evaluation of the study was carried out by several pathologists at different laboratories. Separate reports for the different sacrifice groups were prepared and reviewed at different times. The preliminary draft terminal sacrifice report included data for animals that died on study after the 18-month interim sacrifice along with the data for animals sacrificed at the end of the experiment. It did not include or summarize any data for animals that died prior to these times. This report was reviewed, discussed, and summarized prior to completion of the final IRDC report. When the final report was issued for the entire study many months later, the various reviewers did not go back and incorporate data for animals that died previously.

In addition to problems in tabulating the data, there was a change in the terminology for proliferative liver lesions used during the course of the study that resulted in some mice with hepatocellular tumors being excluded from the reported incidence rates. The diagnosis of neoplastic nodule was used at the 12- and 18-month interim sacrifices, but not at other time periods. This term was proposed by Squire and Levitt (1975) to describe proliferative lesions only in the rat. It was proposed because of the difficulty in differentiating between hyperplastic and neoplastic lesions in the rat on morphologic grounds. The term neoplastic nodule is not typically used to describe mouse tumors, but was used for diagnosing lesions in this study for mice at these two interim sacrifices. Animals diagnosed with neoplastic nodules were not included in the incidence figures reported by MacFarland, NESCAUM, or EPA.

Lesions diagnosed as neoplastic nodules by the original pathologist were reevaluated as part of this review and their morphology was found to be consistent with the diagnosis of hepatocellular adenoma. The morphology of these lesions was also consistent with that of the lesions diagnosed as hepatocellular adenoma at later times in the study and animals with neoplastic nodules should be included in the tumor incidence figures.

REVISED TUMOR INCIDENCE

Tables 2 and 3 present the total number of animals with hepatocellular tumors by tumor type and for all tumor types combined for female and male mice, respectively. The overall revised incidence rates presented for all hepatocellular tumors include all animals considered to be at risk for tumor development (i.e., all animals alive at 12 months) and all animals with primary liver tumors, including those originally diagnosed as neoplastic nodules.

TABLE 2. PRIMARY HEPATOCELLULAR NEOPLASMS IN FEMALE MICE: NUMBERS OF ANIMALS WITH TUMORS AND REVISED INCIDENCE

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
Neoplastic nodule	0	3	0	1
Adenoma	1	4	4[a]	8[a]
Carcinoma	7	6	9	21
Total benign	1	7	4	9
Total benign and malignant				
With neoplastic nodules	8	13	12[b]	29[b]
Without neoplastic nodules	8	10	12[b]	28[b]
Revised incidence	8/79 (10%)	13/76 (17%)	12/79 (15%)	29/78 (37%)

[a] One mouse had an adenoma during the 18- to 24-month study period.

[b] One mouse had an adenoma and a carcinoma and is counted only for the most advanced lesion.

TABLE 3. PRIMARY HEPATOCELLULAR NEOPLASMS IN MALE MICE: NUMBERS OF ANIMALS WITH TUMORS AND REVISED INCIDENCE

	Treatment level			
	0 ppm	*67 ppm*	*292 ppm*	*2056 ppm*
Neoplastic nodule	4	3	5	2
Adenoma	12	4	5	5
Carcinoma	12[a]	14	19[b]	21[a]
Total benign	16	7	10	7
Total benign and malignant				
With neoplastic nobules	27[c]	18[d]	28[e]	27[c]
Without neoplastic nodules	24	15[d]	23[e]	25[e]
Revised incidence	27/75 (36%)	18/70 (26%)	28/71 (39%)	27/77 (35%)

[a]One mouse had a carcinoma at the 12 month sacrifice (0 ppm) or during the 12- to 18-month period (2056 ppm).

[b]Three mice had a carcinoma during the 12- to 18-month period.

[c]One mouse had a neoplastic nodule and a carcinoma and is counted only for the most advanced lesion.

[d]Three mice had an adenoma and a carcinoma and are counted only for the most advanced lesion.

[e]One mouse had an adenoma and a carcinoma and is counted only for the most advanced lesion.

The revised incidence rate observed in female mice exposed to 2056 ppm wholly vaporized unleaded gasoline is approximately 25% lower than previously reported. The revised rate is the result of correcting several inconsistencies in tabulating and analyzing data from this study. Inherent problems associated with multiple pathologists conducting the microscopic evaluations further complicated the analysis and should be avoided.

The incorrect incidence data have been used to characterize the potential risks associated with human exposure to gasoline by several regulatory groups (EPA, 1987; NESCAUM, 1989). These risks are based on a treatment-related effect observed only at the high dose level and, therefore, the incidence changes identified in this dose group could alter the estimated risks. This suggests that these risk assessments should be reevaluated.

REFERENCES

BROOKS, P.N., and ROE, F.J.C. (1985). *Digestive System*. T.C. Jones, U. Mohr, and R.D. Hunt (eds.). Springer-Verlag: Berlin, pp. 47–51.

HEALTH EFFECTS INSTITUTE. (1985). Gasoline Vapor Exposure and Human Cancer. Evaluation of Existing Scientific Information and Recommendations for Future Research. Report of the HEI Health Review Committee.

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION. (1983). Motor Fuel Chronic Inhalation Study. Vols. 1–6. Sponsored by the American Petroleum Institute. Sept 15, 1983.

MacFARLAND, H.N. (1982). Chronic gasoline toxicity, in: *The Toxicology of Petroleum Hydrocarbons. Proceedings of a Symposium. May, 1982*. H.N. MacFarland, C.E. Holdsworth, J.A. MacGregor, R.W. Call, and M.L. Kane (eds.): American Petroleum Institute: Washington, D.C., pp. 78–86.

MacFARLAND, H.N. (1984). Xenobiotic induced kidney lesions: Hydrocarbons The 90-day and 2-year gasoline studies, in: *Advances in Modern Environmental Toxicology, Vol. 7, Renal Effects of Petroleum Hydrocarbons*. M.A. Mehlman, C.P. Hemstreet, J.J. Thorpe, and N.K. Weaver (eds.): Princeton Scientific Publishers, Inc.: Princeton, NJ, pp. 51–56

MacFARLAND, H.N., ULRICH, C.R., HOLDSWORTH, C.E., KITCHEN, D.N., HALLIWELL, W.H., and BLUM, S.C. (1984). A chronic inhalation study with unleaded gasoline vapor. J. Am. Coll. Toxicol. 3:231–248.

NORTHEAST STATES FOR COORDINATED AIR USE MANAGEMENT (NESCAUM) (1989). Evaluation of the health effects from exposure to gasoline and gasoline vapors. Air Toxics Committee.

POPP, J.A. (1985). *Digestive System*. T.C. Jones, U. Mohr, and R.D. Hunt (eds.): Springer-Verlag: Berlin, pp. 39–45.

SQUIRE, R.A., and LEVITT, M.H. (1975). Report of a workshop in classification of specific hepatocellular lesions in rats. Cancer Res. 35:3214–3223.

U.S ENVIRONMENTAL PROTECTION AGENCY. (1987) Evaluation of the carcinogenicity of unleaded gasoline Office of Health and Environmental Assessment, Washington, D.C., EPA/600/6-87-001.

Address reprint requests to:
Dr. Judith A. MacGregor
Chevron Research and Technology Co.
P.O. Box 4054
Richmond, CA 94804

THE TOXICOLOGIST

An Official Publication of the Society of Toxicology

and

Abstract Issue of

FUNDAMENTAL AND APPLIED TOXICOLOGY

An Official Journal of the Society of Toxicology
Published by Academic Press, Inc.

978 AN INHALATION ONCOGENICITY STUDY IN RATS OF METHYLETHYLKETOXIME

P E Newton, W L Wooding, W E Rinehart. *Pharmaco LSR, East Millstone, NJ; Industrial Health Foundation, Pittsburgh, PA*

Fischer 344 rats (80/sex/group) were exposed 6 hrs/day, 5 days/week for 26 months via whole-body inhalation to MEKO (methylethylketoxime) vapor concentrations of 0, 15 ± 1 (X ± SD), 75 ± 2 and 374 ± 10 ppm. Survivorship at 26 months (34% in the males; 60% in the females) was unaffected by the MEKO exposures. Microscopically, MEKO related findings included: congestion of the spleen with pigment in reticuloendothelial cells and extramedullary hematopoiesis, hepatocellular carcinoma in the male 374 ppm rats, a dose related increase in hepatocellular adenomas in the males at 75 and 374 ppm, an increase incidence of basophilic foci in males and females at 374 ppm (with increased severity in the males in the 15, 75 and 374 ppm group males and 374 ppm group females). In the nasal turbinates, primarily the dorsal meatus, degeneration of the olfactory epithelium was seen in both males and females at 374 ppm with less severe findings at 75 and 15 ppm. These changes consisted of thinning of the olfactory epithelium with some loss of apical cytoplasm and fewer layers present.

In conclusion, under the conditions of this study, MEKO produced changes in the olfactory epithelium in all MEKO exposed groups and was a liver oncogen in male rats at 75 ppm.

Abstracts of the
34th Annual Meeting
Vol. 15, No. 1, March 95

THE TOXICOLOGIST

CHRONIC INHALATION STUDY OF COMMERCIAL HEXANE IN MICE. W D Daughtrey. Exxon Biomedical Sciences, Inc., East Millstone, NJ. J S Duffy. Texaco, Inc., Beacon, NY. L S Haddock. Unocal Corp., Los Angeles, CA. D W Kelly. Phillips Petroleum Co., Bartlesville, OK. T H Keenan. Ashland Chemical Co., Columbus, OH. W R Richter. Pharmaco LSR, East Millstone, NJ. R A Rhoden. American Petroleum Institute, Washington, DC.

The oncogenic potential of commercial hexane (CH) was evaluated in $B_6C_3F_1$ mice. The CH tested consisted primarily of six-carbon isomers in the following liquid volume percents: n-hexane (52%), methylcyclopentane (16%), 3-methylpentane (16%), 2-methylpentane (13%) and cyclohexane (3%). CH was administered to four groups of 50 animals per sex for 6 hours/day, 5 days/week for two years at target concentrations of 0, 900, 3000, and 9000 ppm in air. There were no significant differences in survivorship between control and CH-exposed groups. Body weight gain was reduced in high-dose females, but not in females of the mid- and low-dose groups nor in males. Microscopic examination revealed a treatment-related increase in hepatocellular neoplasms (adenomas and carcinomas) in females of the 9000 ppm group. There was also an increase in the incidence of pituitary proliferative changes in all groups of CH-exposed females compared to controls. A decrease in the severity and a slight decrease in the incidence of cystic uterine endometrial hyperplasia were observed in the high-dose females. No neoplastic changes were found in CH-exposed males.

Abstracts of the
33rd Annual Meeting
Vol. 14, No. 1, March 1994

THE TOXICOLOGIST

CHRONIC INHALATION STUDY OF COMMERCIAL HEXANE IN RATS. D W Kelly. Phillips Petroleum Co., Bartlesville, OK. J S Duffy. Texaco, Inc., Beacon, NY. L S Haddock. Unocal Corp., Los Angeles, CA. W D Daughtrey. Exxon Biomedical Sciences, Inc., East Millstone, NJ. T H Keenan. Ashland Chemical Co., Columbus, OH. P E Newton. Pharmaco LSR, East Millstone, NJ. R A Rhoden. American Petroleum Institute, Washington, DC.

The oncogenic potential of commercial hexane (CH) was evaluated in Fischer 344 rats. The test sample of CH consisted primarily of the following six-carbon isomers: n-hexane (52%), methylcyclopentane (16%), 3-methylpentane (16%), 2-methylpentane (13%), and cyclohexane (3%). CH was administered to four groups of 50 animals per sex for 6 hours/day, 5 days/week for two years at target concentrations of 0, 900, 3000, and 9000 ppm in air. There were no significant differences in survivorship between control and CH-exposed groups. Body weight gain was significantly reduced in both males and females from the 3000 and 9000 ppm groups. Histological evidence of mucosal irritation was observed in the nasal turbinates and larynx of CH-exposed males and females. The incidence of this finding was generally greatest in animals from the 9000 ppm group. No statistically significant differences in overall or individual tumor incidences between control and exposed animals were observed. Thus, lifetime exposures to CH at levels up to 9000 ppm were not carcinogenic in Fischer 344 rats.

Abstracts of the
33rd Annual Meeting
Vol. 14, No. 1, March 1994

INHALATION TOXICITY OF PHOSPHINE IN THE RAT: ACUTE, SUBCHRONIC, AND DEVELOPMENTAL

Paul E. Newton
Raymond E. Schroeder

Pharmaco-LSR Inc.
East Millstone, New Jersey

Jeremiah B. Sullivan

DEGESCH AMERICA, Inc.
Weyers Cave, Virginia

William M. Busey
Deborah A. Banas

Experimental Pathology Labs, Inc.
Herndon, Virginia

Lethality is the primary hazard of phosphine exposures. All phosphine-related effects seen at sublethal exposure levels were relatively small and completely reversible either during the exposure or during a recovery period. Acutely, phosphine exposures were lethal to female Fischer 344 rats at a cumulative concentration–time product of about 180 ppm-hr if the concentration were greater than 5–7 ppm. For daily 6-hr exposures, the median lethal times were 3 days at 10 ppm and 4 days at 7.5 ppm. Thirteen daily 6-hr exposures to 5 ppm were not lethal. Decreased erythrocytes, lung congestion, and increased kidney weights with coagulative necrosis of the tubular epithelium in the outer cortex were seen in the 10 ppm rats only. The effects were more severe in females than in males. Subchronic exposures to 0.37, 1, or 3.1 ppm of phosphine were conducted. Ten animals per sex per group were sacrificed after 4 and 13 weeks of exposure and 4 weeks of recovery. These exposure produced a dose-related decrease in body weight gain at 1 and 3 ppm. Food consumption was decreased at 1 and 3 ppm and transiently in the 0.37 ppm group. Five percent decreases in erythrocytes, hemoglobin, and hematocrit were seen in the 3 ppm group after 13 weeks of exposure. All effects seen in the subchronic study were completely reversible either during the 13-week exposure or the 4-week recovery period. Exposure of pregnant CD^R rats (24 per group) to 0.03, 0.33, 2.8, or 4.9 ppm of phosphine for 6 hr/day over the days 6–15 gestation interval was not maternally or developmentally toxic.

This research was supported by the Metal Phosphide Task Force. The authors gratefully acknowledge the technical assistance of Doris Bowden, Ellen Whiting, Brian Pillsbury, and Albert Couchman.

Requests for reprints should be sent to Dr. Paul E. Newton, Bio/Dynamics, Inc., Box 2360, East Millstone, NJ 08875-2360.

Inhalation Toxicology, 5(2):223–239, 1993

0895-8378/93 $10.00 + .00

INTRODUCTION

Phosphine, or hydrogen phosphide (CAS: 7803-51-2), is a highly toxic colorless gas. Phosphine is flammable, explosive in air, and can autoignite at room temperature. Because of impurities, the technical product has a disagreeable garlic-like or fishy odor.

Phosphine has several different industrial uses. Aluminum or magnesium phosphides are used as fumigants because pellets of these powders can be rapidly hydrolyzed to phosphine in moist conditions. Zinc phosphide is used as a rodenticide. Phosphine is also used in the synthesis of organophosphines and as a dopant in semiconductor production.

Many studies have reported the toxicity of phosphine in both humans and animals. Studies have been reported on this highly toxic gas as early as 1829 (Klimmer, 1969). However, the toxicity of phosphine, and in particular the low-level subchronic or chronic toxicity of phosphine, remains unclear. Divergent opinions about the observed effects, exposure levels that produce effects, and the mechanism of action are partially attributable to flaws in earlier experimental designs. These problems included insufficient numbers of animals, the purity of the test material, and the use of estimated or nominal exposure levels.

The high toxicity of phosphine has resulted in low permissible exposure levels. The current threshold limit value (TLV) for phosphine established by the American Conference of Governmental Industrial Hygienists (ACGIH) is 0.3 ppm. The ACGIH has also established a short-term exposure level (STEL) of 1 ppm for exposures time-weighted over any 15-min period. However, a recent occupational exposure survey reported that during phosphine fumigation, worker exposure levels frequently exceed this STEL (Zaebst et al., 1989).

To characterize the inhalation toxicity of phosphine, a series of studies was conducted. The results of an acute, subchronic, and developmental toxicity study are reported here.

MATERIALS AND METHODS

Exposure Levels

For the single 6-hr acute exposures, 0, 2.5, 5, and 10 ppm were selected based on a published 4-hr LC_{50} of 10 ppm (Waritz and Brown, 1965, Muthu et al., 1980). Fifteen animals per sex per group were exposed, with 5 per sex per group scheduled for sacrifice after the exposure and 10 per sex per group after a 2-week observation period. Based on both minimal effects seen in the acute exposure results and reports in the literature, which indicated that exposures to 3 ppm would be tolerable for a 13-week study, the subchronic exposure levels were set at 0, 0.3, 1, and 3 ppm. Thirty animals per sex per group were exposed with scheduled

sacrifices of 10 animals per sex per group after 4 and 13 weeks of exposure and 4 weeks of recovery. However, because there was no effect seen in the interim sacrifice group after 4 weeks of exposure, concern was raised over whether the high exposure level was close to a maximum tolerated dose for the 13-week study. Therefore, satellite groups of 10 animals per sex per group were added at target concentrations of 0 and 10 ppm. Four of 10 females died after the third exposure. As a result, the 10 ppm exposures were stopped and satellite groups at target concentrations of 0 and 5 ppm were added. All of these animals survived until the scheduled sacrifice 13 days later. For the subsequent developmental study, 24 pregnant females per group were exposed to target concentrations of 0, 0.03, 0.3, 3, 5, or 7.5 ppm. Because of deaths at the high exposure level, this group was terminated.

Test Material

In order not to exceed the lower explosive limit during exposure atmosphere generation, the phosphine was supplied as 1% in nitrogen (AIRCO, Riverton, NJ). Impurities identified in the phosphine used to generate the 1% phosphine in nitrogen mixture were as follows (PPM): nitrogen (0.72), oxygen and argon (0.22), carbon monoxide and dioxide (3.61), arsine (<2), total hydrocarbons (<0.5), other phosphines (<50), and moisture (0.6). Chemical similarity of the phosphine in the test cylinder with phosphine generated from a metal phosphide was confirmed by gas chromatography.

Exposure System

All exposures were conducted for 6 hr/day in 10,000-L chambers. The stainless steel and glass chambers (Harford Metal Products, Inc., Aberdeen, MD) had pyramidal tops and bottoms. Chamber airflow entered tangentially into the turret at the top of the chambers to facilitate mixing. The chambers were operated dynamically at flow rates of 2000 L/min. This provided one complete air change every 12 min and a 99% equilibration time of 23 min. All chambers were operated at a slight negative pressure (-0.5 cm H_2O) relative to the surrounding area. Chamber airflow, temperature, and relative humidity were recorded hourly during the exposures. In the subchronic study, each test animal's location was rotated on a daily basis throughout the chamber.

For the exposures, the 1% phosphine in nitrogen mixture was delivered to the chambers from the cylinder through a stainless steel regulator equipped with a vent and purge valve. The flow of the mixture to each chamber was monitored by a digital mass flowmeter (Sierra Instruments Inc., Monterey, CA). The animals remained in the chambers for 30 min following each exposure. During this time the chamber was cleared

using room air at the same airflow rate used during the exposure. Control animals were subjected to the same procedures as the test-exposed animals, except that they were exposed to clean air only during the 6-h in-chamber period. As a safety precaution, from the time the phosphine cylinder was opened until it was closed and the chambers flushed out, all technicians wore fresh-air-supplied positive-pressure suits.

Exposure Atmosphere Analysis

Samples for GC determination of the phosphine exposure levels were withdrawn hourly through a septum in a line being flushed with chamber air from the breathing zone of the animals. Analyses were performed on a Hewlett-Packard model 5890A GC/NPD. 100/120 Chromosorb 102 packing was used in a 6-ft × 2 mm ID glass column. The nitrogen carrier gas flow rate was 20 ml/min. The air and hydrogen detector gas pressures were 50 and 20 psi, respectively. The temperature settings of the GC were injector 150°C, detector 300°C, and column 120°C. Each day the GC was calibrated before exposure initiation and checked after each series of hourly measurements using certified gas standards (AIRCO, Riverton, NJ).

Animals

Male and female Fischer 344 rats, 4 weeks of age at receipt, were obtained from Charles River Breeding Laboratory (Kingston, NY) and used for the acute and subchronic studies. Sprague-Dawley derived CD® rats, 6 weeks of age at receipt, were obtained from the Portage, Michigan facility of this supplier and used for the developmental toxicity study. The animals were acclimated for at least 12 days during which time they were examined by a veterinarian. Animals were doubly housed in elevated, stainless steel, wire mesh cages during the first week of acclimation and were individually housed thereafter. Tap water (via automatic watering system) and Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) were provided ad libitum except during the actual exposures. The no. 5001 diet was used for the acute and subchronic studies and no. 5002 diet was used for the developmental toxicity study. The animals were maintained on a 12:12 hr light/dark cycle. The environment in the animal's quarters was maintained at 68–74°F and 40–60% relative humidity to the maximum extent possible. The animals were individually ear-tagged for identification and randomly sorted for equal body weights among all the exposure groups. To provide mated animals for the developmental toxicity study, females were cohoused nightly with males (1:1) of the same strain from an in-house breeding colony. Females were observed each morning for evidence of a vaginal plug and/or microscopic observations of sperm in the vaginal rinse. The day evidence of mating

was seen was identified as day zero of gestation. Females were at least 9 weeks of age at initiation of mating (186–281 g).

Clinical Laboratory Tests

In the subchronic study, hematological and clinical chemistry indices were evaluated after 4 and 13 weeks of exposure and after a 4-week recovery period. Blood samples were obtained from fasted rats via venipuncture of the orbital sinus under light ethyl ether anesthesia. Hematological indices including hemoglobin, hematocrit, erythrocyte count, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, and total leukocyte count were determined on an ELT-8 Hematology Analyzer (Ortho Diagnostic Systems, Westwood, MA). Differential leukocyte counts and erythrocyte morphology were determined manually. Serum biochemical evaluations including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, blood urea nitrogen (BUN), fasting glucose, total protein, albumin, and chloride were determined using a CentrifiChem System 500 (Union Carbide, Rye, NY). Sodium and potassion determinations were performed using an Electrolyte E2A Analyzer (Beckman Instruments, Brea, CA).

Animal Observations

In both the acute and subchronic studies, animals were observed during each exposure for viability and overt signs of toxicity. Detailed observations were conducted weekly. In the subchronic study, body weights and food consumption were measured twice pretest and weekly thereafter. Fasting body weights were measured at termination. Ophthalmoscopic examinations were performed on all animals pretest and on the day before their scheduled sacrifice. In the developmental toxicity study, each animal was given a detailed physical examination on days 0, 6–15, and 20 of gestation. Body weights were recorded on days 0, 6, 10, 12, 16, and 20 of gestation. Food consumptions were recorded over the day 0–6, 6–10, 10–16, and 16–20 gestation intervals.

Gross Necropsy and Histopathology

In the acute and subchronic studies, complete gross postmortem examinations were performed on all animals. The external surface, all orifices, the cranial cavity, carcass, the external and sectioned surfaces of the brain and spinal cord, nasal cavity and paranasal sinuses, the thoracic, abdominal, and pelvic cavities and their viscera, and the cervical tissues and organs were examined. The following tissues were preserved in neutral buffered 10% formalin and following routine processing and hematoxylin-eosin staining were examined microscopically: adrenal

gland, aorta, sternum, brain, esophagus, eyes, heart, cecum, colon, duodenum, ileum, jejunum, kidneys, liver, right lung lobes, lymph nodes, mammary gland, larynx, nasal turbinates, nerve, ovaries, pancreas, pituitary, prostate salivary glands, seminal vesicles, skin, spinal cord, spleen, stomach, testes with epididymides, thymus, thyroid/parathyroid glands, trachea, urinary bladder, uterus, vagina, and any tissues with gross lesions.

Maternal Evaluations (Developmental Toxicity Study)

On day 20 of gestation, all surviving dams were sacrificed by exsanguination under ethyl ether anesthesia. Immediately following sacrifice, the ovaries and uterus were excised intact and weighed. Along with recording the number of corpora lutea on each ovary, the numbers and relative locations of live and dead fetuses, early and late resorptions were recorded for each uterine horn. If no uterine implants were grossly apparent, the uterus was stained with ammonium sulfide (Salewski, 1964). When foci were visualized following staining, the females were considered pregnant in the calculation of the pregnancy rate; however, the number of foci was not included in the calculation of uterine implantation data.

Fetal Evaluations

Each fetus was individually identified, weighed, sexed, and examined for external malformations/variations to include observation for palatal defects. Approximately one half of the fetuses in each litter were evaluated for visceral malformations/variations using a microdissection procedure similar to that described by Staples (1974). The heads of these fetuses were fixed in Bouin's solution and processed for evaluation using a razor blade sectioning technique. The remaining fetuses in each litter were processed for staining of the ossified skeletal structures with alizarin red S. These fetuses were then evaluated for skeletal malformations and ossification variations. Fetal visceral and skeletal evaluations were performed under a dissecting microscope.

Statistical Analyses

Statistical evaluation of equality of means was made by the appropriate one-way analysis of variance (ANOVA) technique, followed by a multiple comparison procedure if needed. First Bartlett's test was performed to determine if groups had equal variance. If the variances were equal, parametric procedures were used; if not, nonparametric procedures were used. The parametric procedures were the standard one-way ANOVA using the F distribution to assess significance. If significant differences among the means were indicated, Dunnett's test was used to

determine which means were significantly different from the control. If a nonparametric procedure for testing equality of means was needed, the Kruskal–Wallis test was used. If differences were indicated, a summed rank test (Dunn) was used to determine which treatments differ from control.

A statistical test for trend in the dose levels was also performed. In the parametric case, standard regression techniques with a test for trend and lack of fit were used. In the nonparametric case, Jonckheere's test for monotonic trend was used.

Statistical analysis of incidence data was performed using contingency tables. First, a standard χ^2 analysis was performed to determine if the proportion of incidences differed between the groups tested. In keeping with standard statistical practice, if any one cell had an expected value of less than 5, this step was not reported. Second, each treatment group was compared to the control group using a 2×2 Fisher exact test; the significance level was corrected via the Bonferroni inequality to assure an overall test of the stated significance level. Third, Armitage's test for linear trend in the dosage groups was performed. All ratios were transformed via the arc sine transformation prior to analysis.

The test for equal variance (Bartlett's) was conducted at the 1% two-sided risk level. All other statistical tests were conducted at the 5% and 1%, two-sided risk levels.

RESULTS

Acute Exposures

Single acute 6-hr exposures to 0, 2.4, 4.9, and 10 ppm were conducted. All animals survived these exposures. During the exposures, a few animals showed red or mucoidal nasal discharge. During the 14-day recovery period, these observations abated. There was no effect on body weight. Gross postmortem and microscopic examinations revealed no treatment-related findings.

Subchronic Exposures

The exposure concentration results for the subchronic study (Table 1) show excellent agreement between the target, analytical, and nominal results. Distribution measurements performed throughout the exposure chambers showed no significant gradient. Aerosol measurements showed similar particle size and concentration among all the groups (including control), indicating that there was no measurable test substance present as an aerosol. Temperature and relative humidity levels were acceptable.

Table 1. Target, Analytical, and Nominal Exposure Levels (mean ± SD) During the Subchronic and Developmental Inhalation Toxicity Studies of Phosphine

Group	Target level (ppm)	Number exposures	Analytical level (ppm)	Nominal level (ppm)
Subchronic				
I	0	65	0	—
II	0.3	65	0.37 ± 0.38	0.35 ± 0.05
III	1.0	65	1.0 ± 0.24	0.99 ± 0.11
IV	3.0	65	3.1 ± 1.0	3.3 ± 0.6
V[a]	0	3	0	—
VI[a]	10	3	10 ± 0.7	9.1 ± 0.7
VII[b]	0	13	0	—
VIII[b]	5.0	13	5.1 ± 0.6	5.1 ± 1.2
Developmental				
I	0	10	0	0
II	0.03	10	0.03 ± 0.01	0.03 ± 0.01
III	0.3	10	0.33 ± 0.07	0.30 ± 0.02
IV	3	10	2.8 ± 0.6	2.7 ± 0.1
V	5	10	4.9 ± 0.6	4.6 ± 0.2
VI	7.5	10	7.0 ± 0.9	6.8 ± 0.7

[a]Satellite group, started midstudy and terminated after 3 days of exposure due to excessive mortality.

[b]Satellite group, started after termination of the 10 ppm group and sacrificed with groups I–IV animals.

Mortality As described earlier, 4 of 10 females died after 3 days of exposure to 10 ppm of phosphine. This resulted in the premature termination of that group. The concentration-time product ($C \times T$) that produced death was 180 ppm-hr. Females were more sensitive than males to the lethal effect of phosphine in this study.

Physical Observations Weekly detailed physical observations showed only sporadic effects or effects that were seen equally in all groups. There was no indication of any exposure-related effects. There was no indication of any compound-related ocular disease.

Body Weight and Food Consumption There was a statistically significant decrease in body weight gains in both the males and females at 1 ppm and higher (Fig. 1 and 2). However, these body weight effects differed between the sexes. In the males, the decrease in body weight was not statistically significant until the last 2 weeks of exposure. The body weights then recovered toward control weights after termination of the exposures. In the females, the body weights were transiently decreased during only the first few weeks of exposure. The difference in the weight gained during the first 3 weeks between the control and phosphine-exposed females showed a dose-related decrease and is presented in Table 2. After the initial decrease, there was recovery.

The significant decrease in body weight was associated with the sig-

nificantly decreased food consumption seen in both males and females (Figs. 3 and 4). The food consumption effect was more prolonged than the body weight effect and also transiently included the 0.37 ppm group. However, the food consumption effect was also reversible either during the exposures or the recovery period.

Hematology No hematological effects were seen in the females. The males had a 5% statistically significant increase in platelets in the 3.1 ppm group after 4 weeks of exposure. The males also had a 5% statistically significant decrease in hemoglobin, hematocrit, and erythrocyte counts in the 3.1 ppm group after 13 weeks of exposure. These erythrocyte effects appear to be exposure-related as the satellite 10 ppm group, sacrificed after 3 days due to high mortality, also showed decreased erythrocyte counts. The 5.1 ppm group in this study did not show any effects possibly because they only had 13 days of exposure prior to sacrifice. The observed hematological effects were reversible. After a 4-week recovery period all parameters were normal.

Clinical Chemistry The males also showed a few changes in the clinical chemistry parameters. Increased BUN levels were seen after 4 weeks at 3.1 ppm (13%), after 3 weeks at 5.1 ppm (32%), and after 3 days at 10

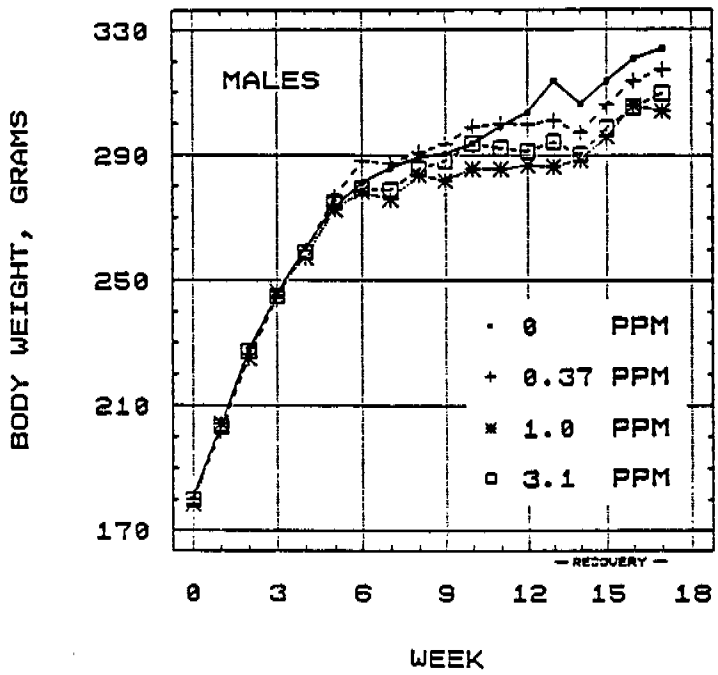


FIGURE 1. Body weight gain of male Fischer 344 rats during a 13-week inhalation toxicity study of phosphine followed by a 4-week recovery period.

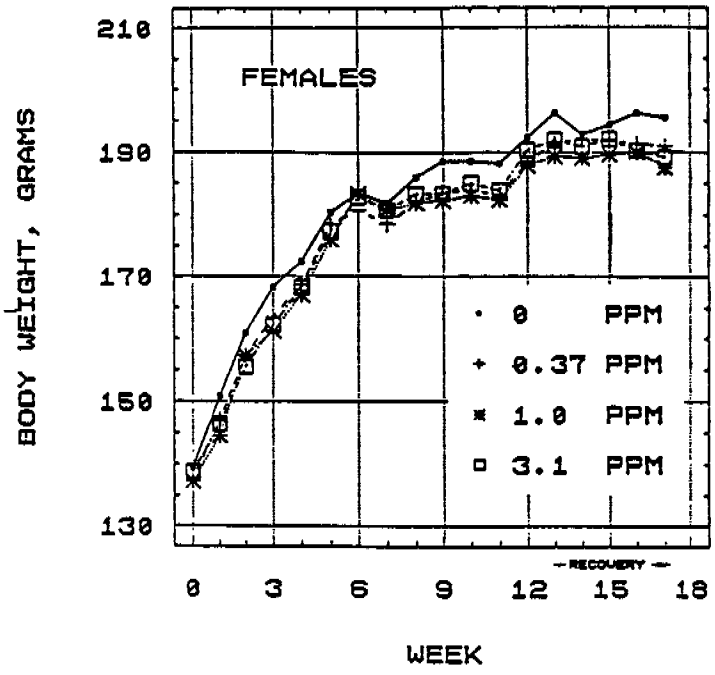


FIGURE 2. Body weight gain of female Fischer 344 rats during a 13-week inhalation toxicity study of phosphine followed by a 4-week recovery period.

ppm (23%). Therefore, these appear to be exposure-related. However, this effect was transient as BUN was not elevated at 3.1 ppm after 13 weeks. A 17% increase in alkaline phosphatase was also seen in the 10 ppm group. The only other change that was possibly exposure-related was a decreased serum glutamic pyruvic transaminase (SGPT) seen in the 3.1 ppm males and females after 13 weeks of exposure. However, there is no known toxicological significance for a decreased SGPT and the effect may have been produced by two unusually high control animal values.

Table 2. Percent Difference in Body Weight Gain Between Control and Exposed Females During a Subchronic Inhalation Toxicity Study of Phosphine

Exposure level (ppm)	Week 1	Week 2	Week 3	Week4
0.37	−1.8	−1.8	−2.9	−1.9
1.0	−4.1**	−1.4	−4.7*	−3.1
3.1	−3.6**	−4.8**	−5.4**	−3.6*
5.1	−2.5	−8.1**	−7.1*	−3.0[a]

[a]First recovery week. *$p \leq .05$; **$p \leq .01$.

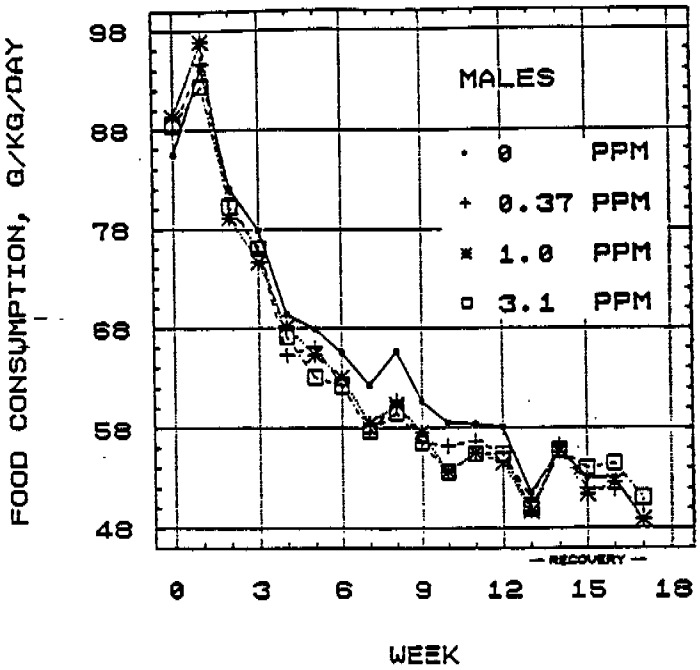


FIGURE 3. Food consumption of male Fischer 344 rats during a 13-week inhalation toxicity study of phosphine followed by a 4-week recovery period.

Other changes occurred sporadically and were not consistent over sex or time.

Terminal Organ and Body Weights and Organ/Body Weight Ratios Significant changes in absolute or relative kidney and liver weights occurred. The 12% increase in kidney weights in the 10 ppm satellite group correlated with the observed microscopic changes. There were also exposure-related, but not dose-related, decreases in male liver weights and ratios in the 0.37, 1, 3.1, and 10 ppm groups. The toxicological significance of decreased liver weights is unclear.

Pathology Gross postmortem observations were random except for an increased incidence of small seminal vesicles that were observed in the 1 and 3.1 ppm groups (6/10 and 5/10) after 13 weeks but not after the 4-week recovery period. This finding may be related to the test substance but because of a lack of a microscopic correlate its significance is equivocal.

Microscopically, treatment-related lesions occurred in the kidneys of animals of both sexes exposed at 10 ppm and consisted of coagulative necrosis of the tubular epithelium of tubules in the outer cortex, presumably the proximal convoluted tubules. These lesions were more se-

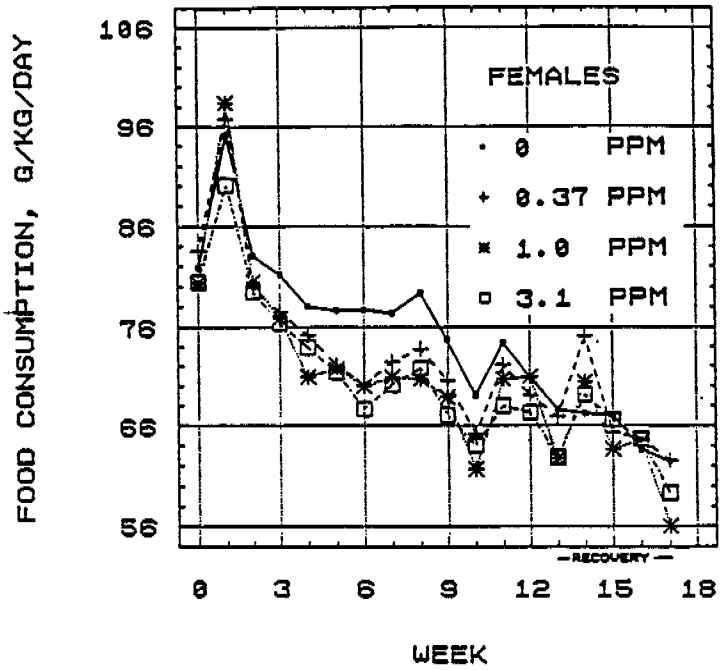


FIGURE 4. Food consumption of female Fischer 344 rats during a 13-week inhalation toxicity study of phosphine followed by a 4-week recovery period.

vere in females than males. Renal lesions were not noted in animals receiving 5.1 or 3.1 ppm of phosphine under the conditions of this study. Congestion was noted in the lungs of the female animals receiving 10 ppm of phosphine and dying during the study.

Following a 4-week recovery period, no treatment-related lesions were noted in any of the tissues examined from animals exposed to 3.1, 5.1, or 10 ppm phosphine. Spontaneous disease lesions and incidental findings were of the usual number and type commonly seen in Fischer 344 rats. They were essentially comparable between control and treated animals.

Developmental Toxicity—Maternal Parameters

Mortality Rates Exposure concentrations for the developmental study are presented in Table 1. No mortality occurred in the control or in the 0.03, 0.33, 2.8, or 4.9 ppm exposure groups. In the 7.0 ppm exposure group, the first 14 females treated died after 3–10 days of exposure (days 8–15 of gestation). Due to this mortality, this group was terminated.

Pregnancy Rates Pregnancy rates for the 0.03, 0.33, 2.8, and 4.9 ppm groups were comparable to control (Table 3).

Body Weight and Food Consumption No adverse effect of treatment at an exposure level to 4.9 ppm was evident from body weights or weight change data over the gestation period (Table 3). Likewise, food consumption data throughout gestation were not adversely affected by treatment to the 4.9 ppm exposure level (Table 3).

Physical Observations No adverse effect of treatment with phosphine at an exposure level to 4.9 ppm was evident from the detailed physical evaluations. Even at the 7.0 ppm exposure level, which was terminated early due to increased mortality, no adverse effect of treatment was evident from physical observation data.

Uterine Implantation Data No adverse effect of treatment with phosphine at an exposure level to 4.9 ppm was evident from uterine implantation data (Table 3). In the low-exposure group (0.03 ppm) the mean number of resorption sites, the mean resorption/implant ratio, and the incidence of females with resorptions were higher than control and these differences were statistically significant. The reason for this increase in resorption data at the 0.03 ppm exposure level was unclear; however, in the absence of similar responses in resorption data at the higher exposure levels evaluated, no adverse effect of treatment was indicated.

Gross Postmortem Evaluations No adverse effect of treatment up to an exposure level of 4.9 ppm was evident from the maternal gross postmortem examinations. Discoloration (reddening) of the lungs and liver was observed in some of the animals that died at the 7.0 ppm exposure level. This was not considered unusual in animals that die and are not exsanguinated before postmortem examination.

Developmental Toxicity—Fetal Parameters

Weight and Sex Distribution Data Mean fetal weights and the ratio of male/female fetuses per group for the phosphine-treated groups were comparable to that of control.

Malformation/Variation Data No adverse effect of treatment with phosphine to an exposure level of 4.9 ppm was evident from the fetal external, visceral, or skeletal evaluations (Tables 4 and 5). In the phosphine-treated groups, low incidences of malformations/variations on a per-fetus and per-litter basis were seen among all group. However, these data did not differ statistically from the control data and, due to the lack of similarity in the types of malformations seen among the phosphine-treated groups, no adverse effect of treatment was indicated.

DISCUSSION

Lethality was the primary hazard of phosphine exposure (7.0 ppm and above). The $C \times T$ product of 180 ppm-hr is similar to the results of

Klimmer (1969) who found a $C \times T$ product of 213-hr for male Wistar rats. Klimmer also found similar $C \times T$ products for cats, guinea pigs, rabbits, turkeys, and chickens. This similar interspecies $C \times T$ product may also hold for humans as Flury (cited in Klimmer, 1969) indicated that a single inhalation of 5–10 min duration should be lethal to humans at 1000 ppm (83–166 ppm-hr).

Table 3. Maternal Body Weight and Food Consumption Data, Uterine Implantation Data, and Fetal Weight Data in Rats Exposed Prenatally to Phosphine

Data	Exposure groups (actual levels, ppm)				
	I (0)	II (0.03)	III (0.33)	IV (2.8)	V (4.9)
No. females:					
Mated	24	24	24	24	24
Pregnant	22 (91.7%)	21 (87.5%)	24 (100%)	23 (95.8%)	23 (95.8%)
Maternal body weight data (g):					
Gestation day (GD) 0	234 ± 17[a]	236 ± 15	236 ± 19	237 ± 19	236 ± 18
GD 20 (actual)	360 ± 21	361 ± 21	360 ± 20	364 ± 24	368 ± 25
GD 20 (corrected)[b]	283 ± 16	288 ± 18	287 ± 18	289 ± 21	290 ± 21
Weight gain (g)					
GD 6–16	44 ± 7	44 ± 8	44 ± 21	42 ± 6	43 ± 15
GD 6–20[b]	20 ± 11	21 ± 9	25 ± 18	22 ± 9	23 ± 9
Maternal food consumption (g/kg/day):					
GD 0–6	84 ± 19[a]	86 ± 13	81 ± 21	87 ± 13	89 ± 22
GD 6–10	74 ± 8	72 ± 6	75 ± 11	72 ± 6	69 ± 10
GD 10–16	78 ± 5	79 ± 6	80 ± 12	78 ± 7	84 ± 18
GD 16–20	83 ± 6	86 ± 9	85 ± 8	86 ± 6	91 ± 8*
Per pregnant female:					
Corpora lutea (CL)	16.0 ± 2.0[a]	16.3 ± 1.9	16.0 ± 2.0	15.9 ± 2.5	16.1 ± 2.3
Implants (I)	15.4 ± 2.0	15.7 ± 1.8	14.7 ± 2.4	15.0 ± 2.8	15.4 ± 2.3
Preimplant loss index (CL-I CL)	0.04 ± 0.05	0.04 ± 0.06	0.08 ± 0.13	0.05 ± 0.11	0.04 ± 0.07
Live fetuses	14.9 ± 2.1	14.1 ± 1.6	14.0 ± 2.3	14.2 ± 2.7	14.7 ± 2.6
Resorptions (R)	0.5 ± 0.9	1.6 ± 1.2**	0.7 ± 1.0	0.9 ± 0.9	0.7 ± 1.0
R/I ratio	0.04 ± 0.05	0.10 ± 0.07*	0.05 ± 0.07	0.06 ± 0.06	0.05 ± 0.07
Litters with one or more resorption (%)	8 (36.4%)	16* (76.2%)	9 (37.5%)	14 (60.9%)	10 (43.5%)
Fetal weights (g)					
Males (M)	3.30 ± 0.23[a]	3.28 ± 0.31	3.31 ± 0.17	3.35 ± 0.25	3.30 ± 0.21
Females (F)	3.20 ± 0.21	3.11 ± 0.22	3.22 ± 0.19	3.20 ± 0.22	3.13 ± 0.22
Both sexes	3.24 ± 0.21	3.18 ± 0.25	3.25 ± 0.17	3.28 ± 0.23	3.22 ± 0.20
Ratio of total M/F:					
Fetuses	1.0	0.9	1.1	1.1	1.0

[a]Mean ± standard deviation.

[b]Corrected day 20 gestation body weight = actual day 20 gestation weight - the weight of gravid uterus.

*$p \leq .05$; **$p \leq .01$.

Table 4. Summary of Malformation Data in an Inhalation Developmental Toxicity Study of Phosphine in Rats

Data	Exposure group (actual levels, ppm)				
	I (0)	II (0.03)	III (0.33)	IV (2.8)	V (4.9)
No. fetuses (litters) evaluated:					
External	327 (22)	296 (21)	335 (24)	326 (23)	338 (23)
Visceral	161 (21)	151 (21)	174 (24)	167 (23)	175 (23)
Skeletal	158 (22)	146 (21)	163 (24)	159 (23)	163 (23)
	No. fetuses (litters) affected				
External examination:					
Filamentous tail	0 (0)	1[a] (1)	0 (0)	0 (0)	0 (0)
Micrognathia and small tongue	0 (0)	0 (0)	1[b] (1)	0 (0)	0 (0)
Edematous with curly tail	0 (0)	0 (0)	0 (0)	0 (0)	1[c] (1)
Visceral examination:					
Microophthalmia	0 (0)	1[d] (1)	0 (0)	1[e] (1)	0 (0)
Distended lateral ventricles of brain	0 (0)	1[d] (1)	0 (0)	0 (0)	0 (0)
Folded retina	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)
Cleft palate	0 (0)	0 (0)	0 (0)	1[e] (1)	0 (0)
Persistent truncus arteriosus	0 (0)	0 (0)	0 (0)	0 (0)	1[c] (1)
Absence of ductus arteriosus	0 (0)	0 (0)	0 (0)	0 (0)	1[c] (1)
VSD	0 (0)	0 (0)	0 (0)	0 (0)	1[c] (1)
Skeletal examinations:					
Absence of vertebrae (L, S, C)	0 (0)	1[a] (1)	0 (0)	0 (0)	0 (0)
Cervical rib	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Cranial bone defects	0 (0)	0 (0)	1[b] (1)	0 (0)	0 (0)
Vertebral defects alone)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
Vertebral rib defects	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
5 L vertebrae	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
Total (%) fetuses affected[f]					
External malformations	0 (0)	1 (0.3)	1 (0.3)	0 (0)	1 (0.3)
Visceral malformations	0 (0)	2 (1.3)	0 (0)	1 (0.6)	2 (1.1)
Skeletal malformations	0 (0)	1 (0.7)	2 (1.2)	3 (1.9)	0 (0)
Total (%) litters affected[f]					
External malformations	0 (0)	1 (4.8)	1 (4.2)	0 (0)	1 (4.3)
Visceral malformations	0 (0)	2 (9.5)	0 (0)	1 (4.3)	2 (8.7)
Skeletal malformations	0 (0)	1 (4.8)	2 (8.3)	3 (13.0)	0 (0)

[a]Same fetus having multiple malformations.
[b]Same fetus having multiple malformations.
[c]Same fetus having multiple malformations.
[d]Same fetus having multiple malformations.
[e]Same fetus having multiple malformations.
[f]No statistically significant differences in comparison to control data.
VSD, ventricular septal defect; L, lumbar region; S, sacral region; C, caudal region.

Table 5. Summary of Variation Data in an Inhalation Developmental Toxicity Study of Phosphine in Rats

Data	Exposure group (actual levels, ppm)				
	I (0)	II (0.03)	III (0.33)	IV (2.8)	V (4.9)
	No. fetuses (litters) affected				
External examination:					
Shiny appearance	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)
Visceral examination:					
Absence of innominate artery (aortic arch)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)
Ureters tortuous	4 (3)	0 (0)	2 (2)	2 (2)	2 (1)
Ureters distended	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Skeletal examination[a]:					
Cranial bones:					
Interparietal I	29 (14)	27 (14)	19 (10)	27 (13)	28 (15)
Supraoccipital I	32 (14)	29 (13)	9 (9)	23 (10)	27 (13)
Hyoid U	28 (10)	16 (7)	14 (9)	21 (13)	19 (10)
Parietal I	5 (4)	2 (1)	1 (1)	4 (2)	1 (1)
Vertebral observations:					
Cr TP-I	5 (4)	12 (7)	12 (8)	8 (5)	16 (9)
Th C-I	22 (10)	25 (13)	19 (10)	23 (14)	21 (13)
S TP-I	30 (16)	29 (10)	13 (10)	22 (11)	27 (14)
C TP-I	65 (20)	50 (17)	43 (17)	38 (15)	51 (21)
C TP-U	13 (7)	15 (7)	12 (8)	12 (6)	14 (7)
Sternebra					
4th I	12 (6)	11 (5)	8 (6)	6 (3)	5 (4)
5th U	59 (18)	54 (19)	50 (17)	51 (15)	61 (22)
6th U	7 (5)	13 (7)	12 (6)	6 (4)	19 (11)
Ribs 1st L rud	2 (2)	3 (3)	5 (4)	4 (4)	2 (2)
Total (%) fetuses with variations[b]					
External	0 (0)	1 (0.3)	1 (0.3)	0 (0)	0 (0)
Visceral	4 (2.5)	0 (0)	2 (1.1)	2 (1.2)	2 (1.1)
Skeletal	115 (72.8)	112 (76.7)	112 (68.7)	104 (63.4)	115 (70.6)
Total (%) litters containing affected fetuses[b]					
External	0 (0)	1 (4.8)	1 (4.2)	0 (0)	0 (0)
Visceral	3 (14.3)	0 (0)	2 (8.3)	2 (8.7)	1 (4.3)
Skeletal	22 (100)	21 (100)	23 (100)	22 (95.7)	22 (95.7)

[a]This is not a presentation of all ossification variation data seen during the study but summarizes those variations seen with greatest frequency among the control and/or treated group fetuses.

[b]No statistically significant differences relative to control.

I, incompletely ossified; U, unossified; Cr, cervical; Th, thoracic; L, lumbar; S, sacral; C, caudal; TP, transverse process(es); C, centrum; Rud, rudimentary structure(s).

The results from this study also confirm Klimmer's conclusion that a threshold or nonlinear lethality effect exists. If Haber's law of $C \times T$ = constant were true, the 5.1 ppm group should have died after approximately six 6-hr exposures. Instead, all animals in the 5.1 ppm group survived and were unremarkable at the scheduled sacrifice after 13 days of exposure. In the developmental study, 7.0 ppm was fatal to pregnant rats after 3–10 days of exposure. The median time to death, 4 days, also gives a $C \times T$ product of approximately 180 ppm-hr. Therefore the lethality threshold for repeated daily 6-hr exposures to phosphine is 5.1–7.0 ppm.

All other phosphine-related effects seen at sublethal exposure levels were relatively small and completely reversible. This included the effect on body weight gain, as also previously reported by Waritz and Brown (1975), and hematological effects, as also previously reported by Klimmer (1969) and Muller (1940). The congestion seen in the lungs and kidneys of animals that died was also similar to that previously reported in major organs by Muller (1940) and in the lungs by Muthu et al. (1980).

The results of this study indicate lethality to be the primary hazard from phosphine exposures. The current TLV of 0.3 ppm gives a safety factor of approximately 20.

REFERENCES

Klimmer, O. R. 1969. Contribution to the study of the action of phosphine (PH_3): the question of the so-called chronic phosphine poisoning. Springer-Verlag reprinted translation of "Beitragzur wirkung des phosphorwasserstoffes (PH_3)." *Arch. Toxicol.* 24:164–187.

Muller, W. 1940. Uber Phosphorwasserstaffvergiftung (Tierversuche). I. Mitt. Akute and Subakute Vergiftung. *Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak.* 195:184–193.

Muthu, M. M., Krishnakuman, M. K., Muralidhara, and Majumder, S. K. 1980. A study on the acute inhalation toxicity of phosphine to albino rats. *Bull. Environ. Contam. Toxicol.* 24:404–410.

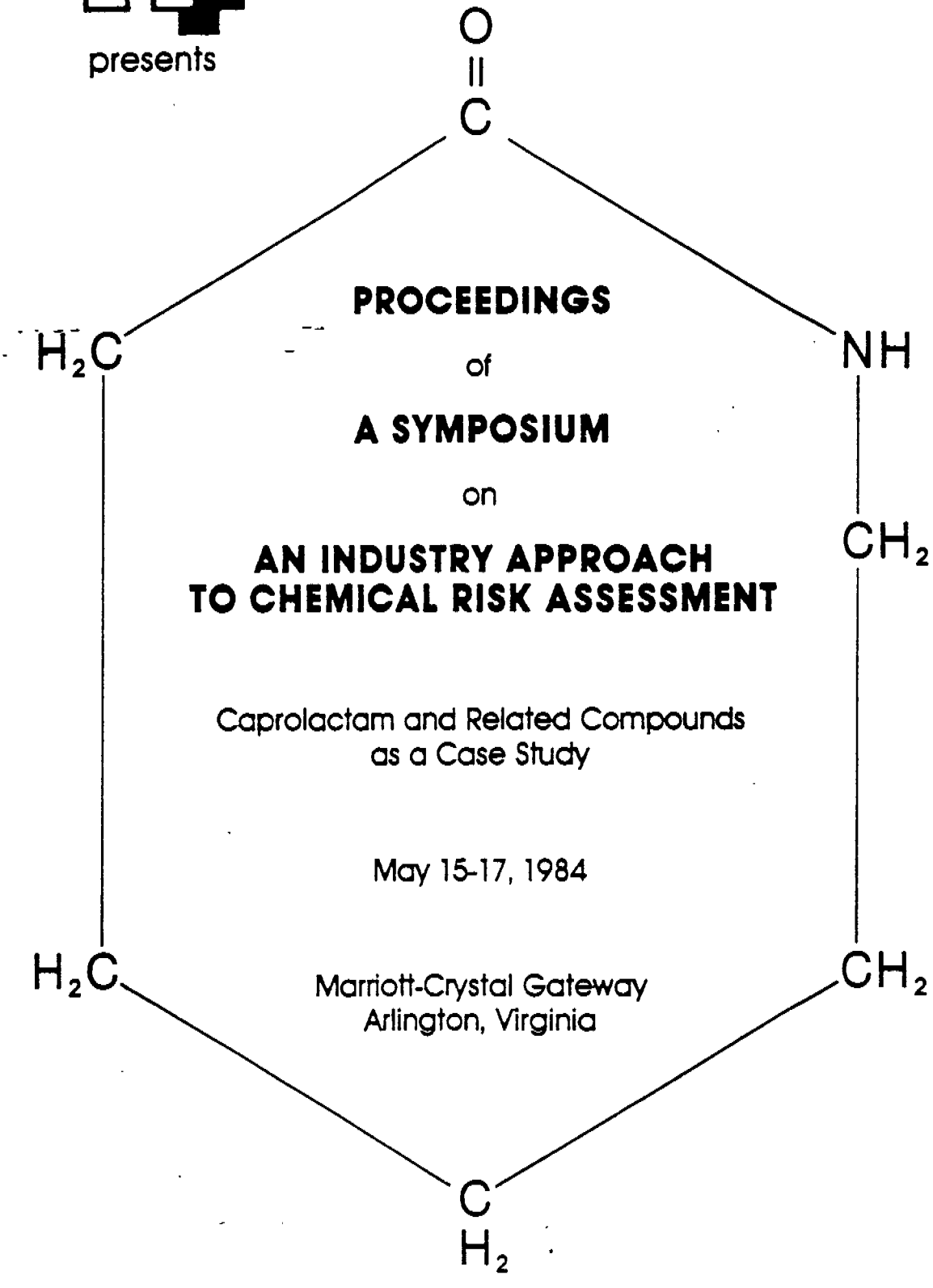
Salewski E. 1964. Farbemethode zum Makroskopischen Machweis von Implantationsstellen am Uterus der Ratte. *Arch. Pathol. Exp. Pharmakol.* 247:367.

Staples, R. E. 1974. Detection of visceral alterations in mammalian fetuses. *Teratology* 9:37A.

Waritz, R. S., and Brown, R. M. 1975. Acute and subacute inhalation toxicities of phosphine, phenylphosphine and triphenylphosphine. *Am. Ind. Hyg. J.* 36:452–458.

Zaebst, D. D., Blade, L. M., Burroughs, G. E., Morrelli-Schroth, P., and Woodfin, W. J. 1988. Phosphine exposure in grain elevators during fumigation with aluminum phosphide. *Appl. Ind. Hyg. J.* 3:145–154.

presents



PROCEEDINGS

of

A SYMPOSIUM

on

AN INDUSTRY APPROACH TO CHEMICAL RISK ASSESSMENT

Caprolactam and Related Compounds
as a Case Study

May 15-17, 1984

Marriott-Crystal Gateway
Arlington, Virginia

INDUSTRIAL HEALTH FOUNDATION
34 Penn Circle West • Pittsburgh, Pa. 15206

INHALATION TERATOLOGY STUDIES ON CYCLOHEXANONE

Raymond E. Schroeder, M.S.,* and Elton R. Homan, Ph.D.**

This inhalation teratology study of cyclohexanone was undertaken pursuant to a negotiated testing agreement with the Environmental Protection Agency and was performed by Bio/Dynamics Incorporated. The study was conducted in rats and mice. Findings with respect to rats are based on the draft report. Results of the mouse study are currently incompletely evaluated and only qualitative results will be presented at this time.

Slide 1

Cyclohexanone was provided by Allied Corporation and was subjected to appropriate analytical and stability evaluation. Vapor was generated using a Laskin generator and diluted with preconditioned room air. Chamber concentrations of cyclohexanone vapor were monitored hourly by infrared absorption.

Experimental animals were female Sprague-Dawley derived CD rats from Charles River Breeding Laboratories. This strain was chosen on the basis of previous experience, the existence of a broad data base and known sensitivity to acetylsalicylic acid, a known rat teratogen. The females were 69 days of age upon receipt and 83 days old at first mating to proven males, two females to one male. Successful mating was determined by vaginal smears. Microscopic finding of sperm permitted definition of Day 0 of gestation. Females were sacrificed on Day 20 of gestation.

Females were exposed to cyclohexanone vapor on gestational Days 6-19, inclusive, for six hours/day. Concentrations of 0, 300, 650 and 1400 ppm were selected based on previous experiments.

Animals were weighed on Day 0, 6, 15 and 20. Animals were examined twice daily for mortality and gross signs of toxicologic or pharmacologic effect and subjected to detailed physical examination on Days 0, 6, 10, 15 and 20. Early in the study, following observations of lethargy, routine evaluation of startle response was initiated while animals were in the chambers before, during and after exposure three times weekly.

Following sacrifice by lethal exposure to diethyl ether vapor on Day 20, all females were subjected to a complete necropsy. The intact uterus and ovaries were removed and weighed. Number and location were recorded for live and dead fetuses, early and late resorptions and implantation sites. The number of corpora lutea were recorded for each ovary.

---

* Manager, Reproduction/Teratology Section, Bio/Dynamics, Inc. , P.O. Box 43, East Millstone, NJ 08373

** Senior Toxicologist, Bushy Run Research Center, Union Carbide Corporation, R.D. #4, Mellon Road, Export, PA 15632

Each fetus was given a gross examination, weighed and sexed. Approximately one half the fetuses in each litter were examined for soft tissue malformations using the Staples microdissection technique. Heads were sectioned serially following fixation in Bouin's solution. The other half of the fetuses in each litter were stained with Alizarin Red S for skeletal malformations and ossification variations. Study results were analyzed statistically using appropriate parametric or nonparametric tests for interval data and incidence data.

Turning to the results, clinical observations included the finding of lacrimation initially on Day 6 in high level females only. The incidence and severity of this finding decreased over the course of the study. Also noted in this group were nasal discharge, lethargy and, on Day 15 only, vaginal discharge in several females. Observations of startle responses made during exposure disclosed a concentration and time related pattern. At 300 ppm there were a few instances of sluggish response. At 650 ppm the incidence was higher and became apparent as early as one hour after beginning of exposure. At 1400 ppm there was a high incidence of lethargy at one hour, and many rats were essentially non-responsive for the duration of daily exposure.

Slide 2

In this slide, and most of those which follow, cyclohexanone concentrations are shown as column headings, evaluation criteria shown on the left. Statistically significant differences from control are indicated by asterisks.

There were 26 females mated in each treatment group. None died during the course of the study and 23 or 24 became pregnant in each group. None aborted or delivered prematurely. At necropsy, the pregnant or non-pregnant status of each rat was confirmed and all pregnant females were found to be carrying viable fetuses.

Slide 3

There were no significant differences between maternal body weights at any time except at the 1400 ppm level. At 1400 ppm the weight differences with respect to controls were statistically significant at Day 15 and Day 20.

Slide 4

In this presentation of maternal body weight changes, the toxic effect of treatment at the 1400 ppm level is apparent from Day 6 on. During the critical period of organogenesis, Days 6-15 in the rat, the weight gain in this group is 44% less than the comparable control group value. For the period of Days 15-20, the high level weight gain is 30% less than control value.

Slide 5

The first row of this table compares the mean weight of the gravid uterus in each group. The reduction in the high level group is significant. In the next row, the corrected maternal body weight was calculated by subtracting the uterine weight from the Day 20 body weight shown on the previous table. Finally, the corrected body weight change for the interval from Day 6 to Day 20 was calculated by subtraction. Again the 1400 ppm group mean is significantly less than the control mean weight gain. Thus

the reduction in weight gain associated with the high level of cyclohexanone, which can be considered a maximally tolerated dose, is the result of reduced gains in both dams and fetuses.

Slide 6

Reproductive data are summarized in this table. No significant differences between treated groups are apparent with respect to numbers of corpora lutea, implantation sites, pups or resorptions. Highly significant reductions are apparent in mean body weights of pups of both sexes in the high level litters. There was no effect on sex ratio, reported conventionally as percent males.

Slide 7

Fetal effects are summarized in this table for the three types of examination used, showing incidences for both fetuses and litters. External malformations were absent or negligible in all groups. Visceral malformations were significantly lower with respect to fetal incidence in the 650 and 1400 ppm groups than in the control group. The predominant finding was slight distention of the renal pelvis. This is regarded as a minor malformation and has been commonly found in low incidence historically in control rats of this strain. These findings indicate no adverse treatment effect from fetal visceral evaluation.

The most commonly noted visceral variation was tortuous ureter. Visceral variations were less frequent in the mid level concentration of cyclohexanone and were absent in the high level group, thus no adverse treatment effect was found in this category.

Skeletal malformations are represented by one fetus with unilateral wavy ribs, one with unilateral fused ribs and thoracic vertebral malformations and one with a misaligned thoracic vertebral centrum. These three fetuses were in different litters and all were from the control exposure group. There were no skeletal malformations in fetuses from any treatment group.

The incidence of fetuses with various ossification variations was considered similar in the control, low and middle concentration groups. In the high concentration group, the incidence of fetuses with incomplete ossification of the cranial bones, and/or ossification irregularities of the hyoid was notably increased from control data. Likewise, at the high concentration, the incidence of incomplete or unossified sternebrae and unossified metatarsals and forelimb phalanges was also increased. Thus, at the high concentration, there was a generalized retardation of ossification.

With respect to fetal examinations, then, evaluation of fetuses recovered from females treated at 300 or 650 ppm of cyclohexanone for external, visceral or skeletal malformations did not reveal an increase in malformations. At the 1400 ppm level, no increase in malformations was evident during fetal external visceral or skeletal examinations. However, the incidence of fetuses with at least one ossification variation was increased at this concentration and some retardation in ossification was indicated, particularly in cranial ossifications, sternebrae and phalanges. These are regarded as evidence of fetotoxicity rather than teratogenicity.

Slide 8

To summarize the results of this study of the teratologic potential of cyclohexanone vapor in rats, there were no treatment effects at 300 or 650 ppm other than transient lethargy. Detrimental effects seen at 1400 ppm included maternal toxicity reflected in significantly reduced body weight gain, and transient lacrimation, lethargy and nasal discharge. Fetotoxicity was indicated by reduced fetal weight gain and an increased incidence of variations in and retardation of ossification processes. There was no evidence of teratogenicity.

Slide 9

This synopsis of an as yet incompletely evaluated mouse teratology study of cyclohexanone shows a brief outline of the experimental design and preliminary findings. CD-1 mice, 30 mated females per group were exposed to control air or 1400 ppm of cyclohexanone for 6 hours/day during gestation Days 6-17 and sacrificed on Day 18.

Preliminary findings include lacrimation, nasal discharge, marked lethargy and a white material, possibly a discharge, on the eyes of the females. Maternal weight was reduced on gestation Day 18, and weight gain in the interval from Day 6 to Day 18. The number of live fetuses per litter was reduced and the number of resorptions per litter increased. Fetal body weight was reduced.

There were no treatment related external or soft tissue abnormalities. Although fused ribs were noted in two fetuses from treated dams, changes of similar severity were seen in control fetuses. There was an increased incidence of ossification variations, representing retarded development. There was no evidence of teratogenicity.

In closing we would like to acknowledge the assistance of Dr. Rochelle W.Tyl, Manager of Teratology, Bushy Run Research Center, in the preparation of this material.

Slide 1

Cyclohexanone Teratology Study in Rats

Experimental Design

Route: Inhalation

Test System: CD Rats (26 females/group)
83 days old at mating
Sacrificed on gestational day 20

Regimen: 0, 300, 650, 1400 ppm
6 hours/day daily on gestational days 6-19

Observations:

In Life - Physical/behavioral signs
Maternal body weight and body weight change

Postmortem - Maternal necropsy

Reproductive system exam:

- Live fetuses
- Dead fetuses
- Resorptions
- Implantation sites
- Corpora lutea

Fetal exam:

- Body weight
- Sex
- External
- Soft tissues (Staples)
- Skeleton

Slide 2

Cyclohexanone Teratology Study in Rats

Maternal Survival and Pregnancy Status

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
Females mated	26	26	26	26
Died	0	0	0	0
Pregnant	24	23	24	23
Aborted	0	0	0	0
Delivered premature	0	0	0	0
Examined at scheduled necropsy	26	26	26	26
Nonpregnant	2	3	2	3
Pregnant	24	23	24	23
With viable fetuses	24	23	24	23

Slide 3

Cyclohexanone Teratology Study in Rats

Mean Maternal Body Weight During Gestation

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
Day 0	273 g	274 g	273 g	274 g
Day 6	307	310	309	310
Day 15	350	357	346	333 *
Day 20	420	432	418	383 **

* $p < .05$
** $p < .01$

Slide 4

Cyclohexanone Teratology Study in Rats

Mean Maternal Body Weight Change During Gestation

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
Days 0-6 Preexposure	34 g	36 g	36 g	36 g
Days 6-15 Organogenesis	43	47	37	24 **
Days 15-20	70	75	71	49 **
Days 6-20	113	122	108	73 **

** $p < .01$

Slide 5

Cyclohexanone Teratology Study in Rats

Mean Uterine Weight and Net Maternal Body Weight Change

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
Gravid Uterine Weight (GUW)	80.2 g	87.0 g	80.0 g	59.9 g **
Corr BW (BW - GUW)	339.5	346.6	338.9	323.0
Corrected BW Change D6-20	32.8	35.5	29.3	13.2 **

** $p < .01$

Slide 6

Cyclohexanone Teratology Study in Rats

Summary of Reproductive Data

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
Corpora lutea per dam	15.9	17.4	16.3	15.8
Implant sites per dam	14.8	15.8	14.7	14.3
Preimplantation loss	7.1%	9.0%	10.0%	9.6%
Mean litter size	13.8	15.1	13.6	13.3
Resorptions per dam	1.0	0.7	1.1	1.0
Litters with 1 or more resorptions	10	10	16	15
Mean body weight (g)				
Viable fetuses	3.67	3.66	3.68	3.73 **
Male fetuses	3.77	3.80	3.76	3.83 **
Female fetuses	3.56	3.53	3.58	3.64 **
Percent males	48.6	48.3	53.4	52.9

** $p < .01$

Slide 7

Cyclohexanone Teratology Study in Rats

Summary of Fetal Effects

	Cyclohexanone Concentration			
	0 ppm	300 ppm	650 ppm	1400 ppm
External malformations (% affected)				
Fetuses	0.0	0.6	0.0	0.0
Litters	0.0	8.7	0.0	0.0
Visceral malformations (% affected)				
Fetuses	6.4	4.4	0.6 **	0.6 **
Litters	29.2	26.1	4.2	4.3
Visceral variations (% affected)				
Fetuses	5.8	5.0	0.6	0.0 **
Litters	29.2	17.4	4.2	0.0 **
Skeletal malformations (% affected)				
Fetuses	1.9	0.0	0.0	0.0
Litters	12.5	0.0	0.0	0.0
Skeletal variations (% affected)				
Fetuses	84.2	89.3	87.3	97.9 **(a)
Litters	100.0	100.0	100.0	100.0

* $p < .05$
** $p < .01$

(a) Incompletely ossified cranial bones, unossified or incompletely ossified sternebrae and unossified metatarsals and forelimb phalanges.

Slide 8

Cyclohexanone Teratology Study in Rats

Summary of Effects

No significant treatment effects at 300 or 650 ppm

Detrimental effects seen at 1400 ppm:

- Maternal toxicity -
  - Reduced body weight gain
  - Lacrimation, lethargy and nasal discharge
- Fetotoxicity -
  - Reduced fetal weight gain
  - Increased incidence of ossification variations

No evidence of embryotoxicity or teratogenicity

Slide 9

Cyclohexanone Teratology Study in Mice

Experimental Design:

CD-1 mice

Inhalation concentrations 0, 1400 ppm

6 hr/day gd 6-17

Sacrifice gd 18

Preliminary findings:

Maternal lacrimation, nasal discharge, marked lethargy, white material on eyes

Maternal body weight reduced gd 18 only

Maternal weight gain reduced gd 6-18

Number live fetuses/litter reduced

Number and % resorptions/litter increased

Fetal body weight reduced

No treatment related external or soft tissue abnormalities

Skeletal abnormalities - fused ribs in 2 fetuses

Increased incidence of ossification variations

No evidence of teratogenicity

ERH/vlt/2240
06/20/84