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Methylene Chloride: A Two-Year Inhalation Toxicity and Oncogenicity Study in Rats and Hamsters[1]

J. D. BUREK,[2] K. D. NITSCHKE, T. J. BELL, D. L. WACKERLE, R. C. CHILDS, J. E. BEYER, D. A. DITTENBER, L. W. RAMPY, AND M. J. MCKENNA

Toxicology Research Laboratory, Health and Environmental Sciences USA, Dow Chemical U.S.A., Midland, Michigan 48640

Methylene Chloride: A Two-Year Inhalation Toxicity and Oncogenicity Study in Rats and Hamsters. BUREK, J. D., NITSCHKE, K. D., BELL, T. J., WACKERLE, D. L., CHILDS, R. C., BEYER, J. E., DITTENBER, D. A., RAMPY, L. W., AND MCKENNA, M. J. (1984). *Fundam. Appl. Toxicol.* **4**, 30–47. A long-term study was conducted to determine the possible chronic toxicity and oncogenicity of methylene chloride. Rats and hamsters were exposed by inhalation to 0, 500, 1500, or 3500 ppm of methylene chloride for 6 hr per day, 5 days a week, for 2 years. No exposure-related cytogenetic effects were present in male or female rats exposed to 500, 1500, or 3500 ppm. Females rats exposed to 3500 ppm had an increased mortality rate while female hamsters exposed to 1500 or 3500 ppm had decreased mortality rates. Carboxyhemoglobin values were elevated in rats and hamsters exposed to 500, 1500, or 3500 ppm with the percentage increase in hamsters greater than in rats. Minimal histopathologic effects were present in the livers of rats exposed to 500, 1500, or 3500 ppm. Decreased amyloidosis was observed in the liver and other organs in hamsters exposed to 500, 1500, or 3500 ppm. While the number of female rats with a benign tumor was not increased, the total number of benign mammary tumors was increased in female rats in an exposure-related manner. This effect was also evident in male rats in the 1500- and 3500-ppm exposure groups. Finally, male rats exposed to 1500 or 3500 ppm had an increased number of sarcomas in the ventral neck region located in or around the salivary glands. Therefore, in this 2-year study, some effects were observed in male and female rats exposed to 500, 1500, or 3500 ppm of methylene chloride. In contrast, hamsters exposed to the same exposure concentrations had less extensive spontaneous geriatric changes, decreased mortality (females), and lacked evidence of definite target organ toxicity.

Methylene chloride (CH_2Cl_2) is widely used in a variety of medical, industrial, and commercial applications. Major uses include paint stripping, solvent extraction in food processing, flexible polyurethane foam production, and as an aerosol propellent.

Inhalation toxicity studies of methylene chloride have been performed in a variety of laboratory animals (Heppel *et al.*, 1944; Heppel and Neal, 1944). Several workers have shown that continuous exposure to methylene chloride produced adverse effects at markedly lower levels than in intermittent exposures (Thomas *et al.*, 1971; Weinstein *et al.*, 1972; Bullock *et al.*, 1971; Haun *et al.*, 1971, 1972; McEwen *et al.*, 1972) and in most of these studies, the liver was the main target organ.

The fate of ^{14}C-labeled methylene chloride in rats and mice, has also been studied by several investigators following intraperitoneal injection (DiVincenzo and Hamilton, 1975), oral administration (McKenna and Zempel, 1980; Yesair *et al.*, 1977), or inhalation ex-

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[2] Present address: Merck Sharp & Dohme Research Laboratories, West Point, Pa. 19486. To whom correspondence should be addressed.

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posure (McKenna *et al.*, 1979). The data from these studies indicate that methylene chloride is primarily metabolized to carbon monoxide and carbon dioxide, both of which are excreted in expired air. Metabolism of methylene chloride to carbon monoxide takes place in the microsomal fraction of the liver and requires oxygen and NADPH for optimal activity (Kubic *et al.*, 1974; Kubic and Anders, 1975; Ratney *et al.*, 1974). Repeated administration of methylene chloride does not affect the rate of metabolism to carbon monoxide or subsequent carboxyhemoglobin formation (Kubic *et al.*, 1974; Hogan *et al.*, 1976). Furthermore, Stevens *et al.*, (1980), have shown that biotransformation of methylene chloride to carbon monoxide *in vitro* is unaffected by common inducers of mixed-function oxidase metabolism.

The biotransformation of methylene chloride to carbon dioxide has been studied (Anders *et al.*, 1977). The proposed metabolic pathway for this process involves the cytosol fraction of the liver and requires glutathione as a cofactor. These authors have postulated the existence of transient intermediates which include formaldehyde and formic acid.

Stewart *et al.* (1972a,b), reported that inhalation exposure to methylene chloride resulted in increased carboxyhemoglobin levels in man. Subsequent studies (Stewart *et al.*, 1974; McKenna *et al.*, 1980) of human volunteers under controlled exposure conditions have demonstrated that metabolism of methylene chloride to carbon monoxide is a dose-dependent phenomenon in man, and a maximum of only 10–12% carboxyhemoglobin is achievable with exposures up to 500 ppm methylene chloride for as long as 7½ hr. No evidence for accumulation of carboxyhemoglobin levels was found in humans repeatedly exposed to methylene chloride (Stewart *et al.*, 1974).

Methylene chloride was not teratogenic in either rats or mice at exposure concentrations as high as 4500 ppm (Schwetz *et al.*, 1975; Hardin and Manson, 1980).

A human epidemiology study revealed no adverse health effects or increased frequency of tumors in employees occupationally exposed to methylene chloride (Friedlander *et al.*, 1978).

The mutagenic potential of methylene chloride to salmonella tester strains has been studied extensively. Methylene chloride was reported to be mutagenic in the Ames' test with two tester strains (TA97 and TA100) of *Salmonella typhimurium;* however, metabolic activation with rat liver homogenates did not appear to alter the number of the mutations observed (Green, 1980). Bacterial metabolism of methylene chloride by an oxidative pathway was the primary cause of the positive result; methylene chloride is metabolized to an intermediate which is so unstable that in the Ames' test this intermediate is effective only when produced in the bacterial cell. The mutagenic potential of methylene chloride has also been studied in *Drosophila* by the recessive lethal test (Abrahamson and Valencia, 1978; Filippova, 1967) with no demonstrable mutagenic potential.

No data on the chronic toxicity or oncogenicity of methylene chloride were found in the literature. This 2-year inhalation study was conducted in rats and hamsters to assess the chronic toxicity and oncogenic potential of methylene chloride following inhalation exposure to 0, 500, 1500, or 3500 ppm for 6 hr/day, 5 days/week. Preliminary results were previously presented (Rampy *et al.*, 1979). This report summarizes the final results of that study which was cosponsored by Diamond Shamrock Corporation, Dow Chemical U.S.A., Imperial Chemical Industry Ltd. (U.K.), Stauffer Chemical Company, and Vulcan Materials Company.

METHODS

Experimental design. A total of 1032 rats (129/sex/exposure concentration) and 866 hamsters (107 to 109/sex/exposure concentration) were exposed by inhalation to 0, 500, 1500, or 3500 ppm of methylene chloride. Exposures were 6 hr/day, 5 days/week, excluding holidays, for 2 years. Approximately 95 rats and hamsters/sex/exposure concentration were part of the chronic toxicity

and oncogenicity portion of the study. The remaining animals were sacrificed as part of the cytogenetic studies (rats only) or for one of the interim kills at either 6, 12, 15 (rats only), or 18 months.

Test material, vapor generation, and chamber analysis. Technical grade material was used throughout the exposure and was determined by gas chromatographic analysis to be greater than 99% pure methylene chloride.

The nominal concentration of methylene chloride vapor in the chamber was determined from the rate at which the liquid methylene chloride was dispensed and the total chamber air flow. The concentration of methylene chloride was measured at least 3 times/day for each chamber. The daily time weighted average concentration for each chamber was calculated.

Animals and animal husbandry. Male and female Sprague–Dawley rats (Spartan SD rats, SPF derived, from Spartan Research Animals, Inc., Haslett, Mich.) and male and female Golden Syrian Hamsters [Ela: Eng (Syr) from Engle Laboratory Animals, Inc., Farmersburg, Ind.] were used in this study. When they arrived, the rats and hamsters were approximately 6 weeks of age. They were acclimated for approximately 2 weeks and were assigned to exposure groups using a computer-derived randomization procedure. All animals were placed in wire-bottom stainless-steel cages. Hamsters were placed 3 to 4 per cage for the first year, then housed individually for the remainder of the study. Rats were housed no more than three per cage.

The chambers used were $8 \times 8 \times 8$-ft rooms (14.5 m^3) with stainless-steel ceilings in the shape of a regular quadrangular pyramid and walls and floors coated with epoxy resin. They were operated under dynamic air flow conditions with temperature and humidity controlled air and were maintained on a 12-hr light/dark cycle.

During the nonexposure periods of the study, the animals were kept in the same chambers with a filtered air supply. The controls for the study were also kept in a similar chamber, but were exposed to filtered air only. Food (Purina Laboratory Chow, Ralston Purina, St. Louis, Mo.) was removed from all animals during exposure, but was provided *ad libitum* during the nonexposure periods of the study. Water was provided *ad libitum* throughout the study by means of an automatic watering system.

In-life observations and palpable masses. Rats and hamsters were observed daily during the work week for general health status and signs of possible toxicity. Moribund and dead animals were culled daily. In addition, all rats and hamsters were palpated monthly for palpable masses starting by the third month of the study and continuing thereafter for the duration of the study.

Body weights. A subgroup of 50 rats and hamsters/sex/exposure concentration were weighed weekly for the first 8 weeks of the study and monthly thereafter. In addition, animals designated for the 6-, 12-, 15- (rats only), or 18-month interim kills, as well as all animals killed at the termination of the study, were also weighed after fasting overnight prior to necropsy.

Hematology. Blood samples for hematologic determinations were collected from the severed cervical vessels of hamsters at the time of necropsy and from the tail veins of rats prior to necropsy at the 6-, 12-, 15- (rats only), 18-, and 24-month kills. The hematological determinations included packed cell volume (PCV), total erythrocyte count (RBC), total and differential leukocyte counts (WBC), and hemoglobin (Hgb) concentration. These techniques were performed using either automated[3] or manual procedures.

The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated on all animals using the total erythrocyte count (RBC), hemoglobin (Hgb) concentration, and packed cell volume (PCV) data. Additionally, a reticulocyte count was performed on all animals from the 18-month kill and from a maximum of 10 animals/sex/dose from the 24-month kill.

Clinical chemistries. Blood for serum was collected from rats and hamsters following decapitation at necropsy from all the animals at the 6-, 12-, 15- (rats only), and 18-month kills. Serum samples from a maximum of 10/sex/dose were saved at the 24-month kill. Alkaline phosphatase (AP) and serum glutamic pyruvic transaminase (SGPT) activities and blood urea nitrogen (BUN) levels were determined on all samples.[4] In addition, total protein and albumin were determined on all hamster samples from the 12-, 18-, and 24-month kills.[5] Serum was obtained by orbital sinus puncture after 30 days of exposure from 10 rats/sex from the control and 3500-ppm exposure groups for determination of SGPT activity.[5]

Urinalysis. Urinalysis was performed on all animals from the 6-, 12-, 15- (rats only), and 18-month kills, and from a maximum of 10 animals/sex/dose from the 24-month terminal kill. Specific gravity,[5] pH, glucose, ketones, bilirubin, occult blood, protein, and urobilinogen were determined on all samples.[6]

Carboxyhemoglobin determinations. Blood samples for carboxyhemoglobin determinations were obtained from the tail veins of 4 rats/sex/exposure level following 6, 11, 18 (females only), and 21 months (except the 1500-ppm males) of exposure. Orbital bleeding was used to obtain blood samples for carboxyhemoglobin determinations from 4 hamsters/sex/exposure level following 22 months of exposure. Because of differences in male and female hamster carboxyhemoglobin values, additional hamsters were ordered and exposed to one 6-hr exposure at the

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[3] Coulter Counter Model ZBI, Coulter Electronics, Hialeah, Fla.

[4] Technicon AutoAnalyzer, Technicon Corp., Tarrytown, N.Y., and CentrifiChem System 400, Methods File, Union Carbide, Rye, N.Y.

[5] T. S. Meter, American Optical Company, Buffalo, N.Y.

[6] Ames Bililabstix, Ames Company, Elkhart, Ind.

same methylene chloride concentrations and in the same chambers as the 2-year animals. Blood was obtained by orbital bleeding from a maximum of 6 hamsters/sex/exposure concentration. Blood carboxyhemoglobin was determined spectrophotometrically (Amenta *et al.*, 1963).

Cytogenetic studies. Bone marrow cells were collected from 5 rats/sex/group for cytogenetic evaluation after 6 months of exposure. Bone marrow samples were processed by conventional techniques and examined for evidence of cytogenetic effects.

Pathological examinations. All animals were necropsied. Moribund rats and those at scheduled sacrifices were killed by decapitation following clamping of the trachea under methoxyflurane anesthesia. The eyes from all rats were examined by gently pressing a wet glass slide against the cornea and examining the eye under bright fluorescent illumination. The lungs and trachea from all animals were removed as a unit and expanded with phosphate-buffered 10% Formalin. All tissues were fixed in phosphate-buffered 10% Formalin (except as noted below for eyes).

At each of the interim and terminal kills, the eyes from a minimum of 5 animals/sex/exposure concentration were preserved in Zenker's fixative with the remaining eyes from all of the animals fixed in phosphate-buffered 10% Formalin. Prior to fixation, the weights of the brain, heart, liver, kidneys, and testicles were recorded from animals sacrificed at the interim and terminal kills.

Representative sections of organs and tissues were processed using conventional methods and were stained with hematoxylin and eosin. Special stains and electron microscopic evaluations were conducted on selected tissues or lesions as needed.

Statistical evaluation. Hematology, clinical chemistry, organ weight, and body weight data were evaluated using analysis of variance and the Dunnett's Test (Steel and Torrie, 1960). Statistical evaluation of tumor incidence was performed by Fisher's Exact Probability Test (Siegel, 1956). In addition, palpable mass data were evaluated using a modified Wilcoxon Test (Haseman and Hoel, 1974). The level of significance chosen for all cases was $p < 0.05$, the overall level of statistical significance is unknown because of the large number of statistical comparisons. The final interpretation of numerical data considered statistical analyses along with other factors such as dose–response relationships and whether the results are plausible in light of other biological and pathological findings.

RESULTS

Concentration of methylene chloride vapor in chambers. Rats were exposed to analytical concentrations of methylene chloride of 510 ± 27, 1511 ± 62, and 347 ± 145 while hamsters were exposed to 510 ± 27, 1510 ± 62,and 3472 ± 144 for the desired concentrations of 500, 1500, and 3500 ppm, respectively. Close agreement was observed between daily nominal and analytical values indicating minimal losses of the test material during vaporization and no detectable decomposition.

In-life observations. During the first week of exposure to 3500 ppm methylene chloride, the rats exhibited a slight decrease in physical activity; this effect disappeared after approximately 1 week. No other exposure-related effects due to methylene chloride were observed. However, a disease consistent with sialodacryoadenitis (SDA virus), a transient viral infection, was observed in male and female rats during the first 2 months of the study. This infection did not result in increased mortality and all exposure groups appeared to be affected to the same degree.

No exposure-related effects were observed in any of the male or female hamsters.

Mortality. Female rats exposed to 3500 ppm had statistically significantly increased mortality from the 18th through the 24th month which appeared to be exposure related (Fig. 1). Female hamsters exposed to 3500 ppm had a statistically significantly decreased mortality from the 13th through the 24th month and female hamsters exposed to 1500 ppm had statistically significantly decreased mortality from the 20th through the 24th month (Fig. 2). No exposure-related effects occurred in the other groups of rats or hamsters.

Body weights. There were no exposure-related alterations in body weight in either male or female rats or hamsters.

Organ weights and organ to body weight ratios. Mean liver weights were increased for both male and female rats of the 3500-ppm exposure groups at the 18-month interim kill. Both absolute and relative mean liver weights were statistically significantly increased for males, while only the mean relative weight was statistically significantly increased for females. Mean absolute liver weight was also increased for the 3500-ppm females although the increase was not statistically significant. No other absolute or relative organ weight

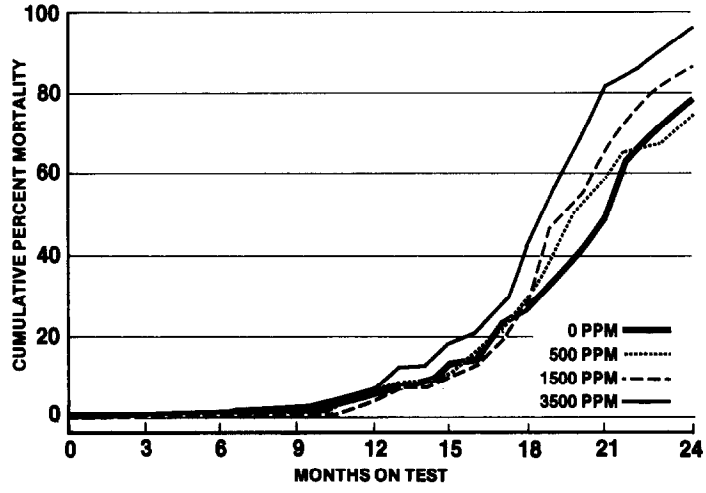

FIG. 1. Cumulative percentage mortality for female rats.

changes were observed that were considered to be related to the methylene chloride exposure.

No exposure-related effects were observed in mean fasted body weights and mean organ weights in male or female hamsters exposed to 500, 1500, or 3500 ppm.

Hematology. Mean hematologic values were determined and mean red blood cell indices were calculated. A few statistically significant increases and decreases were present; they occurred haphazardly among the exposure groups, and most values were within the range expected for these animals.

The red cell indices in rats may have been slightly altered. The MCV and MCH values at 15 months in males were both significantly increased at all exposure levels. In addition, the MCV and MCH values were consistently increased (not statistically) in exposed males and females compared to controls.

The PCV and Hgb data indicated possible exposure-related trends in the values for both male and female hamsters. For example, every group mean Hgb value was minimally increased over their respective control values in males and females from all exposure levels at each kill; however, only 8 of the values were

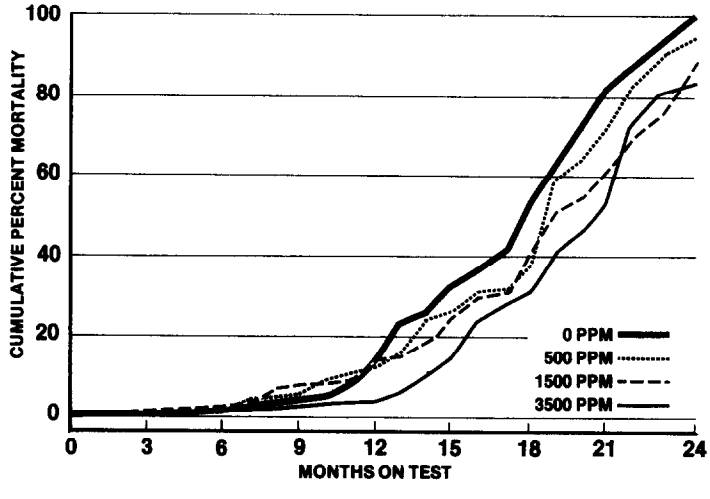

FIG. 2. Cumulative percentage mortality for female hamsters.

statistically significant. Similarly, all the PCV values for males and females (except for males exposed to 500 ppm and killed at 12 months) were elevated over their respective control values at all scheduled sacrifices.

Clinical chemistries and urinalysis. No exposure-related effects in clinical chemistry or urinalysis values were observed for any of the parameters evaluated throughout this investigation.

Carboxyhemoglobin. Male and female rats and hamsters exposed to 500, 1500, or 3500 ppm all had statistically significantly elevated carboxyhemoglobin values; however, there was no dose–response relationship (Table 1).

Cytogenetics. No increased cytogenetic aberrations were observed in rats exposed to 500, 1500, or 3500 ppm of methylene chloride for 6 months when compared to their respective control groups.

Pathologic observations in rats. In view of the voluminous nature of the pathology data, only selected histopathologic findings are summarized in Table 2.

Effects on the liver were observed in both males and females at all exposure levels at 12 months and beyond. All exposure groups had increased hepatocellular vacuolization (Fig. 3) which was consistent with fatty change. The number of rats with this change increased in an exposure-related fashion in both males and females. Male rats had 17, 38, 45, and 54% in the 0-, 500-, 1500-, and 3500-ppm exposure groups, respectively, while the females had 34, 52, 59, and 65%, respectively.

Multinucleated hepatocytes in the centrilobular region of the liver occurred in control and methylene chloride exposed females (Fig. 4). The cells were large with from a few to dozens of nuclei per hepatocyte. Ultrastructurally the cells had increased numbers of mitochondria that were larger than normal and contained intra-mitochondrial inclusions (Figs. 5 and 6a, b). Inhalation of methylene chloride resulted in a statistically significant increase in the number of female rats with these cells, but with no evidence of a dose-related increase in incidence or severity.

The number of foci and areas of altered

TABLE 1

SUMMARY OF CARBOXYHEMOGLOBIN VALUES IN RATS AND HAMSTERS

Exposure level (ppm)	Sex	Percentage carboxyhemoglobin (mean ± SD)								
		Rats					Hamsters			
		N	6 month	11 month	18 month	21 month	*N*	1 day	*N*	22 month
Control	Male	4	5.1 ± 4.0	0 ± 0	N.D.[a]	0.2 ± 0.4	6	3.0 ± 2.7	4	3.3 ± 3.5
500	Male	4	8.9 ± 0.6*	9.1 ± 0.3*	N.D.	13.0 ± 4.3*	6	24.1 ± 2.2*	4	28.4 ± 5.9*
1500	Male	4	10.1 ± 1.0*	9.3 ± 0.9*	N.D.	N.D.	6	33.5 ± 4.6*	4	27.8 ± 2.9*
3500	Male	4	8.8 ± 1.2*	8.7 ± 1.8*	N.D.	11.4 ± 3.7*	5	28.5 ± 5.6*	4	30.2 ± 4.9*
Control	Female	4	2.5 ± 0.9	1.4 ± 0.7	5.3 ± 2.0	0.4 ± 0.7	6	0.3 ± 0.8	4	4.0 ± 5.7
500	Female	4	9.5 ± 1.9*	12.3 ± 2.7*	20.4 ± 2.4*	12.8 ± 2.6*	4	22.2 ± 5.6*	4	23.6 ± 8.2*
1500	Female	4	7.7 ± 2.6*	14.0 ± 7.6*	19.1 ± 2.7*	14.8 ± 4.4*	6	27.7 ± 2.3*	4	30.2 ± 6.9*
3500	Female	4	9.1 ± 0.9*	10.5 ± 1.7*	18.2 ± 3.7*	12.2 ± 5.7*	6	23.2 ± 6.0*	4	34.6 ± 5.4*

[a] N.D. = No data.
* Significantly different from control value by Dunnett's test, $p < 0.05$.

TABLE 2

SUMMARY OF THE MAJOR EXPOSURE-RELATED HISTOPATHOLOGIC OBSERVATIONS IN RATS

	Males				Females			
Exposure concentration (ppm):	0	500	1500	3500	0	500	1500	3500
Number of rats examined:	92	95	95	97	96	95	96	97
Liver								
Individual hepatocellular necrosis	2	8	10*	11*	—[a]	—	—	—
Vacuolization consistent with fatty change	16	36*	43*	52*	33	49*	56*	63*
Coagulation necrosis	—	—	—	—	1	0	2	7*
Foci of altered hepatocytes	—	—	—	—	35	36	27	50*
Foci of altered hepatocytes, basophilic	—	—	—	—	3	0	4	10*
Area of altered hepatocytes	—	—	—	—	19	24	28	35*
Multinucleated hepatocytes	—	—	—	—	7	36*	34*	29*
Kidney								
Chronic progressive glomerulonephropathy								
Very slight or slight	4	11	16*	21*	32	46*	42	28
Moderate	19	18	24	30	25	15	13*	15
Severe	70	62	53*	39*	5	3	4	5
Total with any degree of chronic progressive glomerulonephropathy	93	91	93	90	62	64	59	48*
Pituitary								
Adenoma in anterior portion	—	—	—	—	34	24	30	16*
Mammary gland								
Total number of rats with a benign mammary tumor	7	3	7	14	79	81	80	83
Total number of benign mammary tumors**	8	6	11	17	165	218	245	287
Salivary gland region								
Sarcomas	1	0	5	11*	—	—	—	—

[a] —, No exposure-related effects.

* Significantly differed from control when analyzed by Fisher's Exact Probability Test, $p < 0.05$.

** Data could not be analyzed by Fisher's Exact Probability Test, $p < 0.05$.

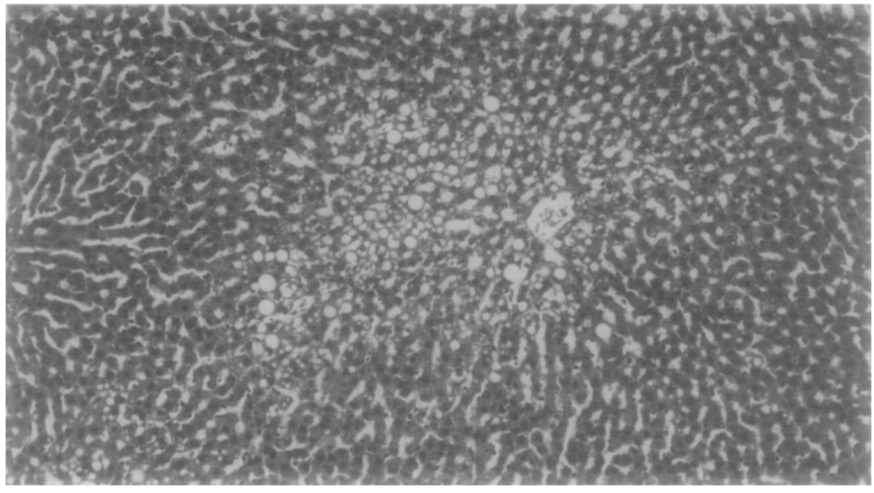

FIG. 3. Vacuolization in the liver of a female rat exposed to 3500 ppm. H&E stain, ×10.

hepatocytes (Fig. 7) were statistically significantly increased in female rats exposed to 3500 ppm, but were not increased in the groups of females exposed to 500 or 1500 ppm or in males exposed to 500, 1500, or 3500 ppm. The foci and areas were apparent after 12 months and their number and size increased during the exposure period, but the number of neoplastic (hyperplastic) nodules (Squire and Levitt, 1975), or hepatocellular carcinomas, was not increased in any exposure group.

Males exposed to 1500 or 3500 ppm had an increased incidence of individual hepatocellular necrosis, and females exposed to 3500

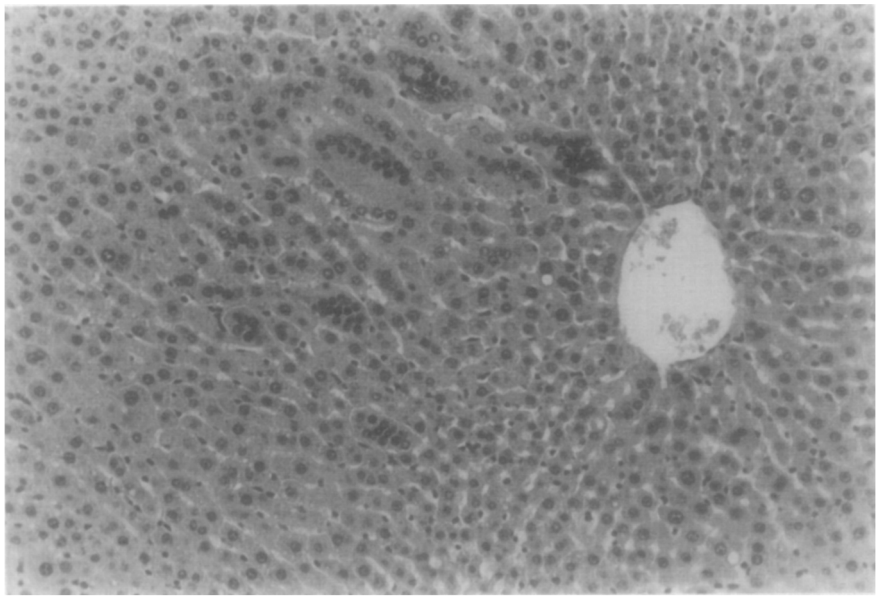

FIG. 4. Multinucleated hepatocytes in the liver of a female rat which had been exposed to 3500 ppm. H&E stain, ×20.

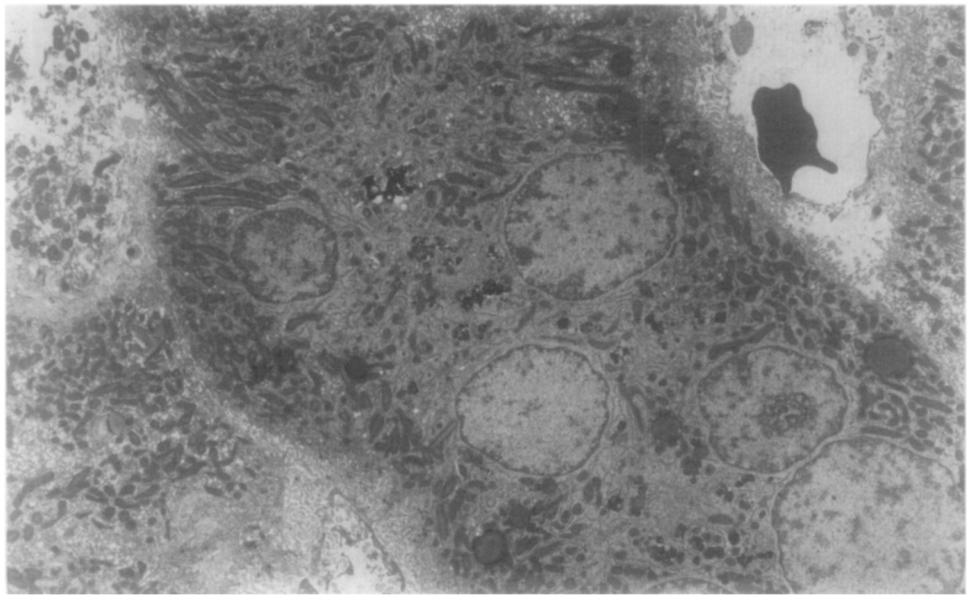

FIG. 5. Ultrastructural appearance of a multinucleated hepatocyte from a female rat exposed to 3500 ppm illustrating multiple nuclei and enlarged mitochondria. ×1620.

ppm had a slight increase in the number of cases of coagulation necrosis. Also, the livers of some male and female rats exposed to 1500 or 3500 ppm and some females exposed to 500 ppm for 12 months appeared to have slightly more hemosiderin pigment than controls.

Both male and female rats exposed to 3500 ppm and males exposed to 1500 ppm had either a decreased incidence (females) or decreased severity (males) of spontaneously occurring age-associated chronic renal disease (chronic progressive glomerulonephropathy) compared to controls. Furthermore, the nonrenal lesions (i.e., uremic pneumonitis, mineralization of multiple organs and blood vessels, periarteritis, parathyroid hyperplasia, fibrous osteodystrophy, brain malacia, and myocardial degeneration) that normally occurs secondarily in rats with severe renal disease (Burek *et al.*, 1979) was also reduced in the males exposed to 3500 ppm.

Males exposed to 1500 or 3500 ppm had a statistically significantly decreased incidence of grossly observed pancreatic nodules. Histologically, fewer islet cell adenomas, decreased blood vessel mineralization because of the decrease in severe renal disease, or less pancreatic acinar atrophy were observed especially in the 3500-ppm exposure group. As a result, there was a definite decrease in the total number of pancreatic lesions in the males exposed to 3500 ppm compared to controls with 39% of the controls not having any recognized histopathological lesion compared to 70% of those exposed to 3500 ppm.

Male rats exposed to 3500 ppm had a statistically significant increase in the total number of cases of increased extramedullary hematopoiesis (EMH) in the spleen and both males and females exposed to 3500 ppm had a significant decrease in the number with hematogenous pigment (hemosiderin) in the spleen.

Groups of female rats exposed to 500, 1500, or 3500 ppm of methylene chloride all had increased numbers of benign mammary tumors (Fig. 8) per tumor-bearing rat. The increase was based on palpable mass data and on gross examination. Histopathologic data

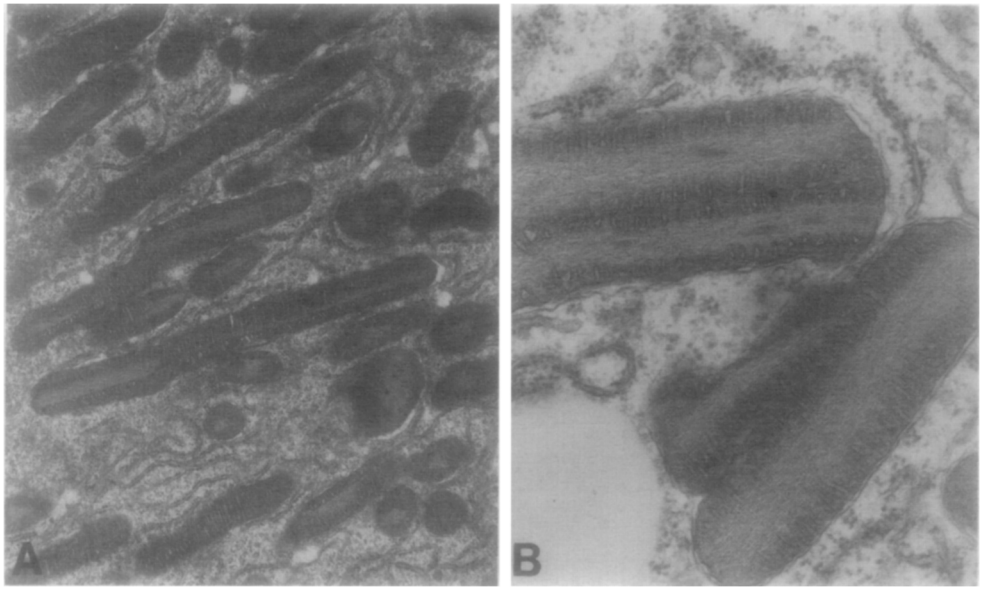

FIG. 6. Enlarged mitochondria in a multinucleated hepatocyte from a female rat exposed to 3500 ppm. Note the material separating the cristae and the parallel lamellar arrangement of the material (A) ×8300, (B) ×80,000.

provide the numbers of masses that were confirmed to be of mammary origin. The total number of female rats with a benign mammary tumor was not statistically significantly increased in any exposure group. However, the total number of benign mammary tumors increased in an exposure-related manner. The average number of benign mammary tumors per mammary tumor-bearing female rat increased from 2.1 in the control rats, to 2.7 in rats exposed to 500 ppm, to 3.1 in those exposed to 1500 ppm, and to 3.5 in rats exposed to 3500 ppm.

The benign mammary tumor response was present in males, but to a lesser extent than in females. There was an increase (not statis-

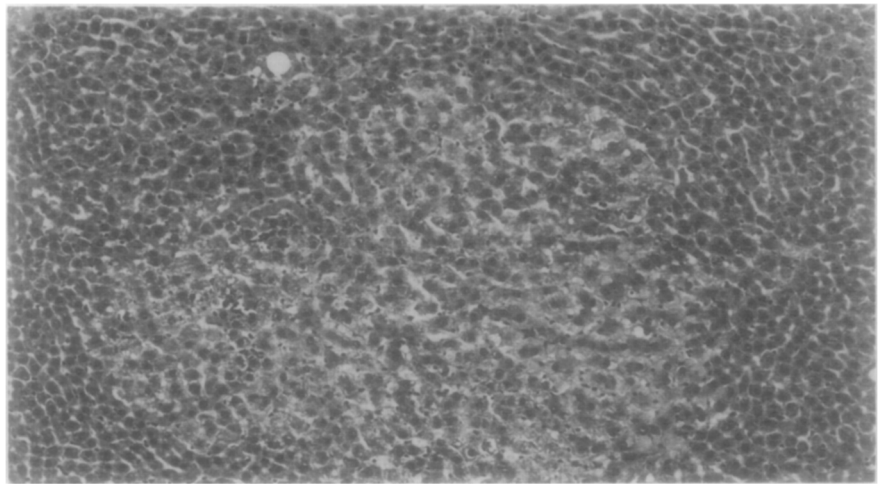

FIG. 7. Focus of altered hepatocytes in the liver of a female rat exposed to 3500 ppm. ×10.

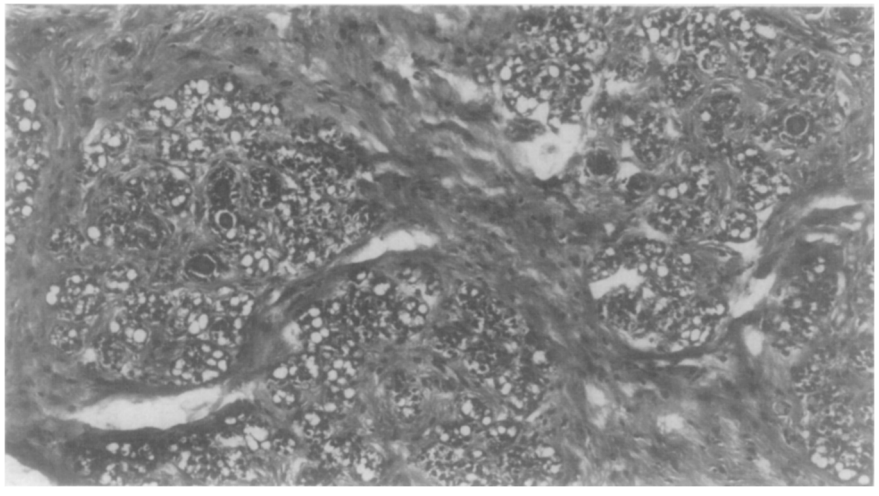

FIG. 8. Fibroadenoma of the mammary gland of a female rat exposed to 3500 ppm. ×10.

tically significant) in the number of rats with a benign mammary tumor in males exposed to 3500 ppm (14 compared to 7, 3, and 7 in the 0, 500, or 1500 exposure groups). There was a slight increase in the total number of benign mammary tumors in males exposed to 1500 or 3500 ppm. As was the case for females, these effects in males exposed to 1500 or 3500 ppm were exposure related.

Despite the increased numbers of benign mammary tumors, there was no indication of an increased number or incidence of malignant mammary tumors in either male or female rats exposed to methylene chloride, and thus no indication for progression of benign to malignant mammary tumors.

A second tumor type occurred in the ventral midcervical region, in and around the salivary gland, in male rats. There was 1 tumor in 124 male control rats compared to 0 of 124, 5 of 124, and 11 of 124 in the 500-, 1500-, and 3500-ppm exposure groups, respectively. These tumors were large (several centimeters in diameter), cystic, necrotic, or hemorrhagic. They appeared to invade all adjacent tissues in the neck region and often completely replaced the salivary glands. Histologically, all were sarcomas (Fig. 9). They were composed of round to spindle cells. Mitotic figures were frequently observed as were necrosis and local invasion into adjacent tissues. Most tumors had remnants of normal salivary acini or ducts within the area of cellular proliferation. Most tumors had some areas that morphologically resembled one cell type (i.e., fibrosarcoma), while other areas resembled another cell type (i.e., neurofibrosarcoma), and still other tumors had cell types that were undifferentiated or pleomorphic. In some, one cell type was predominant, while in others, areas of all of the above cell types were present depending on the area of the tumor examined. Furthermore, the origin of each of these tumors remains questionable. All appeared to be arising in the midcervical region and all involved the salivary glands. Two were relatively small masses and appeared to be arising in the interstitial and capsular tissue of the salivary glands. The rest were larger, invasive, and destructive tumors that clearly involved the salivary glands as well as adjacent tissues and could have been growing either into or out of the salivary glands.

Special stains (periodic acid–Schiff, Mallory's phosphotungstic acid, hematoxylin, Gordon and Sweet's reticulum, and Mayer's mucicarmine) showed no evidence of secretory material, but small thin reticulum-like fibers

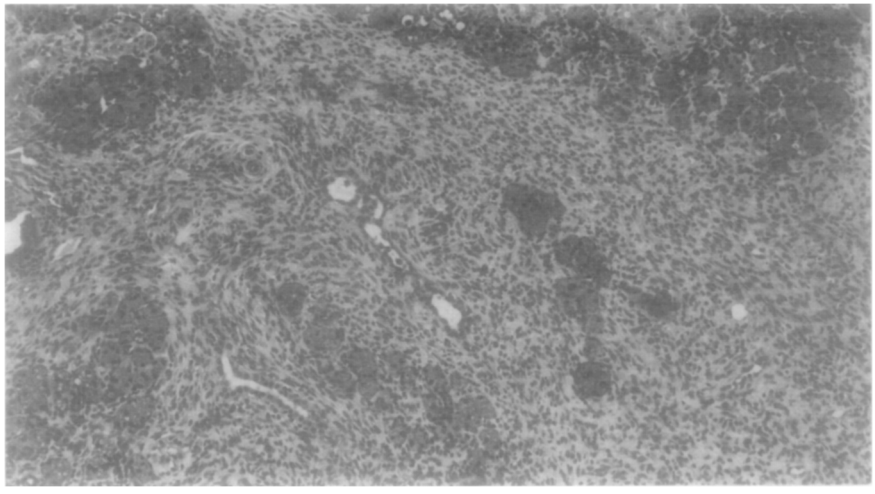

FIG. 9. Sarcoma in the salivary gland region of a male rat exposed to 3500 ppm. Normal acinar structures are present with tumor cells infiltrating through the gland. H&E stain, ×4.

were present around many, but not all, of the individual cells.

In addition, three sarcomas were examined ultrastructurally. Formalin-fixed tumor tissue was prepared for electron microscopy from the one male rat of the control group and from two males from the 3500-ppm exposure group. Ultrastructurally, the cells most resembled mesenchymal cells and contained prominent rough endoplasmic reticulum, polyribosomes, variable amounts of cytoplasmic fibrils, and a few mitochondria. Collagen was present between many of the tumor cells. As expected, the Formalin fixation interfered somewhat with the ultrastructural evaluation. However, prominent secretory elements were not a part of these cells and desmosomes were not found connecting tumor cells to each other.

Groups of females exposed to 500, 1500, or 3500 ppm had incidences of pituitary tumors of 24, 30, and 16%, respectively, compared to 34% in the controls. Decreased pituitary tumors may represent an exposure-related effect in female rats exposed to 500, 1500, or 3500 ppm.

The total number of rats with a tumor, the total with benign tumors, and the total with malignant tumors were evaluated. The cumulative numbers of all tumors, either benign or malignant, was no different in control or exposed groups of female rats.

Pathologic observations in hamsters. In view of the voluminous nature of the data, only selected findings are summarized in Table 3. All hamsters had some hemosiderin in the liver. At the 6-month kill, 5 of 5 males exposed to 3500 ppm had a larger amount of pigment compared to 0 of 5, 1 of 4 and 3 of 5 in the 0-, 500-, and 1500-ppm groups, respectively. This trend was not apparent in exposed females. The results at 12 months were similar and seemed to indicate a trend for more pigment in males exposed to 3500 ppm, since 5 of 5 males in the 3500-ppm group had a larger amount of pigment compared to 1 of 5, 1 of 5 and 3 of 5 in the 0-, 500-, and 1500-ppm groups, respectively. The toxicological significance of the increased hemosiderin is unclear; however, it is probably a slight exposure-related effect in the liver of male (but not female) hamsters exposed to 3500 ppm for 6 or 12 months.

Amyloid deposits were common in control hamsters, especially in females. The lesions and distribution were similar to reports of other investigators (Renshaw *et al.*, 1975; Dunham and Herrold, 1962; Fortner, 1957;

TABLE 3

SUMMARY OF THE MAJOR EXPOSURE-RELATED HISTOPATHOLOGIC OBSERVATIONS IN HAMSTERS

	Males				Females			
Exposure concentration (ppm):	0	500	1500	3500	0	500	1500	3500
Number of rats examined:	92	94	93	92	91	92	91	91
Liver								
Amyloid	48	22*	20*	15*	82	74	71*	64*
Biliary cyst(s)	42	37	30	29	47	33	37	36
Kidneys								
Total with any degree of chronic glomerulonephropathy probably due to amyloid	26	23	14*	18	66	57	54*	57
Adrenal gland								
Amyloid	47	19*	20*	10*	77	64*	65*	52*
Increased hemosiderin	30	46*	53*	53*	2	7	18*	12*
Foci of altered cells or spindle cell hyperplasia	23	37*	39*	38*	—[a]	—	—	—
Thyroid								
Amyloid	33	8*	14*	7*	68	55*	46*	39*
Spleen								
Total with any degree of amyloid	37	13*	15*	8*	79	57*	55*	57*

[a] —, No exposure-related effects.

* Significantly different from control when analyzed by Fisher's Exact Probability Test, $p < 0.05$.

Gleiser *et al.*, 1971; Russfield, 1965). In excess of 50% of the control males and 90% of the control females had amyloid in some organ. The severity varied greatly and many organs were commonly affected, including liver, kidneys, spleen, adrenal glands, and thyroid gland. Occasionally, amyloid was found in other tissues, including the gastrointestinal tract and lymph nodes.

Hamsters exposed to methylene chloride had less amyloid than controls (Table 3). The exposed hamsters had a decreased incidence as well as a reduced severity when compared to controls. This was evident in most of the exposure-related gross and histopathologic lesions, and usually with a dose–response relationship. Furthermore, control hamsters with severe amyloidosis often had many secondary lesions including atrophy of the genitalia, loss of body fat, and liver failure (including ascites, hydrothorax, pulmonary edema, and subcutaneous edema). These parameters were also decreased in exposed hamsters, probably because of the decreased severity of amyloidosis.

In addition to decreased amyloid, male and female hamsters exposed to methylene chloride had fewer biliary cysts in the liver. Male hamsters exposed to 3500 ppm had decreased histopathologically observed mineral deposits in the renal cortex or medulla. On the other hand, females exposed to 500, 1500, or 3500 ppm had increased mineral deposits in the kidneys. The significance of these findings is unclear. Male (500-, 1500-, and 3500-ppm groups) and female (1500- and 3500-ppm groups) hamsters had increased hemosiderin pigment in the adrenals. This pigment is common, but often becomes difficult to recognize if amyloid deposits are present. Males in all three exposure groups had increased small hyperplastic foci in the adrenal cortex. Amyloid was decreased in these groups and, as a result, may have made these lesions more easily recognized. However, the decreased amyloid may have also altered the occurrence of these lesions in the adrenals.

The total number of hamsters with a tumor, the number with a benign tumor, and the number with a malignant tumor were evaluated and no exposure-related differences were recognized in male hamsters. A statistically significant increase in the total number of benign tumors was observed in females exposed to 3500 ppm, but this was considered to be secondary to the increased survival of this group.

DISCUSSION

Inhalation exposure of rats and hamsters to 0, 500, 1500, or 3500 ppm of methylene chloride resulted in alterations in several of the parameters that were evaluated during the course of this 2-year study. Some of the altered values were of minor or equivocal significance while others were of greater toxicological significance. Furthermore, some were adverse effects while others could be considered beneficial or protective effects.

Exposure to 3500 ppm resulted in increased mortality in female rats during the last 6 months of exposure compared to control values. The mortality in the female rats exposed to 3500 ppm was probably caused by the numerous benign mammary tumors in this group. Even though these tumors were benign, they were often large and often became ulcerated. However, mortality was decreased in female hamsters exposed to 1500 or 3500 ppm and appeared related to the decreased incidence and severity of amyloidosis in exposed animals compared to controls.

The liver was the major target organ for toxicity in rats since exposure-related effects were present in male and female rats exposed to 500, 1500, or 3500 ppm of methylene chloride. Definite hepatic effects were present at the 12-month interim kill and beyond.

Male and female rats exposed to methylene chloride had increased numbers of benign mammary tumors per animal. Despite the increased number of benign mammary tumors, there was no indication of an increased number or incidence of malignant mammary tumors in either male or female rats exposed to

methylene chloride. The significance of this benign mammary tumor response is unknown. However, the Sprague–Dawley rats used in this study normally have a high incidence of mammary tumors. The incidence varies from study to study but normally exceeds 80% in females and about 10% in males in our laboratory. Therefore, the predisposition of this strain to these mammary tumors plus the high methylene chloride exposure concentrations may have resulted in an increased benign mammary tumor response.

Male rats exposed to 1500 or 3500 ppm had an increased number of sarcomas in the ventral midcervical area in the region of the salivary glands with 1, 0, 5, and 11 sarcomas in the 124 male rats per group exposed to 0, 500, 1500, or 3500 ppm, respectively. Based on routine sections, special stains, and ultrastructural evaluation, these tumors appeared to be of mesenchymal cell origin; although a myoepithelial cell origin of these cells could not be ruled out. All appeared to be arising in the midcervical region and all involved the salivary glands. Only 2 tumors were small enough to be localized within the salivary gland. The rest were larger tumors that clearly involved the salivary glands as well as adjacent tissues and could have been growing either into or out of the salivary glands. However, all probably arose within the salivary glands based on the two small tumors noted above and their similarity to tumors of this type which have been documented as arising in the salivary glands of rats (Glucksmann and Cherry, 1973) and mice (Dawe, 1979).

The relevance and toxicological significance of the increased incidence of sarcomas in the salivary gland region, in light of the presently available toxicity data on methylene chloride, are uncertain. There are several reasons for this uncertainty.

Studies of chronic methylene chloride exposure at high levels in a wide variety of laboratory animal species and strains have established the liver as the primary target organ. The present indication of an apparent relationship between methylene chloride exposure and the salivary gland is unusual and appears to be inconsistent with other existing data. Further, this particular tumor type (sarcoma) appears to arise from mesenchymal (connective) tissue rather than the epithelial components of this organ which further confounds the interpretation.

Present knowledge of the metabolism, pharmacokinetics, pharmacodynamics and toxicologic response to methylene chloride in both laboratory animals and man provides no evidence for appreciable sex differences in biological response to this material either acutely or chronically. However, the increased incidence of sarcomas was specific to male rats with no increase in tumors of this type detected in female rats or in hamsters of either sex. Therefore the high degree of sex and species specificity for this response is inconsistent with the rest of our knowledge about the biology of this material.

The rats in this study had a common viral disease (sialodacryoadenitis) early in the treatment period. The findings were similar to those reported by other investigators (Jonas *et al.*, 1969; Bhatt *et al.*, 1972; Jacoby *et al.*, 1975; Lai *et al.*, 1976; Weisbroth and Peress, 1977). This infection primarily affects the salivary glands and was present in both control and methylene chloride exposed rats. The combination of viral infection and exposure to high concentrations of methylene chloride may have been associated with the tumor response.

Finally, the present findings also appear to be inconsistent with the current knowledge of the biology and toxicology of methylene chloride in man. In workers occupationally exposed to methylene chloride there was no evidence of an association between exposure to methylene chloride and a tumorigenic response, nor was there evidence of a sex-specific response (Friedlander *et al.*, 1978).

Several naturally occurring (geriatric) pathologic alterations were decreased in an exposure-related manner in both rats and hamsters. For example, male and female hamsters had decreased amyloid in methylene

chloride exposed animals compared to controls. The decrease was most prominent in females and appeared to have resulted in increased survivability of the females exposed to 1500 or 3500 ppm. Another example was in male rats exposed to 1500 or 3500 ppm and females exposed to 3500 ppm which had less age-associated chronic progressive kidney disease than their respective controls. Other examples of possible exposure-related decreases in a naturally occurring lesion included decreased biliary cysts in methylene chloride exposed male and female hamsters, and decreased pancreatic lesions in methylene chloride exposed male rats, and decreased pituitary tumors in methylene chloride exposed female rats. The toxicological significance of these exposure-related decreases is unclear.

Female hamsters exposed to 3500 ppm had a statistically significant increase in the total number of females with a benign tumor. However, the females exposed to 3500 ppm had statistically significantly better survival than did the controls. In addition, the incidence and severity of amyloid was decreased in females exposed to 3500 ppm. The increased survival alone could explain this slight increase in benign tumors. However, the decreased amyloid in various tissues also made it easier to recognize small benign tissue proliferations, especially in endocrine organs such as adrenal glands. Therefore, the increased number of female hamsters with a benign tumor in the 3500-ppm exposure group was not considered to be the direct result of the methylene chloride.

Carboxyhemoglobin values were elevated in rats and hamsters exposed to 500, 1500, or 3500 ppm of methylene chloride. In the rat, the metabolism of methylene chloride to carbon monoxide exhibits dose dependency at exposure concentrations greater than about 250 ppm. Above 500 ppm there is little change in carboxyhemoglobin levels with increasing methylene chloride exposures (McKenna *et al.*, 1979). As a result, no exposure-related increases would be expected in blood carboxyhemoglobin levels in rats of this study and none were observed. Methylene chloride exposed male and female rats had elevated carboxyhemoglobin values compared to control values with no indications of a dose–response relationship. Both male and female hamsters exposed to 500, 1500, or 3500 ppm had greater carboxyhemoglobin elevations than rats. There are no data on the metabolism of methylene chloride by hamsters. However, in both rats and hamsters, the alteration in carboxyhemoglobin level was independent of duration of exposure. This indicated both a lack of accumulation of carboxyhemoglobin with repeated daily exposures and a lack of demonstrable effect of repeated methylene chloride exposures on this metabolic pathway for methylene chloride to carbon monoxide. Another significant observation in this study was the absence of evidence for cardiac or cardiovascular effects due to prolonged, repeated high level methylene chloride exposure and the attendant carboxyhemoglobin burden in either rats or hamsters.

In summary, in this 2-year study, exposure-related effects were observed in male and female rats exposed to 500, 1500, or 3500 ppm of methylene chloride. In contrast, hamsters exposed to the same exposure concentrations did not have definite adverse effects. Hamsters had less extensive spontaneous geriatric changes, had exposure-related decreased mortality (females), and lacked evidence of definite target organ toxicity.

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