Inhalation Toxicity and Carcinogenicity of 1,3-Butadiene in Sprague-Dawley Rats

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A 2-year inhalation study was conducted in Sprague-Dawley rats with 1,3-butadiene. Groups of 110 male and 110 female rats inhaled 1,3-butadiene at 0, 1000, or 8000 ppm for 6 hr/day, 5 days/week. Interim clinical pathology, neuromuscular, and histopathology investigations were carried out. The study terminated at 20 to 25% survival (105 weeks for females, 111 weeks for males).

Following exposure to 1,3-butadiene there were no effects on hematology, blood chemistry, urine analysis, and neuromuscular function that definitely could be associated with treatment. Treatment was associated with changes in clinical condition, suppression of body weight gain, reduced survival, and increases in certain organ weights and in both common and uncommon tumor types. Although the biological interpretation of the significance of some of the tumor types is equivocal, the evidence suggests that the test article is an oncogen to the rat under the conditions of exposure used in this study, and the mechanism is more likely to be an indirect effect through the endocrine system, rather than a direct effect through the production of reactive metabolites.

Introduction

As a result of 1,3-butadiene being one of the world's major industrial chemicals and the conflicting evidence in the scientific literature regarding the health effects associated with exposure to it, a program of toxicological investigation was initiated.

As part of this investigation, a long-term inhalation study in Sprague-Dawley rats was carried out, since no long-term animal data were available at the time of initiation. A preliminary 90-day experiment in Sprague-Dawley rats established exposure levels for the long-term study. That study used carefully specified 1,3-butadiene and an exposure system that had been shown to give an even distribution throughout the exposure chambers. (2) Despite a wide range of observations, the overall conclusion was that there were no treatment-related effects at concentrations up to 8000 ppm v/v. Exposure levels for the long-term study were therefore established as 0, 1000, or 8000 ppm v/v.

Study Design

Three 8-m³ exposure chambers of glass and stainless steel were used for the study. Each chamber was lit by flash-proof lights via two strip glass windows that were inserted into the stainless steel roof. Each chamber had a drain at the lowest point for cleaning purposes. The

main air flow into the chamber was drawn from the room through a filter (efficiency 95% at 1 μm). The flow rate was monitored by means of a pitot tube and inclined manometer. The target flow rate was 1000 L/min for all chambers. The chambers were operated at negative pressure, and a separate alarm monitored the differential pressure between the inside and outside of the chambers. The alarm was activated if the airflow rate fell to a level that allowed the production of an explosive atmosphere.

Each chamber had two sets of adjustable runners for easy loading of the cage racks. To avoid the build-up of static the racks were connected by groundwire to the chamber walls. The chambers also had direct ground connections to a ground rod outside the building.

The temperature and relative humidity in the exposure chambers were within the range of 20°C to 25°C and 40 to 80% on 96% of the occasions that measurements were made.

The generation and transfer of 1,3-butadiene gas to the exposure chambers took place in an all-stainless steel system. To help volatilization, the 1,3-butadiene was passed through a water bath at 30°C. Following volatilization the gas passed through traps to remove the inhibitor, tertiary butylcatechol. The atmospheres were routinely monitored by an infrared gas analyzer (Miran, IA, Wilks Scientific Corporation, Ltd.) at hourly intervals. The infrared analyzer was calibrated against a gas chromatograph. An even distribution of the test article was established in both exposure chambers before the start of animal exposures and then confirmed at regular intervals throughout the study.

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Three groups of rats were exposed in the system described for 6 hr/day, 5 days/week for 105 weeks in the case of females and 111 weeks for males at concentrations of 0, 1000, or 8000 ppm. There were 110 males and 110 females in each group: 10 males and 10 females from all groups were killed after 52 weeks; the remainder, when survival was approximately 20%; each sex was treated separately.

The low dose given was the then threshold limit value (TLV) for occupationally exposed personnel. The high-dose concentration was limited by the safety requirements to be below the lower explosive limit of 1,3-butadiene in air.

The animals used on the study were albino, hysterectomy-derived, barrier-maintained rats of the CD strain (Sprague-Dawley origin), obtained from Charles River, UK. The average age of the young animals on arrival was 3 weeks, with body weights ranging from 40 to 50 g.

Before treatment, ten weanlings of each sex and the ten dams were killed and examined to confirm that their health status was appropriate for a long-term toxicity study. Samples from a nasopharyngeal swab, lower gut contents, and a portion of the lower gut were examined for the presence of potentially pathogenic bacteria. The only bacterial flora was *Staphylococcus aureus* in the nasopharynx of three of the animals. In the histopathological examination particular attention was paid to the respiratory tract, but there were no unacceptable findings. It was concluded that the animals were appropriate for use in a long-term toxicity study.

During the acclimatization phase the experimental group allocation was made with the use of a stratified body weight randomization procedure designed to give as near as possible equal group mean body weights and standard deviations. Each animal selected for the dose groups was identified by an individual ear tattoo.

The animals of like sex were housed in groups of five and were treated in stainless steel wire mesh cages that were suspended on racks in the exposure chambers. The position of the racks in the chambers was interchanged at weekly intervals; at the same time, the positions of the cages on the racks were changed according to a predetermined plan.

Pelleted diet (SQC Rat and Mouse No. 1 Expanded, BP Nutrition, Witham, Essex, England) and water were available *ad libitum* during nonexposure periods only. Ten batches of diet were used and, in addition to the data provided by the manufacturer, six of these were analyzed for nitrosamine and total organochlorine content. These diets contained 6 to 104 µg nitrosamines/kg and 59 to 1100 µg total organochlorines/kg.

Test Material

Liquid 1,3-butadiene was supplied in pressurized cylinders, each containing approximately 10 kg. Eight cylinders were supplied (ICI Ltd., Wilton, England) each week during the course of the study. During manufacture, liquid 1,3-butadiene was analyzed semi-

continuously. Analysis showed that it complied with the specifications detailed in Table 1.

The gas supplied to the exposure chambers was analyzed each week for specific impurities: 4-vinyl-1-cyclohexane (dimerization product) and tertiary butyl-catechol (inhibitor preventing polymerization). In general, the concentration of 4-vinyl-1-cyclohexane was below 500 ppm v/v, and the average of the weekly measurements was 413 ppm v/v (SD = 219). There were short intervals when the concentrations were above 500 ppm v/v, but procedures were introduced to ensure that the animals were not exposed to atmospheres containing more than 1000 ppm v/v of the dimer in 1,3-butadiene. The concentration of the inhibitor in the 1,3-butadiene supply was less than 2 ppm v/v on most (93%) of the analyses and between 2.1 and 4.8 ppm v/v in the remaining analyses. Also samples of the gas delivered into the exposure chambers were sent to the supplier for analysis to ensure there were no marked changes occurring during generation.

Observations

All animals were observed twice daily, before and after exposure, and a detailed observation with palpation of superficial masses was performed at weekly intervals. Individual body weights were recorded weekly up to week 13, then every 2 weeks to week 52, and monthly thereafter.

After exposure to the gas for 3, 6, 12, and 18 months blood was withdrawn under light ether anesthesia from the orbital sinus of 20 preselected animals of each sex from each group. Before blood tests, the animals were fasted for 24 hr, but water was available during the last 18 hr of the fast. The blood was used to measure mean cell volume and hemoglobin concentration, and for counts of erythrocytes, leukocytes (total and differential), platelets and reticulocytes. Packed cell volume, mean cell hemoglobin, and mean corpuscular hemoglobin concentration were also calculated. In addition, measurements were made of plasma concentrations of glucose (sample taken from caudal vein), blood urea nitrogen, total protein and protein electrophoresis, as well as activities of alkaline phosphatase, glutamic-oxaloacetic transaminase and glutamate-pyruvate

Table 1. Specification of the 1,3-butadiene used.

Parameter	Value	
1,3-Butadiene, % wt	99.2	min
Peroxides at H_2O_2, ppm wt	5.0	max
Acetylenes (as vinyl acetylene), ppm wt	50.0	max
Sulfur, ppm wt	2.0	max
C_5 hydrocarbons, % wt	0.1	max
Dimer content, % wt	0.05	max
Nonvolatile matter, ppm wt	500.0	max
Carbonyl content (as acetaldehyde), ppm wt	25.0	max
Inhibitor, t-butylcatechol, ppm wt	75–150	

transaminase after exposure for 3, 6, and 12 months. At 12 months, leukocyte counts (total and differential) were made in an additional ten animals of each sex from each group, since a possible treatment-related effect had been seen at the 6-month sampling in the high-dose females.

Individual urine samples were obtained after 3, 6, and 12 months' exposure. Wherever possible, the same 20 males and 20 females were sampled that were used for the blood sampling. The urine samples were obtained during a 4-hr period of food and water deprivation, beginning approximately 2 hr after exposure. During the 2-hr postexposure period all animals were given access to water. The volume and specific gravity of the urine were measured and a semiquantitative assessment was made for glucose, urobilinogen, ketones, bile pigments, blood, protein, and pH. The insoluble constituents of the urine were examined microscopically after centrifugation.

The neuromuscular function of 40 animals of each sex from each dose group was evaluated before and at weeks 1, 4, 15, 26, 52, and 78 of treatment. The time before falling from a rotating cone was recorded as an index of neuromuscular function (1).

All animals were subjected to a postmortem examination. Animals that were to be killed because of their poor condition or as a scheduled examination were not given food but had water *ad libitum* overnight before they were anesthetized with sodium pentobarbitone and then exsanguinated.

The postmortem examination of animals killed at 52 weeks and at the end of the study included weighing the adrenal glands, brain, heart, kidneys, liver, lungs and trachea, spleen and testes. All animals were examined closely for abnormalities and the tissue samples shown in Table 2 were preserved in 10% neutral buffered formalin.

The eyes were placed in Davidsons' fluid, not in formalin. All tissues from the high-dose and control groups were embedded in wax, sectioned (5 μm), stained with hematoxylin and eosin, and examined microscopically. From the low-dose group, the sections shown in Table 3 were prepared and examined.

Femoral marrow smears from all animals in the control and high-dose groups were examined, but the different types of cells present in the smears were not counted.

Transmission electron micrographs were prepared from the livers for five animals of each sex from the high-dose and control groups at termination. Samples (from 10 males and 11 females of each group—one extra female was taken in error) were taken from the left lateral lobe (the right was used if the left was visibly abnormal), fixed in osmium, and embedded in epoxy resin. Survey sections (1 μm) were cut from the five selected control and high-dose animals of each sex, stained with 1% toluidine blue, and examined with the light microscope. Centrilobular and periportal areas were selected and ultrathin sections were prepared and

Table 2. Tissue samples examined.

Adipose tissue	Mammary gland (full thickness of abdominal wall)
Adrenal glands	
Aorta	
Liver (caudate, right median and left lobe)	Esophagus (proximal to the tongue)
Lungs	Pancreas
Femur (including bone marrow)	Parathyroid
Brain	Pituitary
Cecum	Prostate
Colon	Sciatic nerve
Diaphragm	Seminal vesicle
Ears (auditory canal and pinna, with adjacent sebaceous glands)	Small intestine
	Spinal cord (high cervical)
	Thymus
Epididymides	Thyroid
Eyes and optic nerve	Tongue
Gonads	Trachea
Harderian gland	Turbinate bones
Heart	Urinary bladder
Kidneys	Uterus
Larynx	Skin (particularly any showing lesions or abnormalities of hair growth) and vagina
Spleen	
Sternum	
Stomach	All gross lesions
Salivary gland (submaxillary)	Lymph nodes (tracheobronchial, cervical and mesenteric)

Table 3. Sections examined from the low-dose group.

Brain	Urinary bladder and Zymbal gland of both sexes
Gonads	
Liver	Salivary gland
Lungs	Prostate
Lymph nodes (mesenteric)	Seminal vesicles
Pancreas	Epididymides and kidneys in males and of pituitary gland
Spinal cord	
Thyroid	
Spleen	Mammary masses
Skin	Uterus and vagina in females

stained with uranyl acetate and lead citrate. The sections were examined and photographed with a Phillips EM300 operating at 60 KV.

Statistical analysis of the data was undertaken using the following test procedures: survival data and palpable subcutaneous masses were analyzed by the methods of Peto and Peto (8), Peto and Pike (9), and Kaplan and Meier (10). Lesions/tumor incidences were analyzed according to the method of Peto et al. (11); body weights, laboratory investigations, and organ weights were analyzed by using analysis of variance and Student's *t*-test (12).

Results and Evaluation

The daily mean concentration of 1,3-butadiene in the control chamber did not exceed 3.0 ppm v/v on any occasion, and the overall mean was 0.7 ± 0.8 ppm v/v. For the low-dose chamber, 94% of all daily mean con-

centrations were within the range 1000 ± 10% ppm v/v; for the high-dose chamber, 96% of all recorded daily mean concentrations were within the range 8000 ± 10% ppm v/v. The respective means and SD over the course of the study were 999.0 ± 30.8 ppm v/v and 7886.0 ± 704.3 ppm v/v.

An even distribution of 1,3-butadiene throughout each chamber was found when checked on five occasions. The coefficients of variation based on 24 or 27 sample points for the low-dose chamber ranged between 9.19% and 15.69% and for the high-dose chamber, between 3.68% and 7.63%.

Clinical signs that appeared to be related to the treatment were seen in the second until the fifth month of exposure. Some rats in the high-dose group, especially females, showed wet and ruffled fur together with slight limb weakness or incoordination during the first 3 hr of the first exposure day of the week following 2 days without treatment. During the remainder of the week, these signs regressed.

There were only 16 deaths during the first year of the study, but during the second year there was a statistically significant (10) ($p < 0.05$ in males; $p < 0.01$ in females) relationship between increased mortality and the concentration of 1,3-butadiene (Figs. 1 and 2). Comparison of the individual treated groups with the control showed a statistically significant difference ($p \leqslant 0.05$ in males; $p \leqslant 0.01$ in females) only with the 8000 ppm v/v group. Increased mortality in the female animals resulted from the sacrifice of animals with large subcutaneous masses; in males, renal lesions were the likely major cause of the increased death rate.

Over 80% of the subcutaneous masses in the females proved to be mammary tumors; the remainder, fibrous tumors of the skin. Statistical analysis showed that the treated females were more likely to develop mammary tumors than were the controls. The two doses did not differ in this respect. The analysis showed that the median time to develop a mass was 616 days in female control animals, but 546 days in the low-dose group and 576 in the high-dose group.

The body weight gains of both sexes at the high dose and of males at the low dose were slightly but significantly lower than the controls during the first 12 weeks of the study. This was compensated later by an increased gain, so that by the end of the first year the mean body
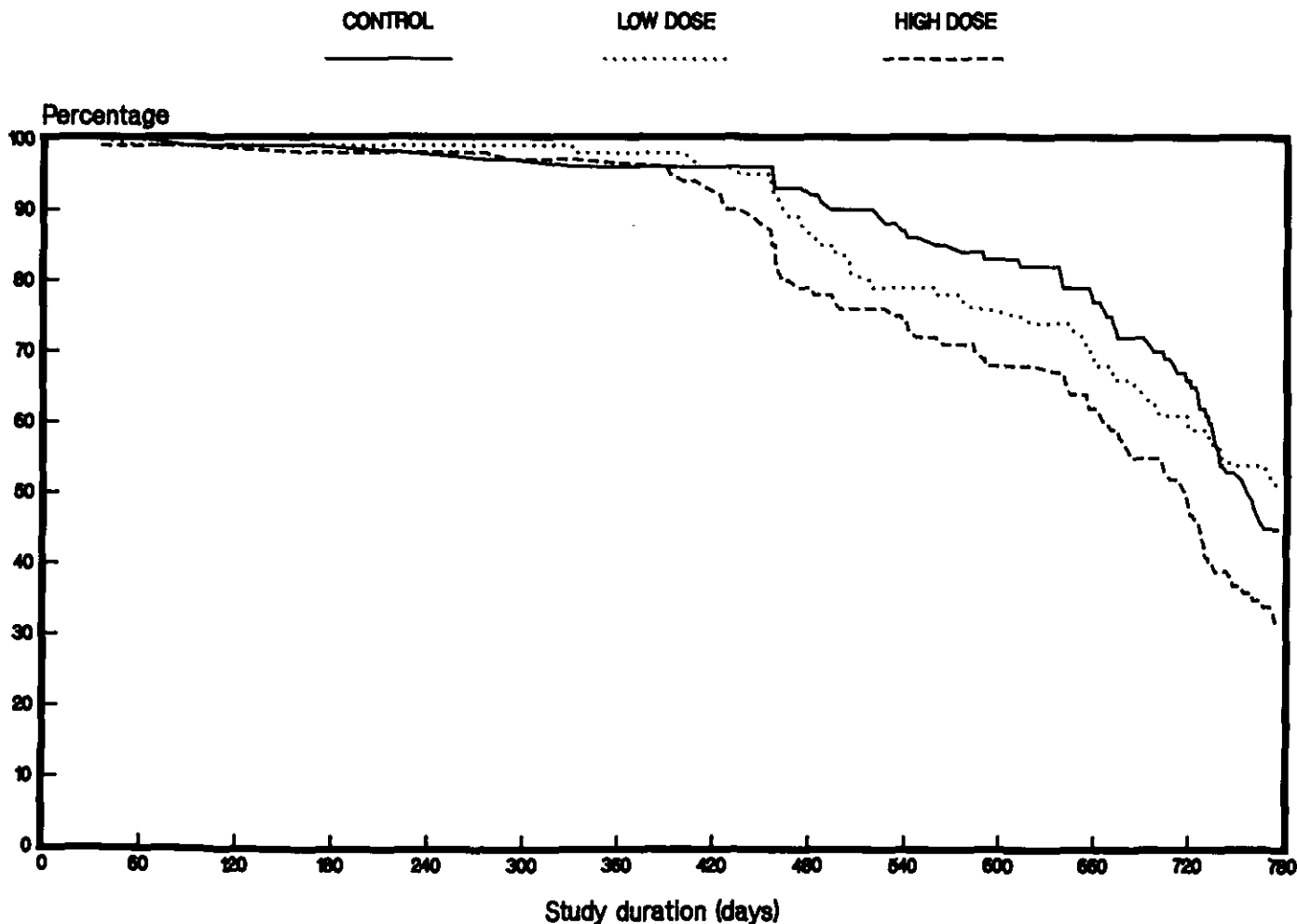

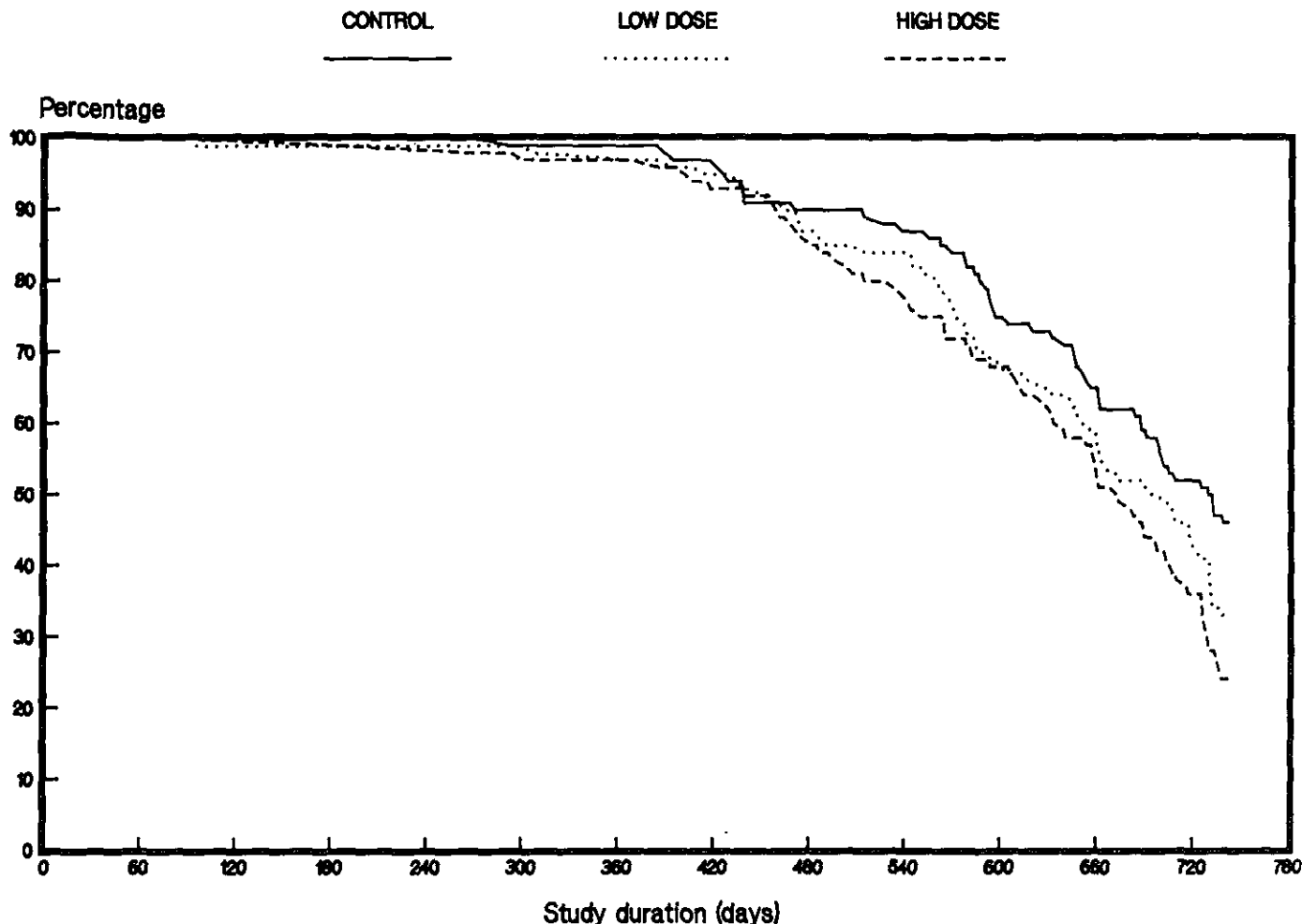FIGURE 1. Survival curves for male animals.

FIGURE 2. Survival curves for female animals.

weights of the control and treated groups were similar. From week 52 onward, there was no significant difference in weight gains for treated animals in comparison with the controls.

The only consistent finding in the hematological examination was higher leukocyte counts in the high-dose females because of increased numbers of lymphocytes during the first year (6.5×10^3/cmm at week 52). This was not seen either at 78 weeks or in the males. In view of this, and since the values were within the range expected for the strain of animals in this laboratory (6.4 to 9.6×10^3/mm^3), they were not considered to be toxicologically significant.

Blood urea nitrogen concentrations were statistically significantly higher than controls in both treated male groups at 13 (19.6 and 20.6 mg/dL compared with 17.1 mg/dL) and 26 weeks (16.7 mg/dL and 16.0 mg/dL, compared with 13.5 mg/dL), but not at 52 weeks. The corresponding values in females were lower than controls (22.9 and 22.2 mg/dL, compared with 27.8 mg/dL) at week 13 and (18.6 and 17.5 mg/dL, compared with 22.6 mg/dL) at week 26. Measurements of the volume and specific gravity of urine collected at 13, 26, and 52 weeks

did not show any treatment-related effects.

Periodic tests for the neuromuscular coordination showed no treatment-related effects in the first 6 months of the study. At 12 months, females from both treated groups were less able than the controls to remain on the rotating cone; in part this may have been due to the presence of mammary masses that could have interfered with the mobility of the animals. Both sexes at the high dose and females at the low dose were less able than the controls to remain on the rotating cone when examined at 18 months. In addition, this observation was made on animals soon after treatment and could not be repeated within 2 to 3 weeks when tested on a day when the animals were not treated. This suggests that either the effects were not reproducible or, if they were due to exposure, recovery was rapid.

Table 4 lists the relative weights of those organs that showed (*12*) a dose-related increase in either sex at either 1 or 2 years. For comparative purposes the values are expressed as a percentage of the control. The relative liver weights of both sexes and both dose levels were elevated at the times of examination with the exception of the high-dose females at week 52. There

Table 4. Relative organ weights (g/100 g body weight).

	Organ weight (% current control)							
	1 year				2 year			
	Male		Female		Male		Female	
Organ	1000 ppm v/v	8000 ppm v/v	1000 ppm v/v	8000 ppm v/v	1000 ppm v/v	8000 ppm v/v	1000 ppm v/v	8000 ppm v/v
Kidneys	104	102	104	102	104	124†	104	102
Liver	105*	124†	116*	110	111†	125†	118†	121
Heart	96	100	103	103	104	116*	97	97
Lung	97	93	105	108	104	114*	106	103
Spleen	92	92	100	93	93	121*	107	133
Number examined	10	10	10	10	50	32	32	24

*Statistically significant $p \leqslant 0.05$
†Statistically significant $p \leqslant 0.01$

was no associated pathological change in the liver, even at the ultrastructural level. The absolute kidney weight and the relative weight were increased in male rats exposed to 8000 ppm v/v for 2 years. This was associated with a more severe nephrosis compared with control males (Table 5), and some slight evidence of functional change were reflected in the amount of protein in the urine. It is possible that the higher relative heart weight in the same group may have been related to blood pressure changes resulting from the developing kidney changes.

There were higher relative lung and spleen weights in the high-dose males at 2 years. In the same males there was a higher incidence of focal epithelialization (metaplasia) in the lungs (5/45 for controls compared with 10/31 for high-dose males killed at the end of the study).

Some tumors were present in larger numbers in the treated animals than in the controls; those with statistically increased incidences are shown in Table 6. Compared with the controls, the incidence of pancreatic

Table 5. Incidence of nephropathy in male rats.[a,b]

| Finding | No. of animals affected | | |
	0 ppm v/v	1000 ppm v/v	8000 ppm v/v
Number examined	100 (45)	100 (50)	100 (31)
Nephropathy			
None	13 (4)	25 (7)	9 (0)
Minimal	29 (12)	32 (20)	11 (1)
Slight	38 (19)	27 (16)	42 (17)
Moderate	10 (7)	7 (4)	11 (2)
Marked	3 (2)	3 (2)	14 (9)
Severe	7 (1)	6 (1)	13 (2)

[a]The figures in parentheses are the values for animals killed at the end of the study.
[b]A statistically significant dose-related trend ($p < 0.05$) for fatal nephropathy was established. "Fatal" was defined for the statistical treatment by the Peto method of analysis.

exocrine adenoma in high-dose males was increased ($p \leqslant 0.001$). For females, uterine sarcoma showed a significant ($p \leqslant 0.05$) treatment-related trend, but the numbers in both treated groups were similar (1 compared with 4 or 5). Zymbal gland carcinoma was confined to the high

Table 6. Total incidence of tumors at selected sites.

| Tissue and tumor | Male | | | Female | | |
	0 ppm v/v	1000 ppm v/v	8000 ppm v/v	0 ppm v/v	1000 ppm v/v	8000 ppm v/v
Pancreas						
Exocrine adenoma	3	1	10	2	0	0
Uterus						
Sarcoma	—	—	—	1	4	5
Zymbal gland						
Adenoma	1	1	1	0	0	0
Carcinoma	0	0	1	0	0	4
Mammary gland						
Benign	0	2	0	32	64	55
Malignant	1	0	0	18	15	26
Total	1	2	0	50	79	81
Thyroid						
Follicular-cell adenoma	3	5	1	0	2	10
Follicular-cell carcinoma	1	0	0	0	2	1
Testis						
Leydig cell tumor	0	3	8	—	—	—
Total number of tumor-bearing rats	84	70	87	97	98	94
Subtotal tumor-bearers						
No tumors	16	30	13	3	2	6
Single tumors	26	32	30	41	24	17
Multiple tumors	58	38	57	56	74	77
Numbers of rats examined	100	100	100	100	100	100

dose, with a significant ($p \leq 0.01$) treatment-related trend in females (0 compared with 4). The incidence of the total mammary gland adenoma and carcinoma in females was increased to a similar extent in both treated groups with a significant ($p \leq 0.001$) dose-related trend (50 compared with 79 or 81). A significant trend ($p \leq 0.01$) was present in females for thyroid follicular-cell adenoma (0 compared with 2 and 10). Leydig cell tumors showed a dose-related increase in incidence ($p \leq 0.001$) (0 compared with 3 and 8 for the low- and high-dose groups, respectively).

It is possible that not all of the lesions classified as pancreatic adenoma were neoplastic since the distinction between adenoma and the hyperplastic focus or nodule in this organ is not clear. The more severe classification was chosen but, in view of the debatable nature of the lesion, the increase—confined to one sex—was considered as doubtful biological evidence of oncogenicity.

The numbers of uterine sarcomas and of Zymbal gland tumors were similar to those normally found in untreated Sprague-Dawley rats at Hazelton Laboratories and do not suggest, in isolation, a treatment-related effect. Most of the Zymbal gland tumors were present in animals killed in a short period (76 to 90 weeks), and none were found at the end of the study. If this cluster of tumors, found relatively early in the exposure period, was due to direct chemical action, it would be expected that others would be found later in the experiment. In the absence of such a time-related effect, it seems unlikely that the Zymbal gland tumors are due to treatment. The mammary gland tumors appeared earlier and in greater numbers in the treated females, suggesting a relation to treatment. The higher numbers of thyroid and testis tumors also are considered to be treatment related, although the highest incidence of testis tumors at the highest dose was close to the historical control range (0% to 6%) seen at Hazelton Laboratories.

It is felt that the effect of 1,3-butadiene is more likely to be as an indirect effect through the endocrine system, rather than a direct effect from the reactive epoxide and diepoxide metabolites. The reasoning behind this opinion is that if the action was simply through the epoxides and diepoxides, then one would expect to see more activity on the liver, whereas in this study the liver suffered only minor changes in weight. Changes in the mammary and pancreatic tumor profiles indicate involvement of the endocrine system.

In summary, following exposure to 1,3-butadiene, there were no effects on hematology, blood chemistry, urine analysis, and neuromuscular function that definitely could be associated with treatment. Treatment was associated with changes in clinical condition, suppression of body weight gain, reduced survival, increases in the weight of certain organs, and increases in both common and uncommon tumor types. Although the biological interpretation of the significance of some of the tumor types is equivocal, the evidence suggests that the test article is a weak oncogen to the rat under the conditions of exposure used in this study, and the mechanism is more likely to be an indirect effect through the endocrine system, rather than a direct effect through the production of reactive metabolites.

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