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LANL Reference 9/5/1980

1,3-DINITROBENZENE: TOXIC EFFECTS *IN VIVO* AND *IN VITRO*

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When 1,3-dinitrobenzene (1,3-DNB) was given to rats orally as a 1% suspension in corn oil, the LD50 was 83 mg/kg with fiducial limits 56–124 mg/kg. The compound was equally toxic in both sexes. Signs of toxicity included reduction in ambulatory motion, ataxia, weakness, dyspnea, rapid heartbeat, cyanosis, coma, and eventual respiratory failure.

When 1,3-DNB was added to the daily drinking water in concentrations of 50, 100, and 200 mg/l for 8 wk, 3 of 6 male rats receiving the highest concentration died during wk 4 and another died during wk 5. Of a like number of females, one died during wk 6 and another during the wk 7. All other animals survived. Growth rate was reduced in both sexes and at 200 mg/l 1,3-DNB in the water supply the animals lost weight. There were mild, consistent reductions in hematocrit and hemoglobin values. Enlarged spleens were present in both sexes at all concentrations; fibrosis with deposition of hemosiderin was present in all rats at 200 mg/l 1,3-DNB. Testicular atrophy was evident but there was no effect on the ovaries. A brown-yellow pigment was deposited in the Kupffer cells of the liver. Hexobarbital-induced sleep time was decreased.

When 3, 8, or 20 mg/l 1,3-DNB was provided daily in drinking water for 16 wk, no signs of acute toxicity were seen. Growth of females receiving 20 mg/l was retarded progressively after 8 wk of exposure; growth was normal in the other groups. Moderate reductions in hematocrit and hemoglobin values occurred in males after 5 and 10 wk of 20 mg/l 1,3-DNB values were normal in the same animals after 16 wk. Spleens were increased in weight and hemosiderin deposits were observed. Testes were sharply reduced in weight among males at the 20 mg/l level.

Behavioral abnormalities, in the form of increased activity in running wheels, were noted in male rats exposed to 3 and 8 mg/l 1,3-DNB in drinking water for 90 d. Other male rats similarly exposed but kept on activity platforms showed increased activity but the increases were not significant.

Minimal effects on cells in vitro *were noted at 3–8 mg 1,3-DNB per liter of medium. Growth reduction by 50% was observed at 20–25 mg/l. All cells were killed when the concentration of 1,3-DNB to which they were exposed exceeded 56 mg/l.*

The authors are indebted to Mary Earley and Kathleen LaDow for their able technical assistance.

This research was supported by the U.S. Army Medical Research and Development Command under contract DADA-17-73-C 3013 and by the National Institute of Environmental Health Sciences under center grant ES-00159.

Portions of this paper were presented at the 18th Annual Meeting of the Society of Toxicology, New Orleans, Louisiana, March 11–15, 1979.

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Journal of Toxicology and Environmental Health, 7:829–847, 1981
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0098–4108/81/040829–19$2.25

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INTRODUCTION

As direct and indirect reuse of water becomes necessary to meet future needs for drinking water and as the public increases its demands for drinking water free of potentially noxious chemicals, biological toxicity assays will probably be required as part of the battery of tests used to ensure public health. A mammalian cell culture assay system such as the one described in this paper is relatively rapid (72 h), simple, and inexpensive for toxicity testing of aqueous samples.

This study is one of a series of toxicological studies of selected chemicals to determine whether a standardized tissue culture assay system can reliably detect chemicals in drinking water at or below the concentration at which biological effects can be detected in intact mammals. By itself, this study does not prove the applicability of the assay. However, the results of toxicological testing with rats and toxicity assessment with the mammalian cell bioassay are presented in the belief that the data on the effects of low concentrations of 1,3-dinitrobenzene (1,3-DNB) in drinking water will be of interest.

Commercial dinitrobenzene usually consists of a mixture of the *o*, *m*, and *p* isomers. It is used in the synthesis of certain dyes and dye intermediates, in the manufacture of explosives, and to some extent in the plastics industry. There has been no differentiation among the three isomers with respect to the industrial hygiene standard (ACGIH, 1978). The isomers are readily absorbed through the skin and their vapors are highly toxic (Hamblin, 1963). Oral LD values of 1,3-DNB have been reported as: cats (MLD), 27 mg/kg; redwing blackbirds (LD50), 42 mg/kg; and starlings (LD50), 100 mg/kg (NIOSH, 1979; Schafer, 1972).

All isomers of dinitrobenzene are methemoglobin formers and, on prolonged exposure, cause anemia (Watanabe et al., 1976). Other symptoms in humans are reduced vision, hearing loss, and ringing ears (NIOSH, 1977). Kiese (1949b) reported hematologic changes, nervous disorders, and convulsions in dogs given daily or intermittent sc injections of 0.1–6.0 mg/kg 1,3-DNB for as long as 144 d. He noted similar effects in dogs given acute doses of 10 and 20 mg/kg sc and reported the sc LD50 to be 10 mg/kg (Kiese, 1949a). Liver damage has been considered a possible effect of exposure to 1,3-DNB, but Watanabe et al. (1976), Kiese and Siems (1949), and Ishihara et al. (1976) did not find proof of hepatotoxicity in rats (100 μmol/kg ip), dogs (10 mg/kg sc), and humans, respectively. 1,3-DNB (0.15 mmol/kg) has been reported to reduce sciatic nerve conduction velocity in rats even after methemoglobinemia has passed (Pankow et al., 1975).

1,3-DNB is soluble in water to 469 mg/l at 15°C and to 3200 mg/l at 100°C (Hodgmen, 1959). It is also relatively nonbiodegradable (Chudoba and Pitter, 1976) and could pose a hazard to humans and animals where drinking water sources are polluted by industrial wastes.

METHOD

The same lot of 1,3-DNB (certified grade; melting point, 88.5–89.2°C; Fisher Scientific, Fair Lawn, N.J.) was used for all experiments reported in this paper.

Toxicology Experiments with Intact Rats

Male and female rats were obtained from Carworth Farms (Charles Rivers, Stoneridge, N.Y.). One lot 8–10 wk old, was used in acute toxicity studies; a second lot, 8–10 wk old, was used in short-term feeding experiments. Weanlings, 3 wk of age, were started on 16-wk exposure experiments. The 8–10 wk rats were housed, 3 per cage, according to sex. Weanlings were housed 4 per cage. Cages were distributed randomly among the 1,3-DNB dosage groups in numbers sufficient to satisfy the design of the experiments. Animals were then marked for individual identification and weighed. Purina Laboratory Chow was the standard diet. Animals were housed in a room reserved for these experiments and maintained at 23 ± 1°C by central air conditioning. They were observed daily for general appearance and behavior and examined and weighed individually once a week.

A weighed quantity of feed was placed in each cage at the beginning of the week; the portion remaining after 7 d was weighed and the difference between the initial and final weights accepted as a measure of the amount of feed consumed in the interim. A weighed quantity of fresh drinking water (or an aqueous solution of 1,3-DNB) was provided to each cage on Monday, Wednesday, and Friday. The unused portions of water were weighed and discarded. Glass water bottles, rubber stoppers, and drinking stems were washed and sterilized weekly.

In the acute toxicity study, groups consisting of 6 male and 6 female rats were given one of six doses of 1,3-DNB by intubation. Doses ranged from 36 to 180 mg/kg and were given as a 1% solution in corn oil. Signs of toxicity were noted and fatalities recorded daily during 3 wk after administration of the dose.

In 8-wk experiments, three groups of 6 male and 6 female rats were supplied with drinking water containing 50, 100, or 200 mg/l 1,3-DNB; a fourth group of 12 males and 12 females was provided with tap water. The tap water was not analyzed for trace chemical impurities, but samples were refrigerated for future chemical analyses should they be considered necessary.

In 16-wk experiments, four groups of 20 male and 20 female weanling rats were provided with distilled water containing 0, 3, 8, or 20 mg/l 1,3-DNB for drinking. Distilled water was used to prepare the 1,3-DNB solutions and was provided to the control group to reduce the possibility of biological effects caused by contaminants in the drinking water. This was thought necessary because of the low concentrations of 1,3-DNB used and the length of the experiment.

Changes in drug metabolic activity were evaluated indirectly by measuring the length of the period of sleep induced in the animals by an ip injection of 125 mg/kg sodium hexobarbital; the timed interval between loss of the righting reflex and recovery was taken as the sleep time. All animals were tested in this fashion during wk 7 of the 8-wk experiments. Also during wk 7, samples of blood were taken from the tail vein of all animals and were analyzed for hemoglobin content, hematocrit, and number of leukocytes per cubic millimeter. Percentage distributions of the differentiated leukocytes were calculated.

After wk 14 of the 16-wk experiments the same 9 animals from each dosage group were tested on alternate days for metabolic activity. Samples of blood were taken from the tail vein of the same five males at each dosage during wks 5, 10, and 14 of treatment. Hematocrits, hemoglobin contents, and leukocyte distributions were determined. At the end of the treatment periods, the animals were anesthetized with pentobarbital sodium, killed by exsanguination, and necropsied. All organs were removed and examined for gross pathological changes. Liver, heart, lungs, kidneys, spleen, brain, gonads, and pituitary and adrenal glands were cleaned of extraneous tissue and weighed.

Tissues were taken from the following organs and fixed in neutral formalin: heart, aorta, lungs, liver, spleen, kidney, stomach, duodenum-pyloris, ileum, colon, urinary bladder, prostate and testes, ovary and uterus, pancreas, thyroids, thymus, adrenals, pituitary, brain, spinal cord, eye, skeletal muscle, and skin. Specimens were embedded in paraffin. Sections cut at 8 μm were stained with hematoxylin and eosin and examined by light microscopy.

Data were examined by conventional methods of analysis of variance and covariance. Appropriate transforms were used when necessary to maintain the assumptions for analysis of variance. The t-test and variance ratios (F-test) were used to ascertain the statistical significance of differences in the data. LD50 values were obtained by probit analysis, using maximum likelihood estimation (Finney, 1971).

Food and water consumption were related to total body weight within the cage on a daily basis. Variations among cages within each treatment group were used to estimate the significance of differences in consumption among the several groups.

Behavioral Toxicology

Changes in spontaneous locomotor activity, measured with both activity wheels and activity platforms, were used to assess behavioral alterations due to consumption of drinking water containing 1,3-DNB. Three groups of 5 male rats (Carworth Farms) each were housed in standard 36-cm Wahmann activity wheels. These units consisted of small home cages (25 X 15 X 12 cm) attached to wire drums, which revolved in response to the animal running. The number of wheel revolutions was

recorded by counters attached to the activity wheels. Rats were allowed to live continuously in the activity wheels for 90 d. During this time, daily activity levels and weekly food and water consumption and body weight were recorded. The three groups received 0, 3, or 8 mg/l 1,3-DNB in the drinking water throughout the experiment. Activity of the animals was recorded continuously, except for a period of approximately 6 h on 1 d each week.

Three additional groups of 10 male rats each were placed on activity platforms. An activity platform consisted of a 30-cm^2 surface on which a plastic home cage (35 X 30 X 15 cm) was placed. Any movement by a rat living in the cage was detected, amplified, digitized, and recorded by appropriate electronic circuitry. These rats were also exposed to 0, 3, or 8 mg/l 1,3-DNB for 90-d. Activity on the platforms was monitored from 5 p.m. to 9 a.m. each day during exposure. Food and water consumption and body weight were recorded weekly.

Tissue Culture Toxicity

Cells used for tissue culture experiments were a continuous line of mouse connective tissue cells (Earle's L-cell, NCTC clone 929, American Type Culture Collection, Rockville, Md.). The cells were propagated in minimal nutrient medium, consisting of Eagle's minimum essential medium with Hanks' salts (Gibco, Grand Island, N.Y.) containing 0.35 g $NaHCO_3$, 10 mmol Hepes (ICN Pharmaceuticals, Cleveland, Ohio), and 50 mg gentamicin (Schering Corp., Kenilworth, N.J.) per liter. The minimal medium was supplemented with 1 or 5% fetal bovine serum (Gibco). Twice weekly the L-cell cultures were disaggregated with 0.1% trypsin-EDTA solution (Gibco) and subcultured in 75-cm^2 disposable plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) at a density of approximately 27,000 cells/cm^2. The serum concentration was 5% in the culture medium. Tissue cultures were kept at 36.5°C in tightly closed flasks.

Minimal media containing 1,3-DNB were freshly prepared for each experiment by dissolving weighed quantities of 1,3-DNB in medium supplemented with fetal bovine serum (1% v/v). Stock media containing 1,3-DNB were diluted with minimal medium plus 1% serum to achieve the lower concentrations used in the experiments. Control cultures were treated with minimal medium lacking 1,3-DNB but containing 1% serum.

Cytotoxicity assays were carried out as follows. Disposable tissue culture tubes (16 X 150 mm; Demuth Glassware Division, Parkersburg, W.Va.) were seeded with 200,000 L-cells in 2 ml minimal medium supplemented with 1% serum. Tubes were tightly stoppered and placed on their sides at a slight angle for 72 h at 36.5°C. After 72 h the cells had multiplied to become confluent sheets. The medium was withdrawn from the culture tubes and replaced with medium containing various concentrations of 1,3-DNB. One set of cultures was exposed to medium lacking

1,3-DNB, and another set was removed from the experiment after the first 72-h incubation and used to determine cell protein and DNA at the onset of exposure to 1,3-DNB. The treated and control culture sets were returned to the 36.5°C incubator for 72 more hours. Cultures were examined daily by microscopy. After the exposure period, the medium was removed from each culture and the cultures were rinsed twice with 2 ml phosphate-buffered saline solution (pH 7.0). Culture tubes containing rinsed cultures were then inverted to drain and air dry. Protein and DNA contents of the dried cultures were determined by the method of Oyama and Eagle (1956) and the indole method (Ceriotti, 1955), respectively. Five or six replicate cultures were used at each experimental point. Each experiment was repeated several times.

RESULTS

Acute Toxicity

Ingestion by rats of sufficient amounts of 1,3-DNB resulted in toxicity that was characterized by reduction in ambulatory motions, ataxia, weakness, dyspnea, and rapid heartbeat. Paws, ears, lips, and nostrils appeared cyanotic. Eyes were protuberant, presumably congested, and dark in color. As intoxication progressed, respiratory failure and coma ensued. Death occurred 1-26 h after treatment. Mortality and severity of intoxication were generally about the same for both sexes at each dose level. The LD50 of 1,3-DNB was estimated to be 91 and 81 mg/kg for males and females, respectively. The 95% confidence limits of both LD50 values were approximately 50 and 150 mg/kg. The LD50 value of 1,3-DNB for both sexes combined was 83 mg/kg with 95% confidence limits of 56 and 124 mg/kg. For rats given sublethal doses of 1,3-DNB signs of toxicity were less severe and there was a temporary loss of body weight; the severity of the response was related to the magnitude of the dose. Recovery was prompt; the animals were normal in body weight, appearance, and actions after 1 wk.

Effects on Rats Associated with 8 wk of Ingestion of 1,3-DNB

Three of six males exposed to 200 mg/l 1,3-DNB in drinking water died during wk 4 and one died during wk 5; one female died during wk 6 and another during wk 7. The remaining rats at this level and all rats consuming 50 or 100 mg/l 1,3-DNB in drinking water survived.

Other than mortality, the predominant effect was a reduction in growth rate (or loss in body weight), which was generally related to the amount of 1,3-DNB ingested in the drinking water. Table 1 shows weekly average body weight in conjunction with average daily intake of food and water; food and water consumptions are expressed as grams consumed per

TABLE 1. Average (±SE) Weekly Body Weight and Food and Water Consumption of Rats Drinking Water Containing 1,3-DNB Daily for 8 wk[a]

Week	Males: Body weight (g)	Males: Daily intake (g/kg·d) Food	Males: Daily intake (g/kg·d) Water	Females: Body weight (g)	Females: Daily intake (g/kg·d) Food	Females: Daily intake (g/kg·d) Water
			Control			
Start	278 ± 5			189 ± 4		
1	306 ± 6	75 ± 1	139 ± 7	204 ± 4	89 ± 1	164 ± 7
2	326 ± 7	72 ± 1	133 ± 7	214 ± 3	83 ± 1	169 ± 9
3	353 ± 8	67 ± 1	123 ± 2	229 ± 4	70 ± 4	150 ± 4
4	362 ± 9	62 ± 1	118 ± 2	237 ± 5	75 ± 1	153 ± 4
5	374 ± 9	63 ± 2	119 ± 6	238 ± 5	75 ± 1	167 ± 3
6	390 ± 9	56 ± 3	108 ± 5	247 ± 5	67 ± 1	136 ± 3
7	398 ± 10	58 ± 1	114 ± 6	250 ± 6	73 ± 0	156 ± 12
8	404 ± 11	51 ± 2	113 ± 4	253 ± 5	65 ± 1	156 ± 10
			50 mg/l			
S	284 ± 7			178 ± 2		
1	305 ± 10	72 ± 1	110 ± 8	195 ± 5	85 ± 0	155 ± 5
2	318 ± 11	79 ± 0	106 ± 11	196 ± 7*	80 ± 1	142 ± 17
3	329 ± 11	59 ± 3	91 ± 7	207 ± 6**	78 ± 0	117 ± 3
4	339 ± 13	63 ± 1	78 ± 4	214 ± 6**	76 ± 1	112 ± 9
5	349 ± 12*[b]	62 ± 2	100 ± 10	220 ± 8**	75 ± 2	114 ± 7
6	361 ± 13[c]	62 ± 0	89 ± 18	223 ± 8*	68 ± 2	102 ± 10
7	360 ± 16	56 ± 1	93 ± 10	224 ± 9*	71 ± 0	112 ± 2
8	368 ± 15	55 ± 2	88 ± 9	227 ± 8*	64 ± 1	100 ± 3
			100 mg/l			
S	280 ± 8			187 ± 4		
1	293 ± 10	68 ± 1	89 ± 2	191 ± 4*	79 ± 3	110 ± 1
2	292 ± 10	66 ± 1	89 ± 5	189 ± 5[c]	76 ± 0	81 ± 6
3	310 ± 10**	62 ± 1	72 ± 1	197 ± 4	75 ± 1	96 ± 10
4	319 ± 9*	59 ± 0	73 ± 1	186 ± 18	74 ± 1	77 ± 6
5	318 ± 9[c]	60 ± 0	67 ± 0	205 ± 3	75 ± 0	97 ± 5
6	333 ± 12	58 ± 2	63 ± 0	124 ± 4	68 ± 0	92 ± 14
7	336 ± 12	57 ± 3	63 ± 1	212 ± 5	69 ± 1	77 ± 1
8	340 ± 13	55 ± 1	65 ± 0	212 ± 5	63 ± 0	
			200 mg/l			
S	270 ± 9			185 ± 6		
1	242 ± 8[c]	61 ± 0	75 ± 4	167 ± 5[c]	78 ± 1	86 ± 7
2	212 ± 11	49 ± 4	53 ± 3	146 ± 4	63 ± 1	47 ± 0
3	190 ± 14	48 ± 4	54 ± 4	146 ± 6	64 ± 1	55 ± 2
4	216 ± 20[d]	48 ± 10	56 ± 7	160 ± 6	75 ± 0	80 ± 4
5	205 ± 27[e]	56	65	162 ± 5	79 ± 1	72 ± 1
6	211 ± 30	61	60	161 ± 8	72 ± 1	78 ± 2
7	215 ± 32	65	72	166 ± 4[e]	74 ± 2	69 ± 3
8	216 ± 26	66	63	157 ± 9[e]	88 ± 9	90 ± 3

[a]Twelve males and 12 females were used in control group; 6 of each sex were used in all treatment groups.
[b]Significantly different from corresponding controls at (*) $p < 0.05$; (**) $p < 0.01$.
[c]This value and subsequent values in this treatment group are significant at $p < 0.01$.
[d]Three deaths occurred during the week.
[e]One death occurred during the week.

kilogram of body weight per day. Reduction in food and water consumption were associated with losses in body weight. Food intake was not reduced sufficiently to account for losses in body weight, but water intake was reduced sharply ($p < 0.01$) with increasing concentration of 1,3-DNB and dehydration could have been a factor. At these concentrations of 1,3-DNB in the drinking water, adverse effects on growth were more severe among male rats, even though the females consistently ingested a larger daily dose of the compound per kilogram of body weight.

Hematocrits, hemoglobin contents, and leukocyte numbers are shown in Table 2. Hematocrit values were significantly reduced ($p < 0.01$) in females at the 200 and 50 mg/l levels; reductions in those at the intermediate level and in males at all levels were insignificant. Mild but consistent reductions in hemoglobin contents were significant in both sexes. The number of leukocytes and percentage distribution of the different types of leukocytes did not change significantly.

Changes in drug metabolism activity were measured indirectly by the length of the period of sleep induced by a standard dose of sodium hexobarbital. The results are shown in Table 3. One male from each of the four dose groups and two females, one each at 50 and 200 mg/l, did not sleep and were not counted in the evaluations, other than noting their presence. A marked decrease in sleep time of the females was observed. The probability of obtaining such a decrease by chance was <0.01. Males at 100 mg/l and occasionally males at 50 mg/l 1,3-DNB also showed some reduction in sleep time.

Average terminal weights of the body and various organs of the males and females are given in Table 4. In both sexes there was a reduction in final body weight related to the concentration of 1,3-DNB in the water. Enlargement of the spleen occurred in males at 50 and 100 mg/l and in

TABLE 2. Hematologic Values[a] of Rats during wk 7 on Drinking Water Containing 1,3-DNB

1,3-DNB (mg/l)	No. of rats	Hematocrit[a] (vol. %)	Hemoglobin[a] (g per 100 ml)	Leukocytes[a] ($\times 10^3$ mm^{-3})
		Male		
0	12	51 ± 1	15.5 ± 0.2	23.6 ± 1.1
50	6	51 ± 1	14.6 ± 0.1*	26.8 ± 2.3
100	6	49 ± 1	13.9 ± 0.3**	19.2 ± 0.7
200	2	48 ± 1	14.3 ± 0.1**	19.4 ± 1.7
		Female		
0	12	49 ± 1	14.8 ± 0.2	18.7 ± 1.4
50	6	44 ± 1**	12.7 ± 0.1**	23.3 ± 2.9
100	6	47 ± 2	13.7 ± 0.3*	19.5 ± 1.0
200	4	42 ± 3**	12.8 ± 0.4**	14.9 ± 4.2

[a]Average values ± SE. (*) $p < 0.05$, (**) $p < 0.01$.

TABLE 3. Minutes of Sleep Induced by Hexobarbital in Rats Drinking Water Containing 1,3-DNB for 7 wk

1,3-DNB (mg/l)	Average sleep time (min)[a]			
	n^b	Males	n	Females
0	11	38 ± 2	12	123 ± 7
50	5	33 ± 6	5	40 ± 5**[c]
100	5	26 ± 1**	6	64 ± 11**
200	1	42	3	57 ± 12**

[a]Average value ± SE.
[b]Number of rats used is given by n. One additional male rat in each treatment group and one additional female in the 50 and 200 mg/l groups did not sleep. They were not included in the calculations of sleep time. Death of animals consuming 200 mg/l also reduced the number tested at this concentration.
[c](**) $p < 0.01$.

females at all levels. Atrophy of the testes was evident at all three dose levels, but there was no effect on the ovaries at any level. The reduced weights of the testes of the two surviving males at 200 mg/l were only partially related to severe losses in body weight. In proportion to body weight, testicular weight was decreased at this concentration also. Decreases in the ovary weight were directly proportional to losses in body weight and did not appear to be otherwise related to ingestion of

TABLE 4. Terminal Weights of Body and Organs[a] of Rats Given 1,3-DNB in Drinking Water for 8 wk

1,3-DNB (mg/l)	No. of rats	Average weight ± SE[b]				
		Body (g)	Spleen (g)	R^c (mg/g)	Testicles or ovaries (g)	R (mg/g)
			Males			
0	6	419 ± 16	0.92 ± 0.05	2.19	3.57 ± 0.49	8.50
50	6	367 ± 16*	1.22 ± 0.11*	3.28***	1.28 ± 0.06***	3.49***
100	6	333 ± 12**	1.28 ± 0.06***	3.86***	1.31 ± 0.4***	3.95***
200	2	199 ± 30***	0.68 ± 0.19**	3.34	1.20 ± 0.28*	6.03**
			Females			
0	6	272 ± 6	0.74 ± 0.02	2.72	0.14 ± 0.01	0.53
50	6	228 ± 9**	0.93 ± 0.06**	4.10***	0.11 ± 0.01	0.51
100	6	210 ± 6**	0.85 ± 0.06***	4.02***	0.10 ± 0.006**	0.48
200	5	160 ± 8***	0.64 ± 0.09	4.29***	0.080 ± 0.01**	0.52

[a]Variations in weight of liver, heart, lungs, kidneys, brain, pituitary, and adrenals were related to variations in body weight.
[b](*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.
[c]R is the ratio of organ weight to body weight.

1,3-DNB. As evaluated by covariance analyses of the respective organ and body weights, variations in the weights of other organs were related to variations in body weight and there was no evidence of a direct effect of 1,3-DNB.

Histopathological examination of sectioned tissues showed that in all animals ingesting 1,3-DNB in their water, a brown-yellow pigment was deposited in the Kupffer cells of the liver. The deposition was least at 50 mg/l and most severe at 200 mg/l. Sections of testicular tissue from groups exposed to 50 and 100 mg/l revealed evidence of decreased spermatogenesis: the germinal epithelium was not developing into mature sperms and the number of germinal cells was decreased. In sections from the 200 mg/l group more than half of the seminiferous tubules were collapsed. Neither germinal cells nor Sertoli cells were present in these tubules. There were no histopathologic effects on ovaries at any concentration. In the 200 mg/l group, male and female rats showed atrophy and fibrosis of the spleen with marked deposition of hemosiderin. No pathological changes were found in any of the other tissues examined or in tissues from control animals.

Effects on Rats Associated with 16 wk of Ingestion of 1,3-DNB in Drinking Water

Of the 40 rats in each of the 4 groups that were provided daily with water containing 0, 3, 8, and 20 mg/l 1,3-DNB all survived during the 16-wk period.

The average amount of water ingested daily per kilogram of body weight during each of 16 consecutive weeks gradually decreased in all groups irrespective of the concentration of 1,3-DNB. This probably reflected growth of the animals without a proportionate increase in water intake. For both sexes the variations in water consumption among groups receiving the four concentrations of 1,3-DNB was no greater than that among cages within treatment groups.

The average daily intake of 1,3-DNB for the 20 mg/l groups was 3.10 ± 0.425 mg/kg·d for females and 2.64 ± 0.637 mg/kg·d for males. The intake ranged from high values during the first week of 3.88 and 3.98 mg/kg·d for females and males, respectively, to low values of 2.24 and 2.00 mg/kg·d, respectively during the later weeks of the experiment. At the other concentrations the average daily intake values (with standard deviation and high and low values) for females and males, respectively, were: (8 mg/l experiment) 1.32 ± 0.19 (1.73–1.08) and 1.13 ± 0.244 (1.62–0.89) mg/kg·d, and (3 mg/l experiment) 0.48 ± 0.07 (0.63–0.039) and 0.40 ± 0.09 (0.58–0.31) mg/kg·d. On the average, the females consumed about 15% more 1,3-DNB per day throughout each experiment.

Based on average weekly body weights, males and females maintained on the 3 and 8 mg/l levels and males on the 20 mg/l level grew as well as or better than the controls. Females on the 20 mg/l level had reduced

rates of growth after 8 wk, and at the end of the experiment their average weight was significantly lower than that of the control females.

The relation between body weight and food intake, obtained by covariance analysis of the respective log weights, was used to adjust the final body weights of the treated animals to the same food intake as the controls. The adjusted weights indicated that the low weight of the females at 20 mg/l 1,3-DNB was only partly attributable to reduced food intake ($p = 0.02$) and that other factors, probably ingestion of 1,3-DNB, made some contribution. The adjusted weights at other levels of intake did not differ from those of the control groups.

Samples of peripheral blood were taken from the same five male rats in each of the three 1,3-DNB groups and the control group after the animals had been exposed for 5, 10, and 14 wk (Table 5). Hemoglobin values of animals on 20 mg/l 1,3-DNB were moderately depressed after 5 and 10 wk. Despite continued ingestion of the water, the hemoglobin concentrations returned to normal from wk 10 to wk 14. Hematocrit values remained unchanged in all treatment groups throughout the experiment.

A moderate leukopenia, evident in the control rats and those at 20 mg/l during wk 10 ($p < 0.01$), was evident in all animals after 14 wk and

TABLE 5. Hematologic Values[a] of Tail Vein Blood of Male Rats Exposed to Low Concentrations of 1,3-DNB in Drinking Water

1,3-DNB (mg/l)	n^b	Hematocrit (vol. %)	Hemoglobin (g per 100 ml)	Leukocytes ($\times 10^3$ mm^{-3})
		wk 5		
0	5	52 ± 1.5	14.7 ± 0.32	25.1 ± 1.6
3	5	51 ± 0.9	14.5 ± 0.28	25.5 ± 1.1
8	5	52 ± 1.7	15.0 ± 0.06	21.8 ± 2.1
20	5	48 ± 1.3	13.8 ± 0.15*[c]	20.0 ± 2.0
		wk 10		
0	5	52 ± 0.6	15.3 ± 0.12	15.6 ± 1.3
3	5	52 ± 0.8	15.2 ± 0.25	29.7 ± 2.1***
8	5	52 ± 1.6	15.1 ± 0.29	26.0 ± 2.7**
20	5	51 ± 1.6	13.9 ± 0.32**	17.7 ± 1.4
		wk 14		
0	5	54 ± 0.8	15.7 ± 0.25	17.8 ± 2.6
3	5	52 ± 2.1	15.8 ± 0.19	17.6 ± 1.6
8	5	50 ± 1.1	15.7 ± 0.25	16.4 ± 1.4
20	5	53 ± 2.0	15.1 ± 0.32	19.8 ± 1.4

[a] Average values ± SE.
[b] Number of rats.
[c] (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

was not considered dose-related. The percentage distribution of the differentiated leukocytes was normal in all instances.

The average length of the period of sleep resulting from a standard dose of sodium hexobarbital was determined after the rats had been maintained for 14 wk on water containing 1,3-DNB. The length of the period of sleep was prolonged in the males at 8 mg/l 1,3-DNB ($p < 0.01$) (Table 6). No significant difference was noted in the sleeping time of the males at 3 and 20 mg/l or of the females at any level. Because there were no changes in the males at the highest concentration of 1,3-DNB or in the females at any concentration, we believe that the increase in the males at 8 mg/l represents an improbable sampling error or the influence of an undetected factor unrelated to the presence of 1,3-DNB in the drinking water.

Average terminal weights of the body and of various organs for males and females are given in Table 7. In both sexes, the spleens were increased in weight in rats given water containing 8 or 20 mg/l 1,3-DNB. The large spleens in the males at 3 mg/l are attributed to their heavier body weight. The testes were sharply reduced in weight only in males at 20 mg/l. The excess weight of ovaries among females at 3 mg/l was only partly related to increased body weight. After adjustment for body weight, the ovaries were still heavier than expected ($p < 0.05$). In both sexes the weights of other organs varied proportionately with the body weight and showed no change attributable to 1,3-DNB.

Microscopic examination revealed the presence of hemosiderin in the spleens. This was most pronounced in animals that had received 20 mg/l 1,3-DNB in the drinking water. Sections of testicular tissue showed no abnormalities in animals exposed to 3 or 8 mg/l 1,3-DNB. However, there was evidence of a slight to moderate decrease in spermatogenesis in the group receiving 20 mg/l. In all groups an occasional animal had slight

TABLE 6. Minutes of Sleep Induced by Hexobarbital in Rats Drinking Water Containing 1,3-DNB for 14 wk

	Males		Females	
1,3-DNB (mg/l)	n^b	Average sleep time[a] (min)	n	Average sleep time (min)
0	9	31 ± 3	10	109 ± 10
3	8	35 ± 5	8	113 ± 6
8	8	46 ± 3**[c]	8	112 ± 12
20	8	34 ± 2	7	101 ± 10

[a] Average value ± SE.
[b] Number of rats used.
[c] (**) $p < 0.01$.

TABLE 7. Terminal Weights of body and Organs[a] of Rats Given 1,3-DNB in Drinking Water for 16 wk

1,3-DNB (mg/l)	No. of rats	Average weight ± SE[b] Body (g)	Spleen (g)	R^c (mg/g)	Testicles ovaries (g)	R (mg/g)
			Males			
0	20	439 ± 7	0.85 ± 0.02	1.94	3.53 ± 0.09	7.95
3	20	453 ± 6	0.93 ± 0.02**	2.06	3.66 ± 0.05	8.10
8	20	450 ± 6	1.02 ± 0.02***	2.26**	3.74 ± 0.10	8.34
20	20	431 ± 5	1.10 ± 0.04***	2.56***	1.36 ± 0.07***	3.19***
			Females			
0	20	277 ± 4	0.68 ± 0.01	2.48	0.13 ± 0.004	0.44
3	20	281 ± 4	0.69 ± 0.02	2.44	0.14 ± 0.004**	0.51*
8	20	284 ± 4	0.79 ± 0.02***	2.78**	0.13 ± 0.004	0.47
20	20	263 ± 4*	0.84 ± 0.02***	3.19***	0.13 ± 0.003	0.49

[a]Variations in weight of liver, heart, lungs, kidneys, pituitary, and adrenals were related to variations in body weight.
[b](*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.
[c]R is the ratio of organ to body weight.

pneumonitis, which was considered to be an intercurrent disease. There were no pathological alterations in the other organs examined that could be attributed to the experimental exposure.

Behavioral Changes in Rats Given 3 and 8 mg/l 1,3-DNB for 90 d in Drinking Water

Overall, male rats receiving 1,3-DNB showed significantly higher levels of running in the activity wheels than controls ($p < 0.01$). Both groups receiving 1,3-DNB showed elevated activity levels compared to the controls but did not differ between themselves (Table 8). Data from the activity

TABLE 8. Changes in Wheel Running Activity during a 90-d Exposure of Rats[a] to 1,3-DNB in Drinking Water[b]

1,3-DNB (mg/l)	(Avg. revolutions per day)$^{1/2}$
0	45 ± 2
3	62 ± 3 ($p < 0.01$)
8	63 ± 3 ($p < 0.01$)

[a]Five male rats per group.
[b]There were no differences in food or water intake between groups.

TABLE 9. Activity Platform Counts during a 90-d exposure of Rats[a] to 1,3-DNB in Drinking Water[b]

1,3-DNB (mg/l)	(Avg. counts per day)$^{1/2}$
0	229 ± 2
3	234 ± 4 (NS)
8	233 ± 2 (NS)

[a]Ten male rats per group.
[b]There were no differences in food or water intake between groups.

platform studies revealed a trend toward elevated activity in the 1,3-DNB-exposed animals; however, increased activities were not significantly greater than control activities (Table 9). As in the other intact animal studies, exposure of rats to either 3 or 8 mg/l 1,3-DNB in drinking water had no significant effect on food or water consumption or growth.

Tissue Cultures

Seven experiments were conducted to determine the concentration-related response of L-cell cultures to 1,3-DNB. Four are shown in Fig. 1. These experiments had a standard deviation of the mean protein content at each 1,3-DNB concentration that was less than 15% of the control mean. A standard deviation of less than 15% of the control can be routinely achieved in the concentration-response experiments and we use it

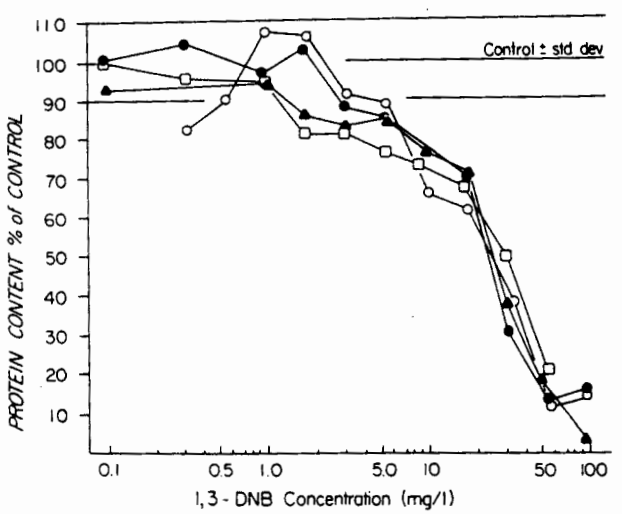

FIGURE 1. Protein content of mouse fibroblast cultures exposed to 1,3-DNB. Each of the four lines connects the means of a separate experiment. The standard deviation of each mean is ≤15% of the control mean.

as a criterion for the acceptability of an experiment. If more than one concentration point in an experiment has a standard deviation of the mean protein content exceeding 15%, the experiment is not included in determining the concentration that causes a 50% reduction in protein content (LC50) or the minimum-effect concentration. The three experiments not shown in Fig. 1 are in agreement with the four shown but do not meet the 15% criterion. The LC50 and the minimum-effect concentration, taken to be the concentration causing a 20% mean reduction in protein content, were determined from Fig. 1 to be 20–25 mg/l and 3.5–8.0 mg/l, respectively.

The response of tissue cultures to 1,3-DNB based on DNA content was the same as that based on protein content (Fig. 2). It appears that the effect of 1,3-DNB is that of a general cell poison, since at all concentrations of 1,3-DNB both protein content and DNA content were reduced in the same proportion.

Microscopic observations of morphological changes in treated cultures and vital staining with erythrosine B showed that cell losses at higher concentrations were due primarily to cell death and destruction and not to detachment of healthy cells from the surface of the tissue culture flask. At lower concentrations of 1,3-DNB, where inhibition of protein and DNA production did not cause complete cell death, cytopathological changes such as excessive granularity of cytoplasm and loss of characteristic fibroblastic shape were observed to be concentration-dependent.

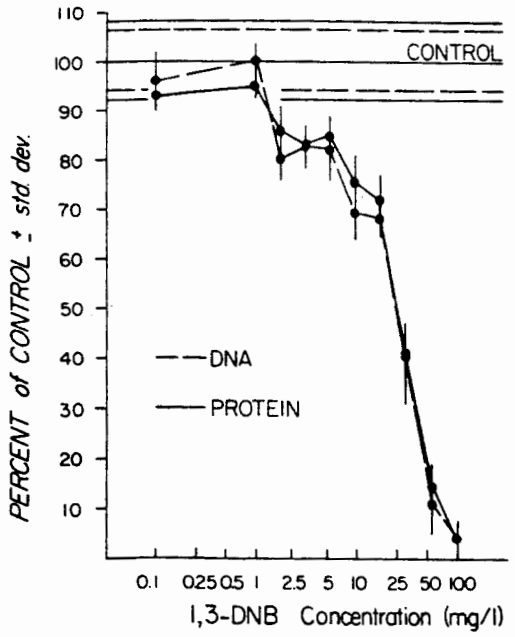

FIGURE 2. Comparison of the effects of 1,3-DNB on protein content and DNA content of mouse fibroblast cultures. The vertical line through each point represents ±1 SD.

Reduction of the pH of the medium, often the earliest sign of a toxic effect, occurred in 1,3-DNB-affected cultures. Although a useful indicator of effect, low pH can also contribute to inhibition of cell growth. Therefore pH was not permitted to drop below 6.8, and precautions were taken to keep it, if possible, between 7.0 and 7.2.

DISCUSSION

Ingestion of moderate to low concentrations of 1,3-DNB in drinking water caused loss of body weight (⩾100 mg/l), a moderate reduction in hematocrit (50 mg/l), a reduction of sleep time in females (⩾50 mg/l), a reduction in hemoglobin content of blood (⩾20 mg/l), atrophy of testes (⩾20 mg/l) but not ovaries, enlargement of spleens (⩾8 mg/l), and an increase in activity (3 and 8 mg/l) as measured in a running wheel). No 1,3-DNB-related increases in leukocyte numbers or changes in distribution were observed at any concentration tested. Similarly, there were no changes in weights or pathological effects in organs, other than those mentioned above, in any treatment group. Toxic effects on tissue cultures—that is, reduction in net production of protein and DNA—were observed at concentrations as low as 3–8 mg/l 1,3-DNB.

1,3-DNB is a lipid-soluble, weakly acidic compound that is easily absorbed through the skin (Ishihara et al., 1976) and probably readily absorbed by the gut. Experiments with ^{14}C-labeled 1,3-DNB showed that in rabbits it is absorbed, metabolized, and excreted in the urine (Parke, 1961). Our acute toxicity data, showing rapid recovery of survivors, and our subacute studies, showing no late appearance of new toxic responses or unusually great increases in severity of toxicity, suggest that in rats neither 1,3-DNB nor its toxic metabolites are retained in the body to an appreciable extent.

Enlargement of the spleen occurred at lower concentrations of 1,3-DNB in drinking water than other responses, with the exception of enhancement of activity. Except at 200 mg/l, where fibrosis was noted, the increases in size and weight of spleens were not accompanied by other pathological signs. Therefore, although the weight increases were seen at 1,3-DNB concentrations below those causing discernible effects on blood, spleen enlargement was probably mainly a secondary response to the methemoglobinemia that results from intake of dinitrobenzenes. Enlargement of the spleen is not uncommon in chemically induced hemolytic anemias (Locket, 1957).

Testes are sensitive to exposure to many chemicals, nitrobenzenes among them. In the case of the aromatic nitro compounds the effect is usually on the primary spermatocyte (Newburgh, 1975). Our results demonstrate that this is the case with ingestion of 1,3-DNB concentrations of 20 mg/l or higher. At 200 mg/l extensive damage to the entire spermatogenesis apparatus is observed. It is apparently not known whether

1,3-DNB or one of its metabolites causes the testicular damage. Cultures of L-cells do not have aryl hydrocarbon hydroxylase activity (Gelboin and Wiebel, 1971; Wiebel et al., 1972) and should therefore be incapable of metabolism of xenobiotics such as 1,3-DNB by microsomal enzyme pathways, and yet toxic effects in tissue cultures demonstrate that cellular damage can occur at concentrations of 1,3-DNB as low as 3–8 mg/l in the bathing fluid. Therefore, unchanged 1,3-DNB could be responsible for the cellular damage that occurs in the testicles at the ingested concentrations. On the other hand, if metabolites of 1,3-DNB are the active compounds, as is thought to be the case in formation of methemoglobin in mammals (Parke, 1968), it is also possible that mutagenesis and/or teratogenesis could occur. It is not known whether any of the metabolites are carcinogens. The possibility of mutagenic or teratogenic effects on the spermatogenic tissues should be considered in further research on this compound.

Metabolism of 1,3-DNB proceeds by enzymic reduction of one or both of the two nitro groups and addition of a hydroxyl group adjacent to one of the amino groups. Conjugation follows, and the metabolites are excreted in the urine. Metabolic studies in rabbits (Parke, 1961) showed that 65–93% of fed 1,3-DNB was excreted in urine, primarily as the metabolites *m*-nitroaniline, *m*-phenylenediamine, 2-amino-4-nitrophenol, and 2,4-diaminophenol during the first 2 d. From 1 to 5% of the dose was excreted in the feces. Carcinogenicity screening of *m*-phenylenediamine and 4-amino-2-nitrophenol yielded indefinite results (NIOSH, 1979).

There was a significant reduction in hexobarbital-induced sleep time in female rats ingesting $\geqslant$50 mg/l 1,3-DNB in their drinking water. This suggests induction of hexobarbital-metabolizing enzymes. Such induction by 1,3-DNB may be explained by a common pathway of metabolism. Hexobarbital is deactivated by hydroxylation (Parke, 1968), which is also a step in metabolism of 1,3-DNB. Enzymatic reactions of this type are usually associated with microsomes of metabolically active cells in liver and, to some extent, kidney, lung, skin, and mucosa.

In male rats, secretions of testicular hormones induce microsomal enzyme synthesis (Kato and Gillette, 1965); thus the sleep time caused by administration of hexobarbital is normally shorter in males than in females. In our experiments, there was no additional reduction in sleep time in males due to 1,3-DNB ingestion except at 100 mg/l (there was a tendency toward sleep time reduction at 50 mg/l). The relation between hexobarbital-induced sleep time and 1,3-DNB ingestion may be confounded in males by alterations in testicular hormone production by the damaged testes.

Our data on increased activity in male rats at 3 and 8 mg/l show a physiological effect on these animals that we were not able to associate with any pathological manifestation. Alterations of behavior are usually due to changes in the central nervous system, although changes in the

peripheral nervous system and non-nervous system changes can also affect behavior.

On the basis of the mechanisms of action of compounds like 1,3-DNB (methemoglobin formers) and the possibility that the active agent might be a metabolite, we speculated before these experiments that the tissue culture assay system under study might not detect concentrations in water as low as those that would affect animals. However, 1,3-DNB was a strong enough cell toxin to give a response in the tissue cultures at concentrations in the same range as those eliciting the most sensitive responses observed in intact rats.

Based on daily consumption per kilogram of body weight, the rats in the 16-wk studies consumed about 6–7 times more water than average adult humans. If humans are affected in the same ways as the experimental animals at a given intake of 1,3-DNB per kilogram of body weight, they could be expected to tolerate 20–50 mg/l in their drinking water before being affected. Allowing a 10-fold safety factor to account for variations in water consumption due to age, health status, climate, work load, and so on, a maximum concentration in drinking water of 2–5 mg/l might be acceptable for humans. Concentrations this high should not occur in adequately treated and monitored public water supplies (EPA, 1975). Chronic exposure to concentrations of 1,3-DNB as high as several milligrams per liter could occur if the drinking water were from an untreated supply contaminated with chemicals, for example, residential wells pumping ground water contaminated with chemicals from an improperly constructed or maintained chemical waste disposal site. The tissue culture assay begins to give a detectable response at 3–8 mg/l 1,3-DNB. If a concentration in excess of 2–5 mg/l appeared in a drinking water supply where the assay was in use, a toxicity response would be noted and appropriate actions could be taken to prevent long-term exposure to a potentially hazardous concentration.

Many compounds with diverse mechanisms of action occur in water supplies. A toxicity assay of general usefulness must respond to many types of compounds that could be present in potentially hazardous concentrations. The lowest concentration detectable by the assay should be low enough to allow action to be taken before harm is done, but it should not be so low that the assay gives too many false warnings. A tissue culture toxicity assay may prove to be generally useful.

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Received July 29, 1979
Accepted September 5, 1980