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LANL Reference 1/1/1984

FUNDAMENTAL AND APPLIED TOXICOLOGY 4, 248–260 (1984)

Subacute and Chronic Toxicity Studies of Trinitroglycerin in Dogs, Rats, and Mice[1]

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Subacute and Chronic Toxicity Studies of Trinitroglycerin in Dogs, Rats, and Mice. ELLIS, H. V., III, HONG, C. B., LEE, C. C., DACRE, J. C., AND GLENNON, J. P. (1984). *Fundam. Appl. Toxicol.* 4, 248–260. Subacute and chronic oral toxicities of trinitroglycerin (TNG) were studied in beagle dogs, CD rats, and CD-1 mice. No adverse effects were seen in dogs given up to 1 mg/kg/day of TNG for 4 weeks, then 5 mg/kg/day for 9 more weeks. Dogs given 25 to 200 mg/kg/day for 5 days had transient and dose-related severe methemoglobinemia, while 200 mg/kg/day produced depression; dogs given 1, 5, or 25 mg/kg/day for 12 months had transient and dose-related mild methemoglobinemia. Rats fed 0.1% TNG for 5 weeks, then 0.5% (230–234 mg/kg/day) for 8 more weeks had decreases in feed consumption and weight gain after the increase in dosage; rats fed 2.5% TNG (1406 or 1416 mg/kg/day for males and females, respectively) for 13 weeks suffered adverse effects, including weight loss, compensated anemia, and testicular degeneration, but they resumed gaining weight as feeding continued. Rats fed 1% TNG (363 or 434 mg/kg/day for males and females, respectively) for 2 years had decreased weight gain, decreased grooming, methemoglobinemia and its sequelae, cholangiofibrosis, hepatocellular carcinoma, and interstitial cell tumors of the testis. A decrease in the naturally occurring pituitary chromophobe adenoma and mammary tumors increased the life span of the females. Some rats fed 0.1% TNG (31.5 or 38.1 mg/kg/day for males and females, respectively) had mild hepatic lesions similar to those seen in rats fed the larger doses. No adverse effects were seen in mice fed up to 0.1% TNG for 3 weeks, then 0.5% for 10 more weeks. Mice fed 1% TNG (1022 or 1058 mg/kg/day for males and females, respectively) for 2 years had decreased weight gain, decreased grooming, and methemoglobinemia and its sequelae, but no obvious cellular changes as found in the rats.

Trinitroglycerin (TNG) became the first "high explosive" in use after Nobel desensitized it by adsorption onto diatomaceous earth, producing dynamite. In recent years, dynamite has been almost entirely replaced by ammonium nitrate-based blasting agents. However, much TNG is still used, in combination with nitrocellulose, to produce double-base solid propellants for firearms and rockets for military and civilian use. A small, but vital, quantity is used as a coronary vasodilator in the management of angina pectoris.

As with other old compounds, few studies of the toxicity of TNG are available. The only adverse effect regularly cited is headache caused by TNGs smooth muscle effects (Ellis *et al.*, 1977; Lange *et al.*, 1972). Most of the animal studies on TNG have been directed at these smooth muscle effects or at the metab-

[1] This research was supported by the U.S. Army Medical Research and Development Command under Contract DAMD 17-74-C-4073.

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olism of TNG (National Institute for Occupational Safety and Health, 1978). Only two carcinogenicity studies have been reported. Takayama (1975) gave rats drinking water containing 0.03% TNG, and found no adverse effects. Suzuki *et al.* (1975) gave mice drinking water containing 0.033% TNG, and found a small, but statistically significant, increase in pituitary tumors in female mice.

This paper reports subacute (13 week) and chronic (1- and 2-year) toxicity studies of TNG in dogs, rats, and mice. A preliminary account of part of this work has been presented (Dacre *et al.*, 1980).

METHODS

Animals

Young healthy beagles[6] (5 to 6 months old), CD rats[7] (200 to 250 g), and CD-1 mice[7] (25 to 27 g) were used. All animals were quarantined for 2 weeks. The dogs were housed 8 to 12 per pen (60 ft^2 of heated inside space) with runs (120 ft^2 of outside space). Two to three rats or four mice were kept in plastic cages with hardwood chip bedding, metal lids, and filter tops. Bedding material was steam-sterilized before use and changed at least weekly. Cages, tops, and water bottles were steam-sterilized and changed weekly. The rodent quarters were maintained, with 10 air changes per hour, at 75 ± 5°F temperature, 50 ± 10% relative humidity, and a 12-hr light cycle. Dog chow, powdered rodent chow[9] and water were available ad libitum.

Material

Commercial TNG, desensitized in lactose as a 10% mixture (SDM No. 17), was purchased.[10] Identification was made by extraction with chloroform and evaporation to a liquid with infrared spectrum identical to that reported for TNG (Hayden *et al.*, 1972). The TNG extract was purity over 99% as determined by gas chromatography. The nominal 10% TNG mixture in lactose contained 9.72 ± 0.09% as analyzed for TNG content (Wells *et al.*, 1970). For the chronic rodent studies, a mixture[11] of 10% TNG on rodent chow[9] was used.

The nominal 10% TNG mixture was used to prepare all doses. For dogs treated with 1 mg/kg or less, a fivefold dilution of the 10% mixture was prepared by dilution with lactose in a ball mill. The dogs were dosed with hard gelatin capsules and at 25 mg/kg/day or greater, more than one capsule was used. The capsules were prepared based on the weekly body weights. For rodents, TNG mixture was added to powdered rodent chow to produce a diet containing the desired concentration and mixed in a wooden box (16 × 16 × 20 in.) no more than half full, rotated about its long axis for 1 hr in a modified cement mixer at a speed of 20 rpm. Lower concentrations of TNG were prepared by successive dilutions. All diets were prepared weekly.

The stability of feed levels of 0.01 or 1% of TNG was determined. TNG was stable when frozen for 8 days. An average of 95 and 89% of TNG remained at the end of 4 and 8 days, respectively, under normal feeding conditions. When the 0.5% TNG feed mixture spiked with [1,3-^{14}C]TNG was stored in an operating fume hood, the loss of TNG was found to be due to evaporation rather than chemical reaction with rat feed components. The average amounts of TNG remaining in feed mixture daily for 7 days (found to be 93%) were calculated from the decay graph, which was used to correct the evaporation loss of TNG in all feeding studies for rats and mice.

Experimental Design

Subacute toxicity studies. One control and three treatment groups were used. Each group consisted of four male and four female beagles or six male and six female rodents. The animals were selected for uniformity of weight within each species. The dogs were given 0, 0.01, 0.1, or 1 mg/kg/day of TNG in capsules. The rats and mice were fed diets containing 0.001, 0.01, or 0.1% of TNG. No adverse effects were observed during the first several weeks. Doses were increased fivefold for all groups of dogs, rats, and mice starting the 4th, 5th, and 3rd week, respectively. The increased doses were 0.05, 0.5, and 5 mg/kg/day for dogs and 0.005, 0.05, and 0.5% in diets for rats and mice. All animals were observed daily for any adverse effects or behavioral changes. Individual body weights were recorded weekly and feed intake for rodents was measured throughout the study.

Blood samples for various tests were collected from the jugular vein of dogs before treatment and at the end of 4, 8, and 13 weeks, and from the abdominal aorta of anesthetized rats and mice at the termination. The hematology tests included erythrocyte counts, reticulocyte,

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[6] Hazleton Research Animals, Cumberland, Va.

[7] Charles River Breeding Laboratories, Wilmington, Mass.

[8] Purina dog chow, Ralston-Purina Company, St. Louis, Mo.

[9] Purina Lab-Blox, Allied Mills, Chicago, Ill.

[10] ICI Chemical Division, ICI America Inc., Chicago, Ill.

[11] Picatinny Arsenal, Dover, N.J.

leukocyte counts, differential and platelet counts, hematocrit, and hemoglobin. The clinical chemistry analyses included fasting blood glucose, serum transaminases, alkaline phosphatase, and blood urea nitrogen. In addition, levels of methemoglobin, Heinz bodies, clotting time, immunoglobin E, serum electrolytes including Na, K, Mg, Ca, and Cl were determined, and bromsulfothalein retention, urinalysis and fecal examination for occult blood were performed for dogs.

When moribund or at terminations, the dogs were killed with phenobarbital sodium and rodents with ether. The liver, spleen, kidney, heart, and brain were removed and weighed; organ weight/body weight and organ weight/brain weight ratios were calculated. These and other organs (respiratory tract, alimentary canal, urogenital organs, salivary gland, pancreas, thymus, thyroids, adrenals, mesenteric lymph nodes, gracilis muscle, pituitary, brain, and rib bone marrow smears), especially those with gross lesions or growths, were processed and stained for microscopic examination.

Because only minimal effects were seen in the initial study, additional subacute studies were performed in dogs and rats at higher concentrations. Four groups of young adult beagles, each consisting of two males and two females, were given 25, 50, 100, or 200 mg/kg/day of TNG in capsules for 5 consecutive days. After each dose, blood samples from each dog were taken at 0.5, 1, 2, 4, 8, 16, and 24 hr for the determination of methemoglobin (Dubowski, 1964) and total hemoglobin concentrations. To study any protective effect on methemoglobin formation, 3 mg/kg of methylene blue injection, USP, was given iv to the dogs 2 hr after administration of 200 mg/kg/day of TNG on the 3rd day. In the second rat study three males and three females were fed 2.5% of TNG in the feed; four males and four females were fed the basal diet serving as controls. Feed intake and body weight were measured throughout. At the end of 13 weeks, blood from the abdominal aorta was collected under ether anesthesia for hematology, clinical blood chemistry, and measurement of serum electrolytes. Heart, liver, spleen, and kidneys were weighed; various tissues were removed for histopathological examination. Since the rats fed 2.5% of TNG also received about 22.5% of lactose as diluent and desensitizing agent, another group of 10 males and 10 females were used to study the effect of lactose. Five males and five females were fed 25% lactose (USP) by weight in feed; five males and five females were fed the basal diet to serve as controls. In addition to the parameters as described above, the femur was weighed, digested in concentrated nitric acid, and analyzed for calcium with a Varian Model 6 atomic absorption spectrophotometer using the resonance line 2399 Å. A nitrous oxide–acetylene flame was used to eliminate any interference from phosphate. A piece of the liver was also weighed, digested in concentrated nitric acid with the addition of 70% perchloric acid, and analyzed for iron with the same spectrophotometer. An air–acetylene flame was used.

Chronic toxicities. The animals were placed [illegible] which were stratified by the animals weights [illegible] tribution into groups. Four groups of 6 male and [illegible] beagles, 38 male and 38 female rats, or 58 ma[illegible] female mice, each, were used. The dogs were [illegible] 5, or 25 mg/kg/day of TNG in capsules. The [illegible] mice were fed diets containing 0, 0.01, 0.1, or 1% [illegible] All animals were observed for adverse effects or be[illegible] changes. Individual body weights were recorde[illegible] and feed intake was measured during the first [illegible] and then for 1 week each month thereafter. [illegible] were terminated at the end of 12 months and th[illegible] at 24 months. After 12 months, interim termina[illegible] performed on eight male and eight female rode[illegible] samples were collected from the jugular vein o[illegible] and from the tail tips of four male and four fe[illegible] before treatment and at the end of 3, 6, 9, 12 [illegible] months for various tests. At terminations, blood f[illegible] abdominal aortae of both rats and mice was co[illegible] under ether anesthesia. When moribund or at termin[illegible] the animals were killed, various organs weighed, and t[illegible] were processed for microscopic examination.

Statistics. Data were analyzed by standard me[illegible] (Steel and Torrie, 1960), with $p < 0.05$ considered [illegible] nificant. Continuous variables were analyzed by Dunnett's multiple comparison procedure or Student's t test. Enumeration, such as tumor incidence, data were analyzed by Fisher's exact probability test.

RESULTS

Subacute Toxicity Studies

In the first weeks of the study, it became apparent that there were no toxic effects in dogs, rats, or mice at the initial doses. As shown in Table 1, all doses were increased fivefold after 3 to 5 weeks of the 13-week study. TNG intake in rodents is shown in Table 2. No adverse effects were seen in any dogs given TNG up to 5 mg/kg/day. Rats fed the high dose of TNG (up to 230 or 234 mg/kg/day for the males and females, respectively) had decreased feed consumption and somewhat lower weight gain after the increase in dosage. There were no toxicologically significant findings in the blood tests, necropsy, and histopathology. Mild to moderate extramedullary hematopoiesis was noted in the livers and/or spleens of some mice given TNG (up to 608 or 561 mg/kg/day for the males and females,

TABLE 1

DOSES OF TRINITROGLYCERIN USED IN VARIOUS STUDIES

	Subacute studies		Additional subacute studies		Chronic studies	
Dose group	Dog[a] (mg/kg/day)	Rat[b], mouse[c] (% in feed)	Dog[d] (mg/kg/day)	Rat (% in feed)	Dog (mg/kg/day)	Rat, mouse (% in feed)
Low	0.01, 0.05	0.001, 0.005	25	—	1	0.01
Middle	0.1, 0.5	0.01, 0.05	50	—	5	0.1
High middle	—	—	100	—	—	—
High	1, 5	0.01, 0.5	200	2.5	25	1.0

[a] Given 4 and 9 weeks, respectively.
[b] Fed 5 and 8 weeks, respectively.
[c] Fed 3 and 10 weeks, respectively.
[d] Given 5 days only.

respectively) for 13 weeks. However, there was no dose relationship, and this lesion does occur spontaneously.

Additional Subacute Toxicity Studies

Dogs. Methemoglobin levels for the dogs given 25, 50, 100, or 200 mg/kg/day of TNG for the first 4 days are shown in Fig. 1; the results on Day 5 were similar. There is a dose relationship for both peak level and duration of methemoglobinemia. The methemoglobin levels of dogs given up to 100 mg/kg/day returned to zero within 24 hr of dosing. Those dogs given 200 mg/kg/day did not completely recover and had higher peak levels on later days. The dogs given two lower doses (25 and 50 mg/kg/day) had no adverse clinical effects. The dogs given two higher doses (100 and 200 mg/kg/day) had cyanosis, starting 2 or 3 hr after dosing and lasting for several hours. The cyanosis corresponded to the period of high methemoglobinemia. In addition, the high dose (200 mg/kg/day) dogs also had decreased activity. Because of the severity of effects, the high dose dogs were given 3 mg/kg of methylene blue intravenously 2 hr after the third dose, and treatment of TNG was discontinued on the later days. This injection of methylene

TABLE 2

TNG INTAKE (mg/kg/DAY) BY RATS AND MICE FED TNG FOR 13 WEEKS AND 2 YEARS

Dose (% in feed)	Males	Females	Males	Females
	Rats,[a] 13 weeks		Mice,[b] 13 weeks	
0.001–0.005	0.8–2.6	0.9–3.1	1.3–6.4	1.3–6.9
0.01–0.05	6.0–24.5	6.4–26.5	11.5–60.2	10.9–58.7
0.1–0.5	59–230	59–234	107–608	95–561
	Rats, 2 years		Mice, 2 years	
0.01	3.04 ± 0.16[c]	3.99 ± 0.18[c]	11.10 ± 0.40[c]	9.72 ± 0.29[c]
0.01	31.5 ± 1.6	38.1 ± 1.6	114.6 ± 4.6	96.4 ± 3.3
0.1	363 ± 10	434 ± 11	1,022 ± 38[d]	1,058 ± 31

[a] The doses 5 weeks and 8 weeks, respectively.
[b] The doses 3 weeks and 10 weeks, respectively.
[c] ± SE of 24 monthly measures.
[d] No unscheduled deaths, only 23 monthly measures.

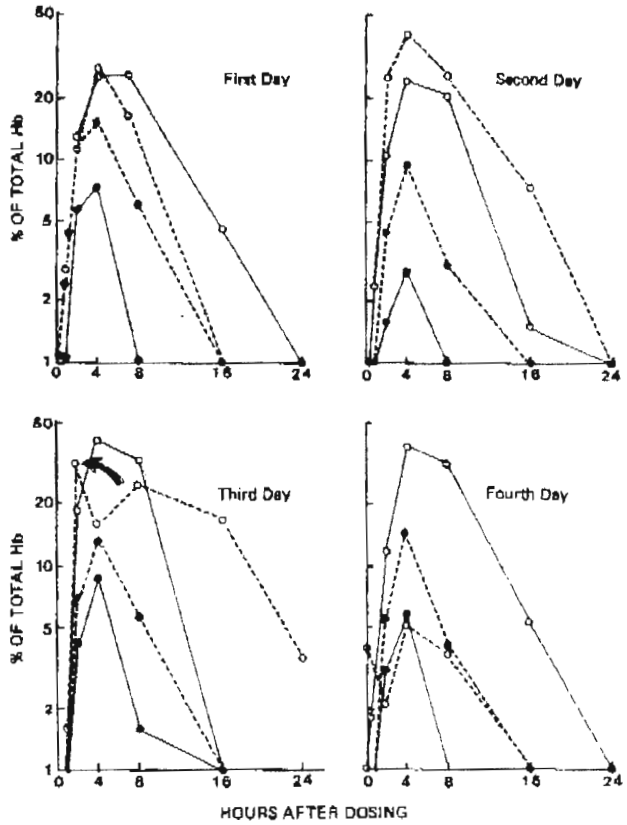

FIG. 1. Methemoglobin concentration in dogs given various doses of TNG: ● —— ●, 25 mg/kg/day; ● - - - ●, 50 mg/kg/day; O —— O, 100 mg/kg/day; O - - - O, 200 mg/kg/day; arrow, 3 mg/kg methylene blue and cessation of TNG treatment.

blue decreased the peak concentration of methemoglobin, but some methemoglobin was detectable nearly 40 hr after dosing.

Rats. Male and female rats fed 2.5% TNG lost weight for 8 weeks but then began to gain weight slowly (Fig. 2). Feed consumption by these rats was half that of the controls during the first 8 weeks; thereafter, the consumption increased. The TNG intake increased throughout the study with an overall average of 1406 mg/kg/day for males and 1416 mg/kg/day for females. The treated rats had a significant increase in erythrocytes, reticulocytes, hematocrit, hemoglobin concentration, and alkaline phosphatase, and a decrease in fasting blood glucose (Table 3). Neither methemoglobin nor other changes were found. The treated rats also had pigment deposits in the liver and spleen and moderate to severe testicular degeneration and/or atrophy with severe to complete aspermatogenesis.

Diets containing 20% or more lactose have been reported to have some adverse effects (Atkinson *et al.*, 1957). Since these rats received 2.5% TNG and 22.5% lactose in their diet, the effects of 25% lactose were studied. No adverse effects were found on body weight gain (Fig. 2), laboratory data (including serum electrolytes), bone calcium, and liver iron content. The lactose caused no lesions, but did increase cecum weight by one-third to one-half. Despite this lack of major adverse effect, TNG was mixed directly with the feed, without lactose, for the chronic studies in rodents.

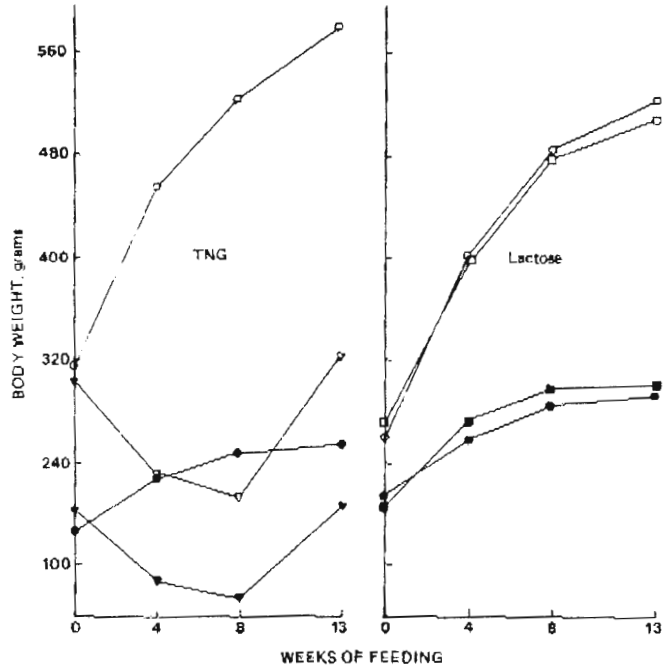

FIG. 2. Body weights of male (top) and female (bottom) rats fed 2.5% TNG (left) or 25% lactose (right). Open figures, males; solid figures, females. Circles, controls; inverted triangles, 2.5% TNG; and squares, 25% control.

Chronic Toxicity Studies

Dogs. The only effect observed in dogs given or 25 mg/kg/day of TNG for 12 months occasional methemoglobinemia, usually than 3%, a level barely detectable. The dence was dose related, with some metoglobin found even in the low dose dogs.

Rats. Weight gain by the high dose (1% in feed) rats lagged significantly from st week of feeding (Fig. 3); all females ome males receiving the high dose lost t during the first week as a result of de- d feed consumption (Table 4). As the continued, feed consumption by the dose rats increased, but it never quite lled that of controls. The body weights high dose rats reached plateaus of about for males and 300 g for females. The s were considerably less than those for ntrol rats, over 800 or 500 g for males males, respectively. These smaller body weights were associated with decreased obesity (especially abdominal fat) in the high dose rats. During the later months of the study, the middle dose (0.1% TNG) males and females were about 60 or 30 g lighter than the controls, respectively. The calculated TNG intakes were 3.04 mg/kg/day for males and 3.99 mg/kg/day for females fed the low dose; 31.5 and 38.1 mg/kg/day, respectively, for those fed the middle dose; and 363 and 434 mg/kg/day, respectively, for those fed the high dose (Table 2).

By Month 3, the decreased weight gain of the high dose rats was obvious on visual inspection. The rats usually had tan fur. There were no obviously localized stains, but the rough, matted appearance indicated a lack of grooming. Occasionally, some high dose rats had bluish skin, especially around the snout.

Unscheduled deaths occurred from pituitary adenomas, ulcerated subcutaneous tumors, and a variety of rarer causes (Fig. 4).

TABLE 3

HEMATOLOGIC VALUES IN RATS FED TNG FOR 13 WEEKS

Hematologic measurement	Dose (% in feed)					
	Male			Female		
	0[a]	1[a]	2.5[b]	0[a]	1[a]	2.5[b]
Erythrocytes	7.52 ± 0.14	8.08 ± 0.24		6.94 ± 0.06	8.10 ± 0.21[c]	
($\times 10^6$/mm^3)	4.14 ± 0.25		5.84 ± 0.23[c]	3.88 ± 0.51		6.12 [illegible]
Reticulocytes (%)	0.96 ± 0.11	2.46 ± 0.52[c]		1.03 ± 0.08	1.14 ± 0.13	
	1.47 ± 0.09		2.47 ± 0.13[c]	1.69 ± 0.11		2.75 [illegible]
Hematocrit (vol%)	48.5 ± 0.5	56.5 ± 2.3[c]	57.3 ± 4.1[c]	46.4 ± 0.9	56.3 ± 2.5[c]	61.0 [illegible]
Hemoglobin (g%)	15.7 ± 0.1	17.3 ± 0.6[c]	17.8 ± 0.8[c]	14.8 ± 0.2	16.9 ± 0.5[c]	18.8 [illegible]
Methemoglobin (%)	0.0 ± 0.0	19.6 ± 4.8[c]	—	0.5 ± 0.5	20.4 ± 2.9[c]	
Platelets ($\times 10^5$/mm^3)	4.8 ± 0.3	5.2 ± 0.5	—	4.8 ± 0.2	5.4 ± 0.4	
Leukocytes ($\times 10^3$/mm^3)	16.1 ± 1.5	16.3 ± 1.7	—	13.5 ± 1.4	12.0 ± 1.3	
Alkaline phosphatase (IU/liter)	49.3 ± 2.8	—	76.3 ± 4.7[c]	25.3 ± 3.4	—	44.0 ± [illegible]
Fasting glucose (mg%)	121.3 ± 4.0	—	82.3 ± 2.9[c]	113.5 ± 5.4	—	83.3 ± [illegible]

[a] Mean ± SE of four rats.
[b] Mean ± SE of three rats.
[c] Significantly different from control by Dunnett's multiple comparison procedure.

The high dose females survived significantly longer than the control female rats. The most common spontaneous tumor in these rats is the pituitary chromophobe adenoma (MacKenzie and Garner, 1973), which accounted for a majority of these unscheduled deaths. The rats with this tumor were readily identified by their partial paralysis (usually unilateral), often combined with hyperexcitability and rapid, sustained weight losses. Subcutaneous tumors were common at milk-line sites in females and were sometimes seen at other sites and in males. The faster-growing tumors often became ulcerated from friction on the taut skin; these rats were then euthanized.

The primary abnormality seen in the hematologic values was methemoglobinemia, typically 10–30% of total hemoglobin, at the end of 3 months in the high dose rats (Table 6). There also occurred reticulocytosis, probably to compensate for methemoglobinemia. The erythrocyte count, hematocrit and hemoglobin concentration were increased, indicating hemoconcentration. No Heinz bodies were found. By 12 months, the erythrocyte count returned to normal, but moderate methemoglobinemia still persisted. After 24 months, methemoglobinemia disappeared. However, the males had elevations of SGOT, SGPT, and alkaline phosphatase. Most of the high dose rats dying at unscheduled times also had methemoglobinemia and/or elevated serum enzymes. No alterations occurred in the low dose and middle dose rats.

The histopathologic effects were seen in rats killed after 12 months feeding. The high dose rats had enlarged livers, more than one and one-half times the liver weight of controls, despite their lower total body weight. All but two high dose rats had cholangiofibrosis, proliferation of the bile ducts, and fibrous tissue. The other lesion was the progressive development of hepatocellular carcinoma, as first described by Reuber (1965) for *N*-2-fluorenyldiacetamide and classified by Squire and Levitt (1975). The early stage, areas and foci of hepatocellular alteration, was found in some rats of all dose groups; there was a dose–response relationship in both incidence and severity (Table 5). The next stage, neoplastic nodules, occurred in one middle dose male,

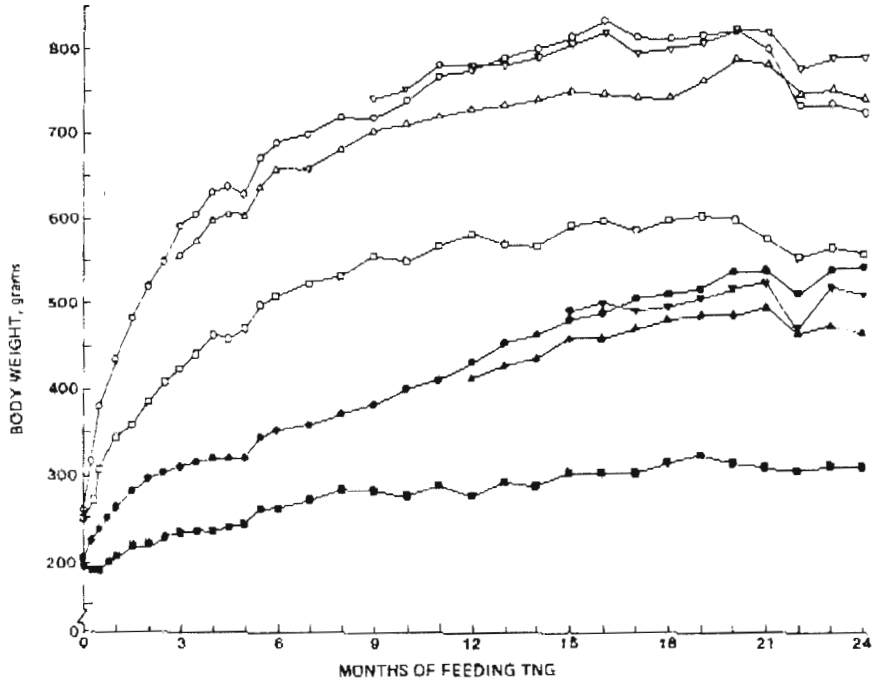

FIG. 3. Average body weights of rats fed various doses of TNG. Open figures, males; solid figures, females. Circles, controls; inverted triangles, 0.01% TNG; triangles, 0.1% TNG; and squares, 1% TNG. Some superimposed data points were omitted.

three high dose males, and one high dose female. The last stage, hepatocellular carcinoma, occurred in one high dose male. The histopathology of these hepatic lesions will be described in a subsequent paper (personal communication from C. B. Hong). There was also excessive pigmentation in the spleens of most high dose rats and in the epithelium of the kidney of many. The pigment resembled hemosiderin, but gave only a weak reaction to Prussian blue; it presumably consisted of denatured hemoglobins.

TABLE 4

FEED CONSUMPTION OF RATS FED TNG

Dose (% in feed)	Feed consumption (g/rat/day)			
	Males		Females	
	First 3 weeks	24 months	First 3 weeks	24 months
0	24.4 ± 0.7[a]	26.8 ± 1.1[b]	16.4 ± 0.4[a]	19.3 ± 0.9[b]
0.01	24.3 ± 1.0	26.7 ± 0.8	17.0 ± 0.4	20.0 ± 0.4
0.1	23.2 ± 0.7	25.9 ± 0.7	14.9 ± 0.2	18.6 ± 0.5
1	16.4 ± 2.2[c]	23.8 ± 0.7	9.0 ± 2.0[c]	15.1 ± 0.5[c]

[a] Mean ± SE of three weekly measures.
[b] Mean ± SE of 24 measures; the first month is the mean of three measures.
[c] Significantly different from control by Dunnett's multiple comparison procedure.

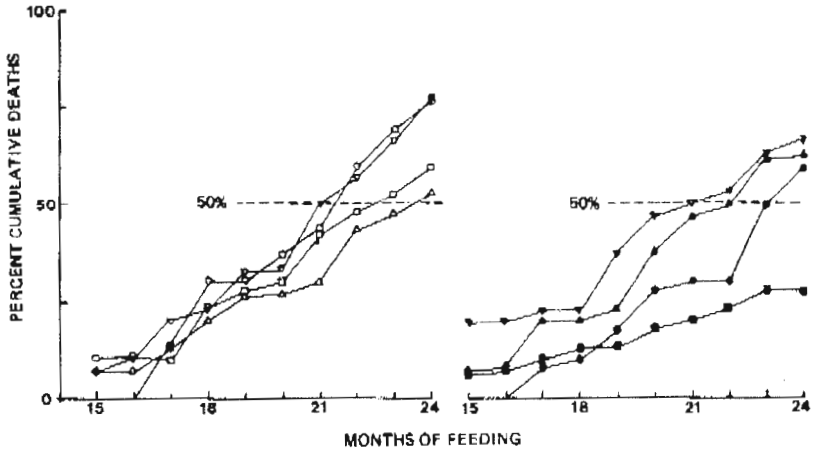

FIG. 4. Cumulative deaths among male (open figures) and female (solid figures) rats fed various doses of TNG. Circles, controls; inverted triangles, 0.01% TNG; triangles, 0.1% TNG; and squares, 1% TNG.

Lesions in rats killed after 24 months were similar to those killed after 12 months, but further developed. Liver weights of high dose rats averaged more than twice the weight of livers from control rats; relative weights (gram liver/gram total body weight) of high dose rats averaged four times those of controls. The rats dying at unscheduled times were similar. The major lesion, seen in most of the high dose rats, was cholangiofibrosis (Table 6). In addition, hepatocellular carcinomas and/or neoplastic nodules were seen in a majority of high dose rats, seven middle dose rats, one low dose rat, and one control rat. Excessive pigmentation was seen in the spleen and in renal epithelium. The incidence increased with increasing doses. In addition, half the high dose males had interstitial cell tumors in one or both testes. The effect of these growing tumors produced an incidence of aspermatogenesis greater than that normally seen in geriatric rats. However, the high dose rats had decreased incidences of mammary gland tumors (primarily fibroadenomas) and pituitary chromophobe adenoma.

TABLE 5

HEPATOCELLULAR ALTERATIONS[a] IN RATS FED TNG FOR 12 MONTHS

	Male rats[b]		Female rats[b]	
Dose (% in feed)	Low degree	High degree	Low degree	High degree
0	1/8	0/8	1/8	0/8
0.01	4/6	0/6	2/7	0/7
0.1	3/7	3/7	1/8	2/8
1	1/8	5/8	0/8	8/8

[a] These alterations are the areas and foci described by Squire and Levitt (1975) as the first stage in development of hepatocellular carcinomas.

[b] Data are rats with lesion/rats with readable slides.

Mice. During the first week of feeding TNG, the high dose mice ate very little and lost weight (Fig. 5). Thereafter, they resumed their weight gain, but lagged behind the controls. The weight plateaus of both the male and female high dose mice were about 6 g less than their respective controls. Feed consumption by high dose mice was only slightly less than controls. The calculated TNG intakes were 11.10 mg/kg/day for males and 9.72 mg/kg/day for females fed the low dose; 114.6 and 96.4 mg/kg/day, respectively, for those fed the middle dose; and 1022 and 1058 mg/kg/day, respectively, for those fed the high dose (Table 2).

As with the rats, after a few months of feeding the high dose mice had a tan matted fur, indicating a lack of grooming. The incidence

TABLE 6

HISTOPATHOLOGICAL LESIONS IN RATS FED TNG FOR 24 MONTHS AND THOSE DYING AT UNSCHEDULED TIMES

	Dose (% in feed)							
	Males				Female			
Lesion	0	0.01	0.1	1	0	0.01	0.1	1
Cholangiofibrosis	0/24	0/28	0/26	18/21	0/29	0/32	0/28	24/25
Hepatocellular carcinoma and/or neoplastic nodules	1/24	0/28	4/26	15/21	0/29	1/32	3/28	16/25
Extensive splenic pigmentation	3/24	1/28	6/26	7/21	1/29	2/32	5/28	16/25
Excessive renal epithelium pigmentation	1/24	0/28	0/26	11/21	0/29	3/32	4/28	31/25
Interstitial cell tumors of testis	2/24	1/28	3/26	11/21	—	—	—	—
Mammary gland tumors	1/24	0/28	1/26	0/21	13/29	19/32	12/28	2/25
Pituitary chromophobe adenoma	14/23	12/25	14/25	5/19	22/28	21/32	21/27	7/24

Note. Data are rats with lesions/rats with readable slides.

of unscheduled deaths was not significantly different between groups, with the high dose rats having slightly fewer survivors than the other groups. Females lived 2 to 3 months longer than males.

High dose mice killed after 12 months had a compensated anemia, normal erythrocyte count, with elevated reticulocyte count (Table [illegible]) as generally seen in rats. However, in mice [illegible] effects were accompanied by Heinz bod[illegible] and methemoglobinemia occurred only in [illegible].

[illegible] only TNG-related lesion was pigment [illegible]ts in most high dose mice and some [illegible] dose mice. There were golden-brown [illegible]llular granules in the liver, with lesser [illegible]ts in the spleen and/or kidneys. These [illegible]ts resembled hemosiderin, but gave only [illegible] Prussian blue reaction, indicating low [illegible]ontent, as seen in the rats. Lesions in [illegible]illed after 24 months, or dying at un[illegible]uled times, were substantially similar to those in mice killed after 12 months.

DISCUSSION

[illegible]re were quantitative and qualitative dif[illegible] to the toxic effects of TNG among species. Oral doses of up to 200 mg/kg/day for 5 days or 25 mg/kg/day for 12 months produced a transient methemoglobinemia with no other serious adverse effects in the dogs. In the rat, lifetime feeding of 363 mg/kg/day for males of 434 mg/kg/day for females caused toxic effects in the liver and blood, and 31.5 or 38.1 mg/kg/day, respectively, caused mild effects in the liver of susceptible individuals. Mice were the least affected. Lifetime feeding of 1022 mg/kg/day for males or 1058 mg/kg/day for females produced a compensated toxic anemia and some pigment deposits.

The only effect commonly seen in all three species was methemoglobinemia, a well-known effect of organic nitrates, including TNG (Kiese, 1974). Methemoglobinemia was transitory, as shown in the 5-day study in dogs. The hematopoietic system was apparently capable of compensating for significant amounts of methemoglobinemia. The usual observed effects were a reticulocytosis and pigment deposits, with little or no decrease and sometimes an increase in erythrocyte level. Heinz bodies sometimes result from methemoglobinemia (Kiese, 1974). We found no Heinz bodies in dogs and rats, but small, consistent numbers in mice. This species difference is unexplained. Another observed species difference was the

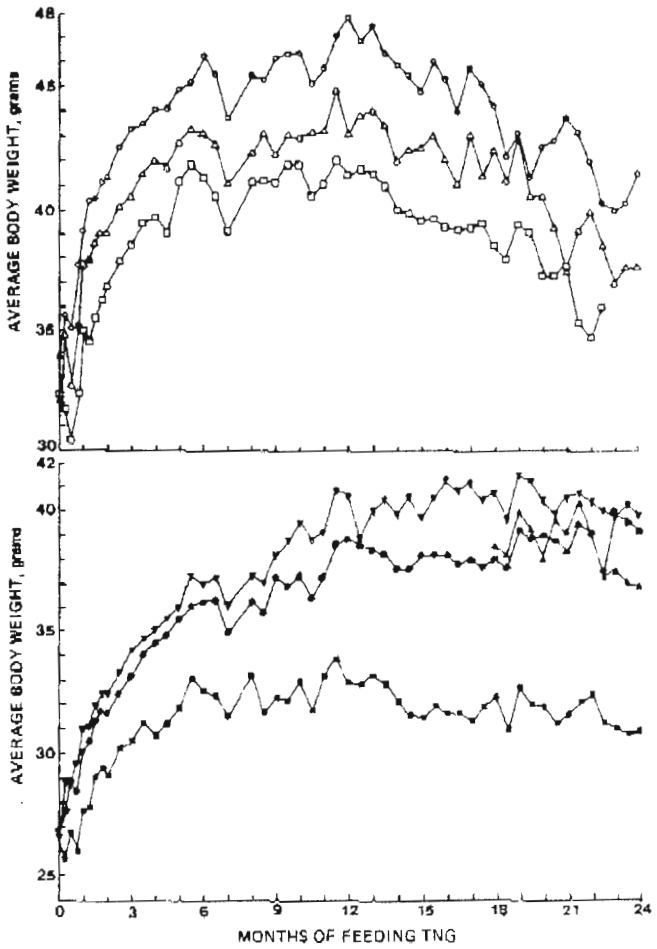

FIG. 5. Average body weights of mice fed various doses of TNG. Open figures, males; solid figures, females. Circles, controls; inverted triangles, 0.01% TNG; triangles 0.1% TNG; and squares 1% TNG. Some superimposed data were omitted.

presence of hemoglobin-derived pigment deposits in rats and mice, but not in dogs, possibly due to the larger doses given the rodents.

Two distinct types of hepatic lesions were seen in rats. They were cholangiofibrosis and hepatocellular carcinoma. The liver was the primary site for the rapid denitration and conjugation of TNG (Needleman *et al.*, 1971; Hodgson and Lee, 1975). In rats, an increase in interstitial cell tumors of the testes also occurred. On the other hand, there were decreases in the incidences of pituitary chromophobe adenoma and mammary tumors, primarily fibroadenomas, the most common tumors in these CD rats (MacKenzie and Garner, 1973). In our experience, these tumors in the pituitary and mammary glands account for most unscheduled deaths of control rats. The decreased death rate in the high dose females was apparently due to the decrease in number of these tumors.

The only reported chronic toxicity study of TNG in rats was that of Takayama (1975), involving rats given TNG in their drinking water. No adverse effects were found at a dose of 31 mg/kg/day (0.03%), comparable to our

TABLE 7

HEMATOLOGIC DATA OF MICE FED TNG FOR 12 MONTHS

Hematologic measurement	Dose (% in feed)			
	Male		Female	
	0[a]	1[b]	0[b]	1[b]
Erythrocytes ($\times 10^6/mm^3$)	7.57 ± 0.28	7.63 ± 0.28	7.39 ± 0.66	7.38 ± 0.32
Heinz bodies (%)	0.00 ± 0.00	2.41 ± 0.12[d]	0.00 ± 0.00	0.93 ± 0.12[d]
Reticulocytes (%)	1.54 ± 0.11	2.78 ± 0.66	3.13 ± 1.57	2.49 ± 0.38
Hematocrit (vol%)	43.0 ± 1.0[c]	45.5 ± 0.9	42.3 ± 2.7	43.5 ± 0.6
Hemoglobin (g%)	13.5 ± 0.4	14.2 ± 0.2	13.6 ± 1.1	13.5 ± 0.5
Methemoglobin (%)	0.0 ± 0.0	2.8 ± 0.0[d]	0.0 ± 0.0	0.3 ± 0.3
Leukocytes ($\times 10^3/mm^3$)	4.4 ± 1.4	5.7 ± 0.6	6.3 ± 2.3	3.2 ± 0.6

[a] Mean ± SE of three mice.
[b] Mean ± SE of four mice.
[c] Mean ± SE of two mice.
[d] Significantly different from control mice by Dunnett's multiple comparison procedure.

middle dose (0.1% in feed). The results of both studies are comparable. The companion study of TNG in C57BL/6Jms mice (Suzuki *et al.*, 1975) found pituitary tumors of unspecified in five females given the high dose of 0.033% in water (58.1 mg/kg/day). results are statistically significant (p 06, exact analysis of contingency table). ver, we found only six pituitary tumors CD-1 mice, all in the low dose group. ymptoms of abnormal liver function ported in humans exposed to TNG. ual adverse effect was headache (Key 977). Occasional chronic effects on the as reported (Carmichael and Lieben, ange *et al.*, 1972; Klock, 1975). The therapeutic dose of 0.2–10.0 mg/man/ *U.S. Pharmacopeia*, 1975) is several or- magnitude smaller than the doses given dogs and rodents. Although TNG has erapeutic effects when given orally to insor and Berger, 1975), the normal therapy is sublingual, which results of the dose initially by-passing the n the other hand, most of the oral dose the dogs and rodents passes through the portal system to the liver, where it is rapidly denitrated and conjugated (DiCarlo *et al.*, 1968; Hodgson and Lee, 1975). The fate of the detached nitro group is unknown.

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