

# Induction of unscheduled DNA synthesis in rat hepatocytes following *in vivo* treatment with dinitrotoluene

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## Abstract

The purpose of this study was to examine the induction of unscheduled DNA synthesis (UDS) by the potent hepatocarcinogen technical grade dinitrotoluene (tgDNT; 76% 2,4-DNT, 19% 2,6-DNT) using the *in vivo-in vitro* hepatocyte DNA repair assay. Male Fischer-344 rats were treated by gavage and hepatocytes were isolated by liver perfusion and cultured with [<sup>3</sup>H]thymidine. UDS was measured by quantitative autoradiography as net grains/nucleus (NG);  $\geq 5$  NG was considered positive. Controls consistently had  $-3$  to  $-6$  NG. A dose-related increase in UDS was observed 12 h after treatment, with 200 mg/kg tgDNT producing 26 NG. A 50-fold increase in the number of cells in S-phase was observed at 48 h after treatment. This increase in S-phase cells could be suppressed in the presence of 10–20 mM hydroxyurea (HU), while the same levels of HU did not affect the level of UDS at 12 h after treatment. 2,4-DNT produced only a weak response, in contrast to 2,6-DNT which was a potent inducer of UDS. Treatment of female rats with tgDNT yielded only modest increases in UDS and DNA replication relative to males. These results are consistent with the carcinogenicity studies and indicate that tgDNT is a potent genotoxic agent, with 2,6-DNT contributing the major portion of the effect.

## Introduction

The important industrial chemical technical-grade dinitrotoluene (tgDNT)\*, a mixture of isomers, has been shown to be a potent hepatocarcinogen in rats following chronic administration (1). Male Fischer-344 rats maintained on a diet of tgDNT (35 mg/kg/day) for 12 months had a 100% incidence of hepatocellular carcinomas while females had a 50% incidence (1). This carcinogenic effect of DNT, however, was not predicted by *in vitro* tests for genotoxicity. In the Ames *Salmonella* assay tgDNT and the individual DNT isomers have been reported as showing no mutagenicity (2,3) or, at best, producing a weak response with specific isomers (4). No increase in mutagenicity was observed in the Chinese hamster ovary cell/hypoxanthine guanine phosphoribosyl transferase mutagenesis assay with tgDNT or its purified isomers with or without metabolic activation (5). Neither 2,4-DNT nor 3,5-DNT produced an increase in mitotic recombination in *Saccharomyces cerevisiae* D3 (3).

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\*Abbreviations: DMN, dimethylnitrosamine; DNT, dinitrotoluene; HU, hydroxyurea; NG, net grains/nucleus; tgDNT, technical grade dinitrotoluene; UDS, unscheduled DNA synthesis.

Neither tgDNT nor any of its purified isomers were capable of inducing unscheduled DNA synthesis (UDS) in primary rat hepatocytes treated *in vitro* (6). Thus, it was probable that additional factors present in the whole animal were playing a role in the mechanism of action of DNT; yet several *in vivo* systems also failed to detect any activity with DNT. Neither 3,5-, 2,4-, nor tgDNT produced dominant lethal mutations in mice (7); 2,4-DNT did not produce dominant lethal mutations in rats (8). Likewise, tgDNT was ineffective in producing coat color mutations in the Russell spot test (7). 2,4-DNT did not induce morphologically aberrant sperm in mice. The purpose of this study was to utilize the *in vivo-in vitro* DNA repair assay (9) to determine whether tgDNT or specific isomers of DNT administered to rats *in vivo* would produce genotoxic effects in the target cells that yielded tumors.

## Materials and Methods

### Chemicals

tgDNT was obtained from Air Products and Chemicals, Inc. (Marcus Hook, PA) and was from the same lot of material employed in the CIIT bioassay (1). The original sample was reported to contain the following proportions of isomers: 2,4-DNT (76.5%), 2,6-DNT (18.8%), 2,3-DNT (1.5%), 2,5-DNT (0.65%), 3,4-DNT (2.4%), 3,5-DNT (<0.1%). Current analysis by gas chromatography showed: 2,4-DNT (71.1%), 2,6-DNT (19.8%), 2,3-DNT (4.3%), 3,4-DNT (4.0%) and other isomers (<1%). 2,4-DNT (98.0% pure) was obtained from Alfred Bader Library of Rare Chemicals, a Division of Aldrich Chemical Co., Inc. (Milwaukee, WI). 2,6-DNT (99.9% pure) was the gift of W. Mayo Smith of Air Products and Chemicals, Inc. (Allentown, PA). [Methyl-<sup>3</sup>H]thymidine (42 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL).

### Animals

Male and female Fischer-344 rats [CDF<sup>®</sup> (F-344)/CrIBR] (130–275 g) were obtained from Charles River Breeding Labs (Kingston, NY) and were maintained on Wayne Lab Blox and water *ad libitum*. Sera from these animals were tested and did not contain antibodies against pneumonia virus of mice, reovirus 3, G.D. VII virus, Kilham rat virus, H-1 virus, Sendai virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus or rat corona virus (Microbiological Associates, Bethesda, MD).

### Treatment

Animals (200–275 g) were administered the indicated doses of DNT in corn oil by gavage. Controls received corn oil. The total volume of the test solution administered was 0.1 ml/100 g body weight.

For a 4-week feeding study, tgDNT was dissolved in acetone and mixed to uniformity with 250 g of NIH-31 feed (Ziegler Brothers, Gardner, PA). This feed was then mixed with ~2 kg of NIH-31 feed in a Kelly-Patterson blender to a final concentration of 1.0 mg tgDNT/g feed. Analysis of feed samples from two separate batches by gas chromatography revealed the actual concentrations to be  $1.04 \pm 0.01$  and  $1.01 \pm 0.04$  mg tgDNT/g feed. Control feed was prepared by blending the same amount of acetone without tgDNT into NIH-31 feed. Male rats (130–150 g) were maintained on water and tgDNT-containing or control feed *ad libitum*. Body weights were measured at the beginning of the study and weekly thereafter. At 1, 2 and 4 weeks after feeding the test diets, representative control and tgDNT-fed animals were analyzed for UDS as described below. Animals were removed from feed no more than 3 h prior to sacrifice.

### Measurement of UDS and DNA replication

Hepatocyte cultures were prepared and UDS measured by autoradiography as previously described (9). The incubation in unlabelled thymidine was changed to 0.25 mM thymidine from the 0.5 mM concentration previously used to facilitate maintenance of the proper pH throughout the incubation. Where cells were incubated with hydroxyurea (HU), all procedures were the same except that the incubations in labelled and unlabelled thymidine were done in the presence of 10 or 20 mM HU.

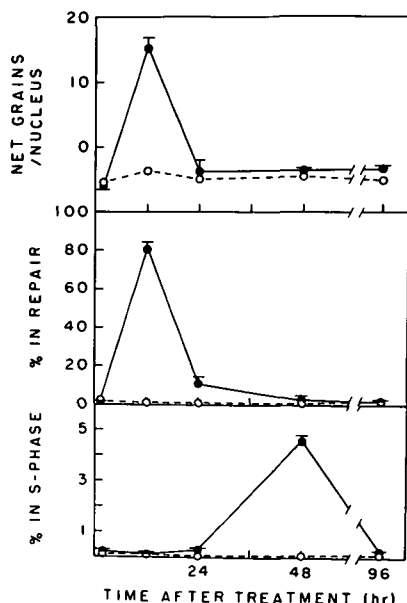
Quantitative autoradiographic grain counts were obtained as previously

described (9) and as utilized by others (6,10). Net grains/nucleus (NG) was calculated as the grains over the nucleus minus the highest of the grain counts in three adjacent nuclear-sized areas over the cytoplasm. Fifty randomly selected, morphologically unaltered cells were scored for each of 3 slides per animal; 2-4 animals were used for every dose of DNT.

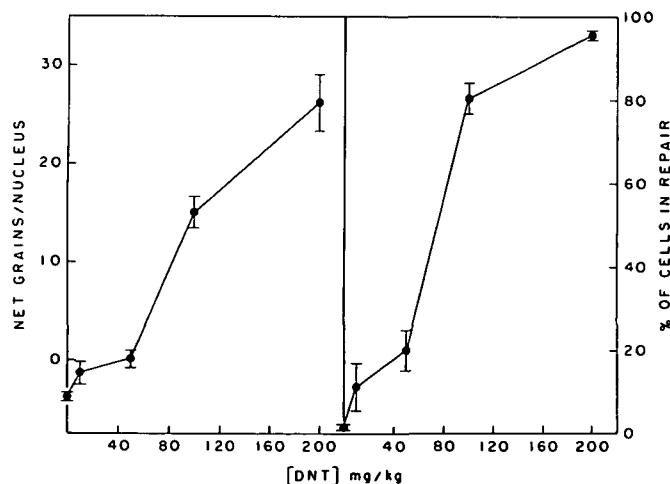
Cells in S-phase exhibit intensely labeled nuclei which are easily distinguished from non-replicating cells, even if the non-replicating cells have a high level of UDS. A total of 1000-2000 cells were scored for each slide and the percentage of cells in S-phase calculated.

**Results**

An examination of the time-course of repair following administration of a single 100 mg/kg dose of tgDNT revealed a peak of activity at 12 h post-treatment (Figure 1). By 24 h the degree of repair had declined substantially, and by 48 h post-



**Fig. 1.** Induction of UDS and DNA replication following administration of 100 mg/kg tgDNT. At times indicated after treatment, rat livers were perfused and hepatocytes isolated as previously described (9). Rats treated with tgDNT are represented by solid lines, controls by dotted lines. At least 3-4 treated animals were used for each point. The standard error for each point is the variation between animals. The % in repair is the percentage of cells with  $\geq 5$  NG.



**Fig. 2.** The dose-response of tgDNT at 12 h post-treatment. Livers were perfused and hepatocytes isolated as previously described (9). The standard error of each point is the variation between animals. 3 animals were used for each point. The % in repair is the percentage of cells with  $\geq 5$  NG.

treatment the level of UDS was the same as controls and remained so for up to 4 days after treatment.

The percentage of cells in S-phase was elevated by 50-fold at 48 h post-treatment relative to controls (Figure 1). The percentage of replicating cells returned to the control value of  $\leq 0.1\%$  by 4 days after treatment.

In the presence of 10 or 20 mM HU, the increase in S-phase cells could be suppressed by  $\geq 95\%$  (Table Ia). At the same levels of HU, however, there was no effect on the level of UDS at 12 h post-treatment (Table Ib).

Dose-related increases in UDS were observed at 12 h after treatment with tgDNT (Figure 2). A very sharp dose-response curve was observed with a marked increase in UDS between 50 and 100 mg/kg.

To examine the relative potency of the two primary isomers of tgDNT, 2,4-DNT and 2,6-DNT were administered to rats and the degree of UDS measured at 12 h after treatment. 2,4-DNT at 100 mg/kg produced only a weak response

**Table Ia**

Effect of hydroxyurea on induction of DNA replication by tgDNT.

HU[mM]	% of cells in S-phase	
	Control $\pm$ S.D. <sup>a</sup>	tgDNT $\pm$ S.D. <sup>a</sup>
0	0.08 $\pm$ 0.03	4.85 $\pm$ 0.44
10	<0.02	0.25 $\pm$ 0.09
20	<0.02	0.17 $\pm$ 0.13

<sup>a</sup>S.D. represents the variation between 3 replicate cultures from the same animal. Rats treated with 100 mg/kg tgDNT or corn oil 48 h prior to sacrifice. A total of 2000 cells scored for each culture.

**Table Ib**

Effect of hydroxyurea on induction of UDS by tgDNT.

[HU]mM	Control		tgDNT	
	Net grains	% in repair	Net grains	% in repair
0	-3.8 $\pm$ 0.4	1 $\pm$ 2	11.5 $\pm$ 0.9	72 $\pm$ 2
10	-3.0 $\pm$ 0.2	1 $\pm$ 2	14.2 $\pm$ 1.5	82 $\pm$ 4
20	-2.8 $\pm$ 0.2	2 $\pm$ 1	12.2 $\pm$ 2.9	77 $\pm$ 13

Rats treated with 100 mg/kg tgDNT or corn oil 12 h prior to sacrifice. Hepatocytes were cultured as described (9) except that incubations of cells in labelled and unlabelled thymidine were done in the presence of 10 or 20 mM HU. 3 slides scored/animal, 50 cells/slide. Standard deviations shown represent the variation between replicate cultures from the same animal. % in repair is the percentage of cells with  $\geq 5$  NG.

**Table II**

Measurement of UDS 12 h after treatment of rats with 2,4-DNT or 2,6-DNT.

Compound	Dose (mg/kg) (n)	NG $\pm$ S.E.	% in repair
Control	- (4)	-3.4 $\pm$ 0.2	2 $\pm$ 1
2,4-DNT	100 (3)	1.5 $\pm$ 0.3	33 $\pm$ 1
2,6-DNT	5 (3)	1.4 $\pm$ 1.2	30 $\pm$ 8
	20 (3)	17.1 $\pm$ 4.1	79 $\pm$ 10
	100 (4)	toxic	toxic

Rats were administered 2,4-DNT, 2,6-DNT or corn oil 12 h prior to sacrifice. (n) is the number of animals treated. S.E.'s shown represent variation between animals. % in repair is the percentage of cells with  $\geq 5$  NG.

Table III

DNA repair, replication and growth parameters of rats maintained on feed containing 0.1% tgDNT.

Group	No. of weeks on feed	Weekly % weight gain <sup>a</sup>	NG ± S.E. <sup>b</sup>	% in repair	% in S-phase
Control	1	24 ± 2	-4.1 ± 0.5	1 ± 1	0.7 ± 0.1
DNT	1	11 ± 2	-2.0 ± 0.7	12 ± 8 <sup>c</sup>	1.2 ± 0.4
Control	2	18 ± 2	-5.2 ± 0.8	1 ± 1	0.4 ± 0.1
DNT	2	9 ± 3	-3.3 ± 0.5	10 ± 3 <sup>c</sup>	1.9 ± 0.4 <sup>c</sup>
Control	3	6 ± 3		—	—
DNT	3	8 ± 6		—	—
Control	4	5 ± 4	-3.2 ± 0.5	2 ± 2	0.03 ± 0.1
DNT	4	5 ± 3	-0.8 ± 0.8	17 ± 6 <sup>c</sup>	2.0 ± 0.5 <sup>c</sup>

<sup>a</sup>Initial body weights were 145 ± 9 g for the DNT group, 139 ± 4 g for the control group. <sup>b</sup>Combined slide-to-slide and animal-to-animal variation, n = 3–6. <sup>c</sup>Significant increase over control level (p < 0.01) by chi-square analysis. Rats were maintained on control or DNT diets prepared as described in **Methods**. The % in repair is the percentage of cells with ≥ 5 NG. UDS was not measured on week 3.

(Table II). Conversely, a dose of 100 mg/kg of 2,6-DNT was so toxic that cells did not survive in culture. A dose of 5 mg/kg 2,6-DNT induced a slight increase in UDS while 20 mg/kg 2,6-DNT induced a response comparable to 100 mg/kg tgDNT.

To determine the effects of chronic DNT administration, rats were maintained on a diet containing 0.1% tgDNT. The degree of UDS was measured at weeks 1, 2 and 4 of the feeding study. A small, but significant, increase in the number of cells in repair was observed at all three time points (Table III). By week 4, 17% of the cells from tgDNT-treated rats were in repair compared to 2% for controls. The strength of the response was modest as shown by the low NG values. Cells from tgDNT-fed animals showed a marked increase in replicating cells with 1.2, 1.9 and 2.0% of the cells in S-phase at 1, 2 and 4 weeks, respectively.

Female rats treated with tgDNT showed a much lower level of UDS compared to males (Table IVa). Treatment of both sexes with dimethylnitrosamine (DMN) produced a very strong positive response. At 48 h after treatment with tgDNT, females showed only a slight increase in the percentage of cells in S-phase relative to males (Table IVb).

## Discussion

DNT is a potent hepatocarcinogen in rats (1), yet tgDNT as well as individual DNT isomers produce little or no response in either bacterial or mammalian cell genotoxicity systems including the *in vitro* hepatocyte DNA repair assay (6). The lack of a response in the latter test is of particular significance because hepatocytes represent the target cells of the carcinogen *in vivo*. This finding is not surprising, however, in that the *in vitro* DNA repair assay has been shown to be unresponsive to nitroaromatic carcinogens (10). Several *in vivo* assays also failed to show any activity with 2,4-DNT (7,8), but these systems did not measure responses in the appropriate target tissue. The dose-related effect (Figure 2) of tgDNT in the *in vivo-in vitro* hepatocyte UDS system and the strong response of 2,6-DNT (Table II) indicate that these chemicals are indeed potent genotoxic agents in rat hepatocytes when administered *in vivo*, and suggests that this system may be of value in predicting potential carcinogenic activity of nitroaromatic compounds.

Other genotoxic carcinogens administered *in vivo* such as DMN and 2-acetylaminofluorene induce UDS within 1–2 h

Table IVa

Induction of UDS in male and female rats following treatment with tgDNT or DMN.

Chemical	Dose (mg/kg)	Sex (n)	NG ± S.E.	% in repair
Control	—	M (4)	-4.2 ± 0.4	2 ± 2
		F (3)	-3.7 ± 0.7	2 ± 0
tgDNT	100	M (4)	15.1 ± 1.6	80 ± 4
		F (3)	4.6 ± 1.9	49 ± 12
DMN	10	M (3)	54.9 ± 4.5	89 ± 5
		F (3)	43.0 ± 6.3	96 ± 1

Male and female Fischer-344 rats were treated with tgDNT in corn oil 12 h prior to sacrifice or with DMN in water 2 h prior to sacrifice. Controls received corn oil. (n) is the number of rats treated. S.E.'s shown represent variation between animals. % in repair is the percentage of cells.

Table IVb

Induction of DNA replication in male and female rats following treatment with tgDNT.

Chemical	Sex (n)	% in S-phase ± S.E.
Control	M (5)	0.08 ± 0.01
	F (3)	0.16 ± 0.08
tgDNT	M (3)	4.57 ± 0.22
	F (3)	0.60 ± 0.05

Rats were treated with 100 mg/kg tgDNT or corn oil 48 h prior to sacrifice. 3 slides scored/rat; 2000 cells/slide. (n) is the number of rats treated. S.E.'s shown represent variation between animals.

post-treatment (9). In contrast, no UDS was induced by tgDNT at this time (Figure 1); instead, UDS induced by tgDNT peaks at 12 h after treatment. This delay in the peak of UDS probably reflects the complexity of DNT disposition. Both the liver (11) and cecal flora (12) are capable of metabolizing DNT. The excretion of reduced metabolites of 2,4-DNT is greatly diminished in germ-free rats (13). This complex pattern of metabolism is the most probable reason that activity is seen *in vivo* and not *in vitro*. Experiments with axenic animals using the *in vivo-in vitro* DNA repair assay have proven that metabolism by cecal bacteria is an

obligatory step leading to the genotoxic activity of tgDNT (14).

The increase in the percentage of cells in S-phase at 48 h post-treatment (Figure 1) is most likely due to the fact that tgDNT is hepatotoxic resulting in necrosis and subsequent replication to replace damaged hepatocytes. tgDNT has been shown to produce liver necrosis following a single 100 mg/kg dose (T. Leonard and J. Popp, personal communication) as well as following chronic exposure (1). This property of tgDNT probably contributes to its potent carcinogenic activity with the initial DNA damage followed by increased replication. These results also underscore a basic problem with DNA repair assays which measure UDS by liquid scintillation counting (15,16). Such assays are unable to distinguish DNA repair from DNA replication, which is easily done autoradiographically.

It has been reported that HU selectively inhibits replicative synthesis but not repair synthesis (17). The ability of HU to suppress the tgDNT-induced increase in S-phase cells (Table I a), but not the tgDNT-induced increase in UDS (Table Ib) clearly demonstrates that the increase in net grains reflects UDS, not an early stage of DNA replication. A small amount of DNA replication might occur under these conditions, but this could not account for the >70% of the cells observed to be in repair.

tgDNT is a complex mixture of isomers with the 2,4 and 2,6 isomers making up >90% of the total. Although 2,4-DNT contributes >70% of the total weight, the degree of UDS induced by 100 mg/kg 2,4-DNT is only 1.5 NG with 33% in repair (Table II). 2,6-DNT at 20 mg/kg yields 17.1 NG with 79% in repair. Therefore, compared to 100 mg/kg tgDNT which yields 15.1 NG with 80% in repair, most of the activity of tgDNT may be accounted for by the 2,6-DNT component.

Two feeding studies have been performed with purified 2,4-DNT. In the first study (18) Fischer-344 rats were administered >95% pure 2,4-DNT in the diet at ~14 mg/kg/day ( $\geq 13.3$  mg/kg/day of 2,4-DNT;  $\leq 0.7$  mg/kg/day of 2,6-DNT) for 18 months followed by a 6 month observation period and 6% of the male animals were reported to have hepatocellular carcinomas. In the second study (19) CD rats were administered 98% 2,4-DNT, 2% 2,6-DNT in the diet at ~34 mg/kg/day (33.3 mg/kg/day of 2,4-DNT; 0.7 mg/kg/day 2,6-DNT) for 24 months and 21% of the male animals were reported to have hepatocellular carcinomas. These results are in sharp contrast to the CIIT study (1) in which Fischer-344 rats were administered tgDNT in the diet at 35 mg/kg/day (26.8 mg/kg/day of 2,4-DNT; 6.6 mg/kg/day of 2,6-DNT) and at 12 months, 100% of the male animals were found to have hepatocellular carcinomas. One obvious difference in these results is that the dose of 2,6-DNT was far greater in the CIIT study compared to the other two studies which employed semi-purified 2,4-DNT. The greater tumor induction in the CIIT study is consistent with what would be predicted from our *in vivo* UDS results. In addition, 2,6-DNT was the only DNT isomer that could initiate  $\gamma$ -glutamyl transpeptidase positive foci in the liver (20). Although many factors contribute to the outcome of long-term carcinogenicity bioassays, taken together these studies suggest that the major portion of the initiating activity of tgDNT resides with the 2,6-isomer.

Rats maintained on a diet containing 0.1% tgDNT exhibited a relatively low, though significant, elevation in the

percent of cells in repair at 1, 2 and 4 weeks (Table III) with only a slight increase in observed NG. Although the amount of DNT consumed in one day, if administered as a single dose, would have yielded a greater increase in UDS, the fact that the dose was spread out over a much longer period may account for the observed low level of DNA repair. The rapid rate of repair of DNT-induced DNA damage (Figure 1) could also contribute to the low degree of UDS observed in the feeding study.

The one week control animals showed an increased number of cells in S-phase (0.7%) compared to the normal number of  $\leq 0.1\%$  seen in adult rats (Table III). This elevated value was probably due to the small size of the rats at the early stages of the study when liver growth was still occurring. Indeed, the number of replicating cells fell in subsequent weeks, declining to the expected value of  $\leq 0.1\%$  by week 4. The steady increase over time in the percentage of cells in S-phase from tgDNT-fed animals is further evidence of the ability of DNT to stimulate DNA replication. The constant pressure of induced DNA damage and repair coupled with the significant elevation in cell replication must surely contribute to the potent carcinogenicity of this chemical.

The incidence of hepatocellular carcinomas in female rats is roughly half that of males (1). This is consistent with the lower level of UDS in females than in males (Table IVa) suggesting that tgDNT is less genotoxic in females than in males. The positive control DMN produced a similar high degree of UDS in both sexes confirming the ability of males and females to carry out high levels of excision repair. tgDNT may also be less hepatotoxic in females as indicated by the much lower level of tgDNT-induced DNA replication (Table IVb). Both the decreased genotoxicity and the decrease in cell proliferation could contribute to the lower tumor incidence in females.

These results indicate that tgDNT is an extremely potent genotoxic agent when administered *in vivo* with 2,6-DNT contributing the majority of the effect. In addition, tgDNT produces an elevation in DNA replication following both acute and chronic administration further increasing the potential carcinogenicity of DNT.

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