EVIDENCE ON THE CARCINOGENICITY OF

2,4,6-Trinitrotoluene

DRAFT

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Reproductive and Cancer Hazard Assessment Branch
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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq*.) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity…if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of carcinogenicity are identified as the members of the Carcinogen Identification Committee (CIC) of the OEHHA Science Advisory Board (per Title 27 California Code of Regulations, section 25102; formerly Title 22, Cal. Code of Regs. §12301).

On September 7th, 2007, OEHHA announced in the *California Regulatory Notice Register* that 2,4,6-Trinitrotoluene (TNT) was a chemical proposed for Committee consideration at their November 19th, 2007 meeting. The September 7th, 2007 notice also marked the start of a 60-day comment period during which interested parties could submit comments to OEHHA that would be forwarded to the members of the CIC prior to the November 2007 meeting. At its November 19th, 2007 meeting, the Committee advised OEHHA to prepare hazard identification materials for TNT. A public request for information relevant to the assessment of the evidence on the carcinogenicity of this chemical was announced on December 12th, 2007, in the *California Regulatory Notice Register*. No information was received as a result of this request.

These hazard identification materials were complied to provide the Committee with relevant information for use in its deliberations. A public meeting of the Committee to discuss this evidence is scheduled for November 5th, 2008. At this meeting it is expected that the Committee will render an opinion on whether TNT has been clearly shown to cause cancer. Written public comments should be submitted to OEHHA by October 14, 2008, in order to be considered by the Committee in advance of the meeting. During the November 5th, 2008 meeting, the public will have an opportunity to present verbal comments to the Committee.

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1 EXECUTIVE SUMMARY

2,4,6-Trinitrotoluene (TNT) is one of the most commonly used explosives for military and industrial applications. It is valued because of its insensitivity to shock and friction, which reduces the risk of unexpected detonations. TNT exposure occurs occupationally during production and use and to the general public through contaminated drinking water, air, soil, foods, or otherwise coming in contact with TNT or TNT-contaminated media.

There are a small number of human studies. An ecologic epidemiological study reported elevated rates of leukemia in a German town where environmental exposures to TNT had occurred. The study was performed because of "an apparently high number of leukemias occurring in the town." Living in the town was significantly associated with increased risks of both acute and chronic leukemia (Kolb *et al*., 1993). A population-based case-control study in the same region of Germany hypothesized that the association in the ecological study was due to exposure to armament wastes. While leukemia was significantly associated with residing in one neighborhood bordering on an open drainage channel receiving wash water during a time of peak TNT production, the association was based on small numbers (just four cases and two controls exposed) (Kilian *et al*., 2001). Similar results were not seen for residences in other TNT-contaminated areas in the study, or for other measures of exposure to TNT. The investigators concluded that their results did not confirm the findings from the earlier ecologic study. A historical cohort study of TNT-exposed munitions workers in China reported a statistically significant increase in the rate of liver cancer (Yan *et al*., 2002), but this study had several significant methodological limitations that reduce the overall confidence that can be placed on study findings. Occupational exposure to TNT has caused liver toxicity and related mortality and there are also some case reports of liver cancer from occupational exposure, as well as leukemia (e.g., Garfinkel *et al*., 1988; Yan *et al*., 2002).

Two-year dietary studies on TNT carcinogenicity have been conducted in male and female Fischer 344 rats and B6C3F$_1$ mice (Furedi *et al*., 1984a, 1984b). Hepatocellular hyperplasia but not cancer was elevated in male rats. In female rats, both benign and malignant neoplasms of the urinary bladder (transitional epithelia) were significantly increased in the high dose group, and with a dose-related trend. This was accompanied by hyperplasia (transitional epithelia of the urinary bladder) providing further support that TNT was carcinogenic in the female rat bladder. In female mice, incidence of leukemia/malignant lymphoma in the spleen was significantly elevated in the high dose group and with a dose-related trend. No significant carcinogenic findings were reported for male rats or mice.

TNT is genotoxic in bacterial and mammalian systems *in vivo* and *in vitro*. It induced both frameshift and basepair substitution mutations in *Salmonella*, mutations in mammalian cells *in vitro* in the Chinese hamster ovary cell hypoxanthine phosphoribosyl transferase (CHO-HPRT) locus assay and the mouse lymphoma thymidine kinase (TK) locus assay. TNT induced oxidative DNA damage in rat sperm *in vivo*, as measured by increased formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). The TNT metabolite 4-hydroxylamino-2,6-dinitrotoluene (4-NHOH-DNT) damaged DNA, increasing the formation of 8-oxodG and cleaving the DNA at sites with consecutive guanines (Homma-Takeda *et al*., 2002). Several

TNT metabolites have also been observed to be genotoxic in the *Salmonella* [2-amino-dinitrotoluene (2-ADNT); 4-amino-2,6-dinitrotoluene (4-ADNT); 2,6-diamino-4-nitrotoluene (2,6-DANT); 2,4-diamino-6-nitrotoluene (2,4-DANT)] and CHO-HPRT assays (4-ADNT; 2,6-DANT weakly positive). Urine from workers exposed to TNT has increased mutagenic activity in the *Salmonella* assay compared to that from unexposed workers.

TNT binds covalently to proteins in humans (hemoglobin (Hb) adducts) and animals (Hb adducts, liver proteins), indicating the potential to bind to DNA. TNT can be metabolized through multiple pathways to form reactive nitroso species and reactive oxygen species (ROS), which may bind covalently with proteins and other macromolecules, induce oxidative stress, and oxidative DNA damage.

Structure activity comparisons with the carcinogenic nitrotoluenes 2,4-dinitrotoluene (2,4-DNT), 2,6-DNT, and 2-nitrotoluene (2-NT) suggest that common pathways of metabolism and similarities in the reactivity of metabolic intermediates with proteins and DNA exist for TNT.

Thus, there is evidence for the carcinogenicity of TNT, in the form of data on the development of benign and malignant tumors of the urinary bladder in female rats and hematopoietic tumors in female mice treated for two years by diet. Further evidence of potential carcinogenicity includes genotoxicity of TNT and metabolites in *Salmonella* and mammalian cells, as well as close structural similarity to 2-NT and 2,4- and 2,6- DNT, all three of which are carcinogenic and listed as such under Proposition 65. Human populations exposed to TNT have been inadequately studied with regard to carcinogenicity.

2 INTRODUCTION

2.1 Identity of 2,4,6-Trinitrotoluene (TNT)

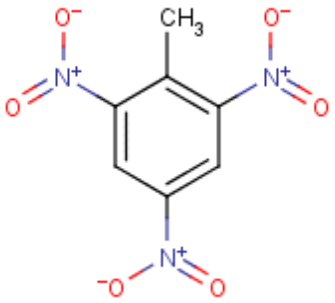

Figure 1. Chemical Structure of TNT.

Molecular Formula: $C_6H_2(NO_2)_3CH_3$

Molecular Weight: 227.1

CAS Registry No. 118-96-7

IUPAC Systematic Name: 2,4,6-Trinitrotoluene

Synonyms: 1-Methyl-2,4,6-trinitrobenzene, 2,4,6-Trinitrotolueen, 2,4,6-Trinitrotoluol, 2-Methyl-1,3,5-trinitrobenzene, Gradetol, TNT, TNT-tolite, Tolit, Tolite, Trinitrotoluene, Tritol, Trojnitrotoluen, Trotyl, Trotyl oil, alpha-TNT, s-Trinitrotoluene, s-Trinitrotoluol, sym-Trinitrotoluol.

Chemical Class: polynitroaromatic hydrocarbon

Chemical Appearance: Yellow needles

Melting Point: 80.35 °C.

Boiling point: 295 °C (decomposition)

Water Solubility: 0.01% at 25°C

2.2 Occurrence and Use

TNT, a yellow, odorless solid, does not occur as a natural product. TNT is available commercially in dry or wetted forms, that contain various portions of water by weight (e.g., <10%, <30% or >30%) (IARC, 1996a).

TNT is one of the most commonly used explosives for military and industrial applications. It is valued because of its insensitivity to shock and friction, which reduces the risk of unexpected detonations. In military applications, it has been used widely for filling shells, grenades and demolition bombs, either as the pure explosive or in binary mixtures. In commercial applications, it has been used in coal and mineral mining, deep-well and underwater blasting, and building demolitions. In chemistry, TNT is used as an intermediate to generate charge transfer salts (Budavari, 1989). Other uses include as a chemical intermediate in the manufacture of dyes and photographic chemicals (Lewis, 1997). U.S. production of TNT has been limited to military arsenals; commercial production is not permitted. In 1985, about 9.2 million pounds of TNT were imported into the U.S. Current data on the amount of TNT produced or imported into the U.S. were not found.

Occupational exposure may occur during production, manufacturing and loading of munitions, and during blasting operations. Environmental contamination with TNT has been recognized as a widespread ecological problem. TNT has been detected in surface and ground water samples collected in the vicinity of munitions facilities and waste disposal sites. An estimated 0.82 million cubic meters of soil in former military installations in the U.S. is contaminated by TNT (Peterson *et al.*, 1998). TNT is a contaminant in at least 20 of the U.S. Environmental Protection Agency's (U.S. EPA) National Priorities List sites (ATSDR, 1995). Individuals may be exposed

to TNT through contaminated drinking water, air, soil, foods, or otherwise coming in contact with TNT or TNT-contaminated media.

3 DATA ON CARCINOGENICITY OF TNT

3.1 Data on Carcinogenicity in Humans

The available literature on the carcinogenic effects of TNT in humans includes several case report publications, a historical cohort study of liver cancer among occupationally exposed workers in China, and one ecological and one case-control study of leukemia in an area of Germany with environmental TNT contamination from munitions plants. Only one of the case studies was published in an English-language journal.

3.1.1 Controlled Studies

Literature search methods for controlled studies

Controlled studies of cancer reporting results for TNT in the PubMed database were identified with the following search string: (tnt OR "tri nitro toluene" OR "2,4,6-trinitrotoluene") AND (epidemiology OR epidemiologic OR cohort* OR control OR controls OR mortality OR morbidity OR incidence OR rate OR rates OR odds OR risk OR ratio OR ratios OR smr OR pmr OR pcmr) NOT (trial OR therapy OR troponin OR coronary OR myocardial OR hypotension OR antibody OR antibodies OR immune OR autoimmune OR "auto-immune" OR macrophage). The PubMed database search was limited to human studies and the topic of cancer in the PubMed limitation feature. This search string identified 20 potentially relevant articles on June 26, 2008.

In the literature search, bias toward finding articles reporting an association between TNT and cancer may have occurred because literature databases at this time generally do not contain the entire contents of articles. For example, PubMed contains only abstracts and selected basic information such as titles and keywords. Bias toward identifying articles that found an association occurs because abstracts are more likely to mention factors found to be associated with a disease than factors not found to be associated. Thus, our literature search may have missed articles in which results for TNT and cancer were reported only in the body of the article.

Issues of validity in controlled studies of cancer and TNT exposure

Validity issues that may be important in controlled studies of TNT exposure and cancer include:

- Confounding bias from uncontrolled risk factor exposure such as tobacco smoke.
- Potential selection bias from nonparticipation.
- Misclassification of exposure, such as classifying subjects with little exposure as exposed.

- Reporting bias within articles due to authors tending to report associations that were found (when they do not report all of their results).

Risk factors for cancer that may be associated with TNT exposure

Tobacco smoke

Respiratory exposure to tobacco smoke is a well-established cause of several types of cancer and is a potential source of bias in controlled studies of TNT and cancer. For example, if a study compares risks of lung cancer in TNT-exposed workers and the general population, and tobacco smoking has been more common among the TNT-exposed workers than among the general population, then lung cancer risk may be higher in the TNT-exposed workers solely due to tobacco smoking. Traditionally, "blue-collar" employment and lower socioeconomic status have been associated with more frequent tobacco smoking and chewing.

Alcohol

Alcohol ingestion, a known cause of several types of cancer, could cause confounding bias in epidemiological studies if TNT exposure was associated with consumption of alcohol. In interpreting studies reporting results for TNT and cancers known to be caused by alcohol ingestion (e.g., liver cancer), it is important to consider whether alcohol might explain associations that have been reported.

Low subject participation

If there was a difference in prevalence of past TNT exposure between subjects who participated in a study and subjects who declined to participate, then non-participation could have led to bias. For example, if it assumed that TNT causes the type of cancer being investigated in a case-control study and if there is less TNT exposure among participants than among non-participants, the strength of the association as measured by the odds ratio will be less than it would have been if all eligible subjects had participated. Surveys must have a respectable response rate to ensure that bias is not introduced by unequal representation of subpopulations within the target population.

Results of controlled studies of TNT exposure and cancer

Controlled studies of TNT exposure and cancer are summarized here and listed in detail in Table 1.

Leukemia

Kolb *et al.* (1993) conducted an ecological study in Germany that compared incidence rates for acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) in Stadtallendorf to the rates in Giessen County, which was not contaminated with TNT. The study was motivated by the observation of an apparently high number of cases of leukemia in the German town of Stadtallendorf, which was the site of large factories that produced TNT during World War II resulting in TNT-contaminated soil and water. Cases of myelodysplastic syndrome (MDS), a condition that can progress to AML, were included in the AML case definition. Cases were

identified at medical centers in the two counties, and the population sizes were estimated from residential registries. Compared to Giessen County, the rate of AML in Stadtallendorf was statistically significantly elevated among both men [rate ratio (RR)=3.5, 95% confidence interval (CI) 1.4-8.5] and women (RR=3.2, 1.4-7.2). The rate of CML was significantly elevated among men (RR=9.1, 3.5-23.4) but not among women (RR=1.3, 0.2-10.3). Limitations of the study included small numbers (e.g., only one exposed female case of CML) and the study's ecological study design in which there were no exposure data on individuals. TNT exposure status was inferred based upon living in Stadtallendorf (exposed) or Giessen County (unexposed).

Kilian *et al*. (2001) carried out a population-based, case-control study to test the hypothesis that the leukemia cluster confirmed by Kolb *et al*. (1993) was caused by "TNT production and the related heavy contamination of soil and water." Historical cases of AML, CML, and MDS diagnosed during 1979-1993 among residents of the towns of Stadtallendorf and Kirchhain were identified from records of local hematological clinics. Stadtallendorf was the location of two large explosives factories that were demolished after World War II, and Kirchhain was a neighboring town through which TNT-laden waste water flowed from the factories in an open channel during the peak production period of 1941-1945. Other contaminants of soil and water were said to have included toluene and coal tar pitch. Controls were randomly selected from the population registers of the towns as of 1994, individually matched to cases on age and gender. Upon interview, controls were required to have resided in one of the two study towns at the time the matching case was diagnosed (in the 1979-1993 time period). In addition to residence, information on occupation, consumption of plants, animal products, and drinking water from contaminated areas, and medical history (e.g., smoking, diagnostic x-rays) was obtained upon interview. Odds ratios, adjusted for age and gender, found that living in Stadtallendorf during the peak exposure period of 1945-1948 was not associated with "all leukemia" (combined AML, CML, and MDS cases) (OR=0.9, 0.3-2.5) or with CML alone (OR=1.2, 0.3-6.1). The odds ratio was more elevated for living in Kirchhain during its peak exposure period of 1941-1945 but was not statistically significant (OR=5.9, 0.6-53.3). When the exposure definition of "living in Kirchhain" was limited to subjects who resided in a neighborhood bordering the open drainage channel (i.e., the "long channel") into which contaminated factory wash water had flowed during the peak phase of TNT production, the odds ratios for both "all leukemia" (OR=5.1, 1.1-23.8) and CML (OR=9.0, 1.1-72.1) were elevated and statistically significant. These findings were based upon very small numbers (for "all leukemia," four cases and three controls exposed; for CML, three cases and one control exposed), but it is unlikely that chance alone could account for the observed five-fold increase in "all leukemia" and nine-fold increase in CML reported for residents near the Kirchhain "long channel." The investigators concluded that their results do not confirm the hypothesis that the increased risk of leukemia in Stadtallendorf is associated with exposure to armament wastes.

One limitation to the Kilian *et al*. (2001) study was its method of choosing controls. The investigators said that, for cost reasons, controls were limited to persons still living in the towns in 1994, when it would have been preferable to select from among all persons living in the towns at the time the cases were diagnosed (1979-1993). Bias could occur if people who moved away were exposed to TNT more or less often than people who did not move away, but there is no evidence that such bias occurred. No information on the mobility of the study area population prior to 1987 was presented; since 1987, six of the 123 individuals contacted as possible controls had immigrated into the area, and 12 had moved out of the community after 1994. Another

limitation was potential selection bias from low control participation (50%, compared to 93% case participation). Low participation could cause bias if participants and non-participants differed in their past TNT exposures. A third limitation was potential misclassification of exposure due to proxy interviews. Only 28% of the interviewed cases (11 of 40) were alive and directly interviewed. For deceased cases, interviews were conducted with spouses, close relatives, and acquaintances. All interviews for controls occurred directly with the controls. While proxy interviews tend to provide less accurate data than direct interviews and can bias odds ratios, the main exposure variable in the Kilian *et al*. study, place-of-residence, was likely to have been fairly accurately reported by proxy subjects.

Liver cancer

Yan *et al*. (2002) reported results of a historical cohort study of cancer morbidity and mortality among male workers at eight weapons factories in China. The cohort consisted of 2,683 TNT-exposed workers and 42,494 TNT-unexposed control workers. While counts, rates, and rate ratios were presented for liver cancer, only counts were presented for 14 other categories of cancer. Liver cancer was the most common type of cancer among the TNT-exposed workers, constituting 32% of all cases. All analyses for liver cancer were adjusted for age and sex. The morbidity analysis for liver cancer was based upon 30 cases diagnosed among TNT-exposed workers over the 1970-1995 study period. In comparison to workers not exposed to TNT, the rate of liver cancer morbidity in TNT-exposed workers was reported to be statistically significantly elevated (RR=3.46, $p<0.01$).

The mortality analysis for liver cancer was also based on 30 cases, because all of the liver cancer cases were deceased (cause of death liver cancer) prior to data collection. Comparisons of rates were made both to TNT-unexposed workers and Chinese national rates for medium and large cities. In comparison to the workers not exposed to TNT, the mortality rate ratio for liver cancer among the TNT-exposed workers was 3.97 (hypothesis test results not stated). It was unusual that the rate ratio of 3.97 was presented only in the abstract of the article. In comparison to Chinese national rates for medium- and large-sized cities, liver cancer mortality in the TNT-exposed workers was again reported to be elevated, with rate ratios of 1.51 ($p<0.05$, based on eight cases) in the 1970-1985 time period, and 3.82 ($p<0.01$, based on 22 cases) in the 1986-1995 time period. The Office of Environmental Health Hazard Assessment (OEHHA) summed the data for the two time periods and estimated an overall liver cancer mortality rate ratio of 2.71 ($p<0.01$) for the comparison to national rates for cities.

The observation of a relatively large three- to four-fold excess risk of liver cancer in the TNT-exposed workers is tempered by several moderating considerations. One consideration is that the risk of cancer was apparently elevated for all types of cancer, which might be real, but which might indicate a methodological problem. For example, there were approximately 21 excess liver cancer cases among the TNT-exposed workers. If the 21 excess liver cancer cases are subtracted from the 94 total cancer cases, then approximately 73 total cancer cases would have occurred among the TNT-exposed workers if there was no excess of liver cancer. OEHHA estimated that with 73 cases the morbidity rate ratio for all cancers combined would have been 1.80 ($p<0.01$). While 1.80 is less than the original all cancer morbidity rate ratio of 2.32 ($p<0.01$) reported by the authors, it is still a nearly doubled risk that is unexplained by liver cancer.

Another consideration is that the Yan *et al*. (2002) study was conducted in the context of the high rate of liver cancer in China, primarily due to hepatitis B virus infection (Bosch, 2004). In general, it is more difficult to detect excess risk of common cancers than rare cancers. Yan *et al*. (2002) did not control for potential confounding by hepatitis B virus or aflatoxin exposure, another established cause of liver cancer observed in Chinese populations. Confounding bias may have occurred if the prevalence of an unknown and unmeasured risk factor for liver cancer differed between the TNT-exposed workers and the comparison populations. Still, in order to solely account for the three- to four-fold excess liver cancer risk, the prevalence of the unknown risk factor (e.g., rates of hepatitis B infection) would need to be three to four times higher in the TNT-exposed workers than in the comparison population (e.g., TNT-unexposed workers in workers in the same factories).

Another limitation of the Yan *et al*. (2002) study was ambiguity about whether liver cancer rates were calculated because liver cancer was an *a priori* hypothesis (in which case the study would be considered hypothesis-testing in nature) or because liver cancer became interesting after it was found to be common among the TNT-exposed workers (in which case the study would be considered hypothesis-generating in nature). While the authors cited reports in the scientific literature of liver cancer cases exposed to TNT (see case reports below), liver cancer was not mentioned among the stated objectives of the study.

Table 1. Controlled Studies of Human TNT Exposure and Cancer, by Type of Cancer (RR = Rate Ratio and OR = Odds Ratio).

	Acute and Chronic Myelogenous Leukemias		Liver Cancer
Study Parameter	**Kolb *et al.* 1993 (Germany)**	**Kilian *et al.* 2001 (Germany)**	**Yan *et al.* 2002 (China)**
Study Design	Ecological (morbidity)	Case-control (morbidity)	Historical cohort (morbidity and mortality)
Case Definition	AML and CML. The AML definition included MDS, which the investigators described "as an early form of AML." Classified by the standardized criteria of the pathologic departments of Gottingen University and Kiel University.	AML, CML, and MDS. Method of classification not stated.	Liver cancer as classified by the cancer coding system of the Ministry of Health of China.
Purpose of Study	To investigate "an apparently high number of leukemias occurring in the town of Stadtallendorf" where soil and water were contaminated with TNT waste from explosives manufacturing and factory demolition.	To test the hypothesis that TNT contamination of soil and water "might be responsible" for the cluster of leukemia in Stadtallendorf confirmed by Kolb *et al*. (1993).	To gain an understanding of the pathogenesis of malignant tumors in arms industry TNT workers.
Hypothesis Testing or Generating for TNT	Generating	Testing	Generating
TNT Exposure	Community exposure in the town of Stadtallendorf, where explosive manufacturing factories contaminated soil and water during manufacturing and subsequent factory demolition.	Community and occupational exposure in Stadtallendorf (where explosive factories were located) and neighboring Kirchhain (through which TNT-laden waste water flowed in an open channel). Other factory contaminants included toluene and coal tar pitch.	Occupational exposure to TNT at eight arms factories (two TNT-producing and six TNT-using).

Study Parameter	Acute and Chronic Myelogenous Leukemias		Liver Cancer
	Kolb *et al.* 1993 (Germany)	**Kilian *et al.* 2001 (Germany)**	**Yan *et al.* 2002 (China)**
Population	Exposed: the town of Stadtallendorf. Non-exposed (control): Giessen County. Case counts: hematological clinics at local medical centers, historical diagnoses 1983-1989, male and female, age >18. Population counts: residential registries.	Cases: hematological clinics in or near the towns of Stadtallendorf and Kirchhain, historical diagnoses 1979-1993, male and female, age >18. Controls: general population of the towns in 1994, randomly selected from population register, individually matched to cases on age and gender. Upon interview, were required to have resided in one of the two study towns when the matching case was diagnosed.	Exposed cohort: male workers who performed at least one year of work involving TNT between January 1, 1970, and December 31, 1995. Identified via employment records. Followed through December 31, 1995. Unexposed cohort (control): same cohort definition as for exposed cohort, except workers did not have contact with TNT. General population (control): national rates of cancer mortality among male residents of all medium- and large-sized cities in China 1973-1975 and 1990-1992.

Study Parameter	Acute and Chronic Myelogenous Leukemias		Liver Cancer
	Kolb *et al.* 1993 (Germany)	**Kilian *et al.* 2001 (Germany)**	**Yan *et al.* 2002 (China)**
Participation	Average numbers of residents Stadtallendorf Male: 9,958, female 10,083 Giessen County Male: 105,396, female 116,276 Numbers of cases: Stadtallendorf Male: 6 AML, 7 CML Female: 7 AML, 1 CML Giesen County Male: 28 AML, 13 CML Female: 29 AML, 10 CML	Cases: 43 occurred, 40 (93%) studied. AML: 12 occurred[1] CML: 21 occurred MDS: 10 occurred Controls: 160 eligible, 80 (50%) studied.	Exposed cohort: 2,683 subjects, 57,390 person-years. Unexposed cohort: 42,494 subjects, 934,184 person-years. The "response rate" in both exposed and non-exposed cohorts was said to be 99%, but it is not clear whether this refers to cohort follow-up success or questionnaire administration success.
Questionnaire Administration Methods	Not applicable (records-based study).	Oral interviews, face-to-face, with subjects (28% of cases, 100% of controls) or proxies such as spouses, relatives, and acquaintances (72% of cases and 0% of controls).	Data on cigarette smoking, alcohol, occupational history, and family history of cancer were ascertained with a questionnaire directly administered to contactable subjects. Methods of questionnaire administration not stated.

[1] The numbers of AML, CML, and MDS cases studied after exclusion of three subjects overall were not stated.

Study Parameter	Acute and Chronic Myelogenous Leukemias		Liver Cancer
	Kolb *et al.* 1993 (Germany)	**Kilian *et al.* 2001 (Germany)**	**Yan *et al.* 2002 (China)**
Results **(rate ratio estimate, 95% confidence interval, and adjustments for potentially confounding variables)**	*Stadtallendorf ("exposed") compared to Giessen County ("unexposed")*: AML Men RR=3.5 (1.4-8.5) Women RR=3.2 (1.4-7.2) CML Men RR=9.1 (3.5-23.4) Women RR=1.3 (0.2-10.3) Adjusted for age.	*Living in Stadtallendorf 1945-1948 within the areas of the factories:* AML, CML, or MDS 28% cases, 28 % controls exposed. OR=0.9 (0.3-2.5) CML only OR=1.2 (0.3-6.1) *Living in Kirchhain 1941-1945:* AML, CML, or MDS 13% cases, 5 % controls exposed. OR=5.9 (0.6-53.3) *Living in Kirchhain 1941-1945 close to liquid waste in an open channel:* AML, CML, MDS 10% cases, 2.5 % controls exposed. OR=5.1 (1.1-23.8) CML only 3 cases, 1 control exposed. OR=9.0 (1.1-72.1) *Occupational exposure at factories:* AML, CML, or MDS 0% cases, 5% controls exposed. OR= not calculable. Adjusted for age and gender.	*Work involving TNT exposure for at least one year:* Compared to non-exposed workers Incidence 1970-1995 RR=3.46 ($p<0.01$) Compared to Chinese national rates Mortality 1970-1985 RR=1.51 ($p>0.05$) Mortality 1986-1995 RR=3.81 ($p<0.01$) Adjusted for age and gender.

Study Parameter	Acute and Chronic Myelogenous Leukemias		Liver Cancer
	Kolb *et al.* 1993 (Germany)	**Kilian *et al.* 2001 (Germany)**	**Yan *et al.* 2002 (China)**
Validity Issues Specific to This Study	*Misclassification of exposure:* This is an ecological epidemiologic study, with no exposure data on individuals. Exposure was inferred for those living in the city of Stadtallendorf, and nonexposure from living in the county of Giessen. Because the study regions were not wholly exposed and unexposed, the rate ratios may have been attenuated (closer to 1.00) in comparison to rate ratios based on wholly exposed and unexposed populations.	*Selection bias:* 1) cases were diagnosed 1979-1993. Instead, controls were persons still living in the towns in 1994. Bias could have occurred if people who moved were exposed more or less often than people who did not move away. 2) Low control participation (50% controls vs. 93% cases) could cause bias if participants and nonparticipants differed in past TNT exposure. *Misclassification of exposure:* only 28% of cases (11 of 40) were alive and directly interviewed. Deceased-case interviews were conducted with proxies (spouses, relatives, acquaintances). All controls were directly interviewed. Proxy interviews tend to be less accurate and can cause bias.	*Data presentation errors and omissions:* 1) Rate ratio for liver cancer incidence in TNT-exposed workers compared to unexposed workers differs between the abstract and body of the paper (3.97 in abstract, 3.46 in the body paper). 2) The Discussion states that liver cancer mortality was 2.3 times higher in TNT-exposed workers than in unexposed workers. Nowhere else is it stated that mortality rates were compared between the exposed and unexposed workers. 3) The study's methods or quantification of its success in determining cancer status in former workers over the 26 year observation period are not described.

3.1.2 Case Reports

Garfinkel *et al*. (1988) were the first to posit the hypothesis that TNT may induce pathological processes related to liver cirrhosis and hepatocellular carcinoma. The authors presented a case study of liver cancer (hepatocellular carcinoma) and liver cirrhosis in a 61 year-old male engineer in Israel who had been occupationally exposed to TNT for 35 years. The individual had no history of viral hepatitis or alcohol abuse (Garfinkel *et al*., 1988).

Yan *et al*. (2002) described five Chinese-language case-report articles as reporting "many deaths from such diseases as liver cancer and leukemia that occurred several years after exposure to TNT." OEHHA was able to obtain and translate the abstracts for four papers and the titles for all five, and confirmed this to be the case. In English, the citations for the Chinese-language case-report articles are as follows:

1. Liu H. Four case reports of TNT intoxication induced hepatitis death from liver cancer. Industrial Hygiene and Occupational Diseases, 1986, 12:297-298.

2. Wang B. Mortality analysis of 16 cases of chronic TNT intoxication. Chinese Industrial Medicine Journal. 1991, 4:49. (Among 170 TNT chronic intoxication cases at a factory, a number of instances of leukemia.)

3. Yang F, Xie X. One case report of chronic TNT intoxication-induced liver disease and death from liver cancer. Chinese Labor Hygiene and Occupational Disease Journal. 1995, 13:114. (A 53 year old who was exposed to TNT for 10 years developed liver enlargement, then cirrhosis, then liver cancer.)

4. Liu C, Li X, Zhang S *et al*. One case report of TNT induced death from acute leukemia. Industrial Medicine Journal. 1995, 8:94-95. (A case of leukemia in a 49-year old who ground and crushed TNT pellets for 15.5 years.)

5. Fu E, Shang B. One case report of chronic TNT intoxication and death from liver cancer. Chinese Industrial Medicine Journal. 1998, 11: 126-127. (A 43 year old worker who packed TNT developed TNT intoxication and died of liver cancer.)

3.2 Carcinogenicity Studies in Animals

A search of the scientific literature regarding carcinogenicity of TNT in experimental animals identified four long-term cancer bioassays of TNT, all of which entailed dietary administration of the compound: one study each in male rats, female rats, male mice, and female mice. Long-term studies of TNT administered via other routes of exposure such as dermal uptake or inhalation were not found in the literature.

3.2.1 Two-year Dietary Studies in Rats

Male and female Fischer 344 rats (75/sex/dose group) received TNT at 0, 0.4, 2, 10 or 50 mg/kg/day in their diet for up to 24 months (Furedi *et al*., 1984a). Ten rats per sex per dose group were sacrificed following six and 12 months, with surviving animals sacrificed at the end

of the 24-month treatment. The doses were selected based on the results of 13-week oral (diet) toxicity studies in Fischer 344 rats. Survival rates and mean survival duration did not differ among control and treated groups in either sex. Dose-related reductions in body weight (5-14% reduction in animals given 10 mg TNT/kg/day, and 30-33% decreases in the 50 mg/kg/day groups) and food intake were observed in both males and females. There was a dose-related increase in the incidence of hepatocellular hyperplasia in male rats (23/54, $p<0.01$; 34/55, $p<0.01$) in the 10 mg/kg/day and 50 mg/kg/day dose groups, respectively. Hyperplasia was elevated in the kidney (10 mg/kg/day: 7/47, $p<0.05$) and urinary bladder (10 mg/kg/day: 12/47, $p<0.01$) in female rats at doses of 10 mg/kg/day or greater. The incidences of benign and malignant urinary bladder tumors were significantly increased in treated female rats in the 50 mg/kg/day dose group, with an incidence of urinary bladder papilloma and carcinoma (combined) of 17/55 ($p<0.01$) as compared to 0/54 in controls (Table 2).

Table 2. Incidence of Hyperplastic and Neoplastic Lesions in F344 Rats Fed TNT for 24 Months (Furedi *et al*., 1984a).

Pathological lesions		**TNT dose (mg/kg/day)**				
		0.0	**0.4**	**2.0**	**10.0**	**50.0**
Male F344 rats						
Liver	Hyperplasia	9/54	9/54	7/54	23/54**	34/55**†
Female F344 rats						
Renal pelvis	Hyperplasia	0/54	0/54	0/55	0/55	7/55*†
Urinary bladder	Hyperplasia	1/54	0/54	0/55	2/55	12/55**†
	Papilloma	0/54	0/54	0/55	1/55	5/55*†
	Carcinoma	0/54	0/54	0/55	0/55	12/55**†
	Papilloma and carcinoma	0/54	0/54	0/55	1/55	17/55**†

Note: Data represent numbers of animals with lesions among the total numbers of animals examined in the same treatment group.
* $p < 0.05$, pairwise comparison with controls by Fisher exact test;
** $p < 0.01$, pairwise comparison with controls by Fisher exact test.
† $p < 0.0001$, Exact test for linear trend.

3.2.2 Two-year Dietary Studies in Mice

Male and female $B6C3F_1$ mice (75/sex/dose group) received TNT at 0, 1.5, 10, or 70 mg/kg/day in their diet for up to 24 months (Furedi *et al*., 1984b). Ten mice per sex per dose were sacrificed following six and 12 months, with surviving animals sacrificed after 24 months. The doses were selected based on the results of four-week oral (diet) toxicity studies in $B6C3F_1$ mice. No deaths were observed in these four-week dose range-finding studies at doses up to 700 mg/kg/day, or 10 times the highest dose administered in the 24-month studies.

In the 24-month studies survival rates were not altered among control and treated groups in either sex. A 10% reduction in body weight gains was observed in the first six to eight months of TNT administration in high dose males and females, increasing to a 15% reduction in body weight gain in high-dose females, and a 20% reduction in body weight gain in high-dose males over the remainder of the treatment period. Increased food consumption was seen in high-dose males and females, and varied over the course of the studies, with increases ranging from 10% to 30% above control levels.

As indicated by an exact test for linear trend, a positive dose-dependent increase in the incidence of malignant lymphoma and/or leukemia of the spleen was observed in females ($p<0.05$) (See Table 3). The incidence of these tumors was significantly elevated in high-dose females ($p<0.01$) compared to controls (Table 3). The observed leukemias were of the granulocytic or lymphatic type and the malignant lymphomas were histiocytic, lymphocytic, or mixed type. These lesions were systemic reticuloendothelial neoplasia, and involved other organs and tissues. No treatment related tumors were observed in male mice.

Table 3. Incidence of Neoplastic Lesions in Female $B6C3F_1$ Mice Fed TNT for 24 Months (Furedi *et al*., 1984b).

Lesion		TNT dose (mg/kg/day)				Exact Test for Linear Trend
		0.0	1.5	10.0	70.0	
Spleen	Leukemia/malignant lymphoma	9/54	15/54	17/54	21/54**	p=0.021
Liver	Adenoma/carcinoma	5/54	11/54	8/54	10/54	p=0.25

Note: Data represent numbers of animals with lesions among the total numbers of animals examined in the same treatment group.
** $p<0.01$, pairwise comparison with controls by Fisher exact test.

3.3 Other Relevant Data

Other relevant data related to the possible carcinogenicity of TNT include studies of pharmacokinetics and metabolism, genetic toxicity of TNT and its metabolites, biomarkers, gene expression, structure activity, pathology, and non-cancer toxicities.

3.3.1 Pharmacokinetics and Metabolism

TNT can enter the body through the gastrointestinal tract, skin, and lungs (Coombs and Schillack, 1998). Due to its hydrophobic properties, the majority of orally administered TNT is absorbed within 24 hours of dosing in rats (Hodgson *et al.*, 1977). Once absorbed, it is distributed primarily to the liver, kidneys, lungs, and fat (ATSDR, 1995). Bader *et al*. (1998) analyzed urine samples obtained from nine workers exposed to TNT at an ammunition-dismantling workshop and from twelve unexposed persons. TNT was detected in the urine in six of the nine TNT-exposed workers at concentrations between 4 and 43 μg/l.

Homma-Takeda *et al*. (2002) administered TNT in corn oil by oral gavage six days per week for two weeks to male Fischer 344 rats, and determined TNT levels within the testis, liver, kidney, thymus, and spleen. One hour after the last dose, TNT was detected in the testis, kidney, thymus and spleen, but not the liver. TNT was detected in the testis up to two weeks after the last dose (Homma-Takeda *et al*., 2002). A study in rats administered ^{14}C-radiolabeled TNT by the oral route, and reported no ^{14}C in expired air (Hodgson *et al*., 1977). Measurements of ^{14}C in the urine indicated that urinary excretion is the primary elimination pathway. The levels of ^{14}C in the bile ranged from 10.3 – 27.3% of the administered dose, suggesting that biliary excretion is an important elimination pathway as well. Results from animal studies comparing the recovery of radiolabeled TNT when administered by different routes found that excretion was most efficient when exposure occurred by inhalation, followed by oral dosing, and then dermal exposure (U.S. Army, 1981). There are no data in humans that are informative as to the effect that route of exposure may have on the rate and extent of TNT metabolism and elimination in humans.

TNT is metabolized by nitroreduction to hydroxylamino derivatives, and by oxidation of the methyl group to benzyl alcohol and benzoic acid derivatives (U.S. Army, 1981). Single electron reduction of nitro groups may be catalyzed by flavoenzyme dehydrogenases-electron transferases, including ferredoxin:NADP+ reductase (FNR) and NADPH:cytochrome P-450 reductase (P-450R), to form anion radicals, initiating redox cycling and generating reactive oxygen species (ROS) under aerobic conditions (Šarlauskas *et al*., 2004). Two electron reduction by DT-diaphorase (NAD(P)H:quinine oxidoreductase; NQO1) or bacterial nitroreductases may also occur to form nitroso and hydroxylamine compounds (Šarlauskas *et al*., 2004).

Figure 2 presents the presumed major metabolic pathways of biotransformation of TNT. Studies in rat liver microsomes indicate that TNT is rapidly reduced to yield 2-hydroxylamino-4,6-dinitrotoluene (2-NHOH-DNT) and 4-hydroxylamino-2,6-dinitrotoluene (4-NHOH-DNT) (Leung *et al*., 1995). *In vivo*, this reduction is thought to occur in the gastrointestinal tract, the liver and in red blood cells (Sabbioni *et al*., 2005). 2-NHOH-DNT and 4-NHOH-DNT either can be reduced to form 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-dinitrotoluene (2-ADNT) (Leung *et al*., 1995), the first stable reductive metabolites of TNT (Grummt *et al*., 2006), or oxidized to form 4-nitroso-2,6-dinitrotoluene, 2-nitroso-2,4-dinitrotoluene, and other reactive metabolites, capable of binding to cellular macromolecules (e.g., proteins) (Bolt *et al*., 2006). 4-ADNT and 2-ADNT can be further transformed to 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT) (Leung *et al*., 1995; Bolt *et al*., 2006). Conjugation of the hydroxyl groups of 2-NHOH-DNT and 4-NHOH-DNT and the amino groups of 4-ADNT, 2-

ADNT, 2,4-DANT and 2,6-DANT with sulfuryl, glucuronide or acetyl moieties can also occur (Sabbioni *et al*., 2007).

4-ADNT and 2-ADNT (either as free or conjugated forms) have been detected in the urine of workers exposed to TNT (Yinon and Hwang, 1985; Ahlborg *et al*., 1988; Sabbioni *et al*., 2005). All reported studies found that 4-ADNT is the major urinary metabolite, followed by 2-ADNT. Analysis of urinary 4-ADNT levels may serve as a biological marker for TNT exposure in occupationally exposed workers (Ahlborg *et al*., 1988), but due to large interindividual variation in levels of 4-ADNT excreted among similarly exposed workers, its utility as a quantitative measure of exposure has been questioned (Liu *et al*., 1995). In addition to 4-ADNT, 2-ADNT, and TNT itself, other TNT metabolites (either as free or conjugated forms) including 4-NHOH-DNT, 2,4-DANT and 2,6-DANT have been detected in human urine (Yinon and Hwang, 1986; Liu *et al.*, 1991; Bader *et al*., 1998).

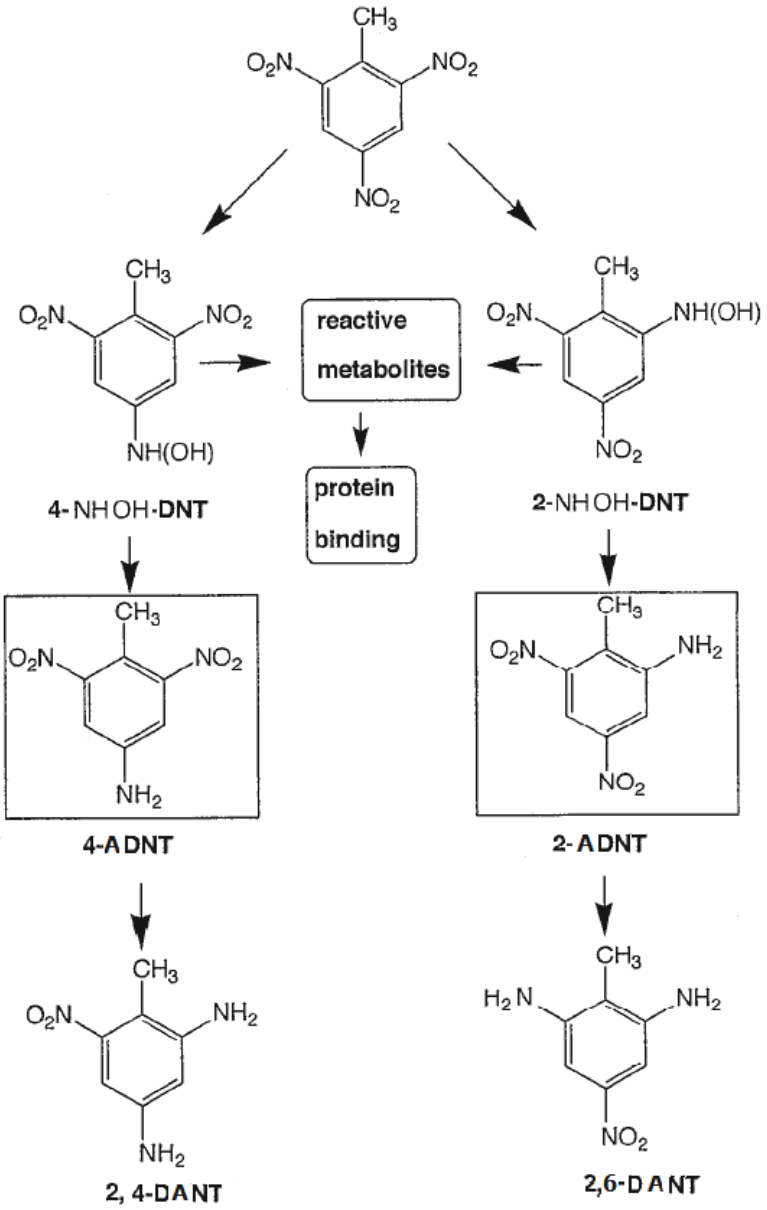

Figure 2. The Major Metabolic Pathways of TNT (from Bolt *et al*., 2006).

3.3.2 Genetic Toxicity

The genotoxic potential of TNT has been investigated in a number of systems, including assays of bacterial mutation, mammalian cell mutation, *in vivo* mouse bone marrow micronuclei, *in vivo/in vitro* in rat liver for unscheduled DNA synthesis (UDS), and *in vitro* and *in vivo* (rat) for oxidative DNA damage. TNT metabolites have been tested in many of these systems as well. There is also one study of chromosomal aberrations in peripheral blood lymphocytes of workers exposed to TNT.

TNT

As summarized by the International Agency for Research on Cancer (IARC) (1996a), TNT induced frameshift mutations in the following *Salmonella typhimurium* strains in the presence and absence of microsomal metabolic activation: TA1537 (Spanggord *et al*., 1982; Whong and Edwards, 1984), TA 1538 (Kaplan and Kaplan, 1982; Spanggord *et al*., 1982; Whong and Edwards, 1984), and TA98 (Won *et al.*, 1976; Kaplan and Kaplan, 1982; Spanggord *et al*., 1982; Whong and Edwards, 1984; Tan *et al*., 1992; Karamova *et al*., 1994), and basepair substitution mutations in strain TA100 (Spanggord *et al.*, 1982; Whong and Edwards, 1984; Tan *et al*., 1992; Karamova *et al*., 1994). Results in strain TA 1535, which also detects basepair substitution mutations, were negative (Spanggord *et al*., 1982; Whong and Edwards, 1984). TNT did not induce mutations in *Salmonella* testing strains deficient in nitroreductase (TA 100NR) or *o*-acetyltransferase activity (TA 100/1,8-DNP), suggesting that these enzyme activities are involved in the biotransformation of TNT to mutagenic species (Karamova *et al*., 1994, as cited by IARC, 1996a). Additional studies in *Salmonella* published since the IARC (1996a) review are generally consistent with these findings. Lachance *et al.* (1999) reported positive mutagenic activity of TNT in the presence and absence of exogenous metabolic activation with rat liver S9; in strains TA 98 and TA 100, using a modified *Salmonella* reverse mutation assay, (i.e., the *Salmonella* fluctuation test). George *et al*. (2001), using the *Salmonella* microsuspension assay, reported that TNT was mutagenic only in the absence of S9 in strain TA 98, and that S9 reduced, but did not abolish the mutagenicity of TNT in strain TA 100. Honeycutt *et al*. (1996) reported mutagenic activity in the presence and absence of S9 in strain TA 98, but not in strain TA 100.

As summarized by Lachance *et al*. (1999), TNT was genotoxic in the *Escherichia coli* SOS Chromotest in the presence of human placenta microsomal fraction, as carried out by Karamova *et al*. (1995), and negative in the *E. coli* SOS Chromotest in the presence or absence of rat liver S9, as carried out by Lachance *et al*. (1996).

TNT produced a positive response in the CHO-HPRT mutation assay (Chinese hamster ovary cell hypoxanthine phosphoribosyl transferase *locus*) in the presence or absence of rat liver S9, at a dose of 40 μg/ml (Kennel *et al*., 2000). TNT was not mutagenic in V79-HGPRT (hypoxanthine-guanine phosphoribosyl transferase) Chinese hamster lung cells in the presence or absence of rat liver S9 (Lachance *et al*., 1999). TNT induced mutations in the P388 mouse lymphoma thymidine kinase (TK) locus mutation assay in the absence, but not the presence of metabolic activation (Styles & Cross, 1983). In discussing their results, Lachance *et al*. (1999) raised the possibility that the V79 cell system might be insufficiently sensitive to detect TNT mutagenic activity, and noted that if the mutagenicity of TNT was mediated by clastogenic events, V79 cells would be unable to detect clastogenicity, as it kills the cells, while such activity is detectable by the mouse lymphoma TK locus assay (Aaron *et al*., 1994).

TNT was negative in the *in vivo* mouse bone marrow micronucleus assay and in the rat liver *in vivo/in vitro* UDS assay (Ashby *et al*., 1985). As summarized by the U.S. EPA, TNT did not induce cytogenetic damage in the bone marrow of Sprague-Dawley rats fed TNT in the diet for 28 days (U.S. EPA, 1993).

TNT induced oxidative DNA damage *in vivo* and the TNT metabolite 4-NHOH-DNT induced oxidative DNA damage *in vitro*. In studies by Homma-Takeda *et al*. (2002), male Fischer 344

rats were administered 300 mg/kg/day TNT by gavage once daily for two days. Under these conditions, TNT induced germ cell degeneration and a dramatic decrease in the sperm number in both testis and epididymis. TNT also increased the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), which is a marker of oxidative DNA damage, in sperm cells. Additionally, *in vitro* studies by Homma-Takeda *et al*. (2002) found that the TNT metabolite 4-NHOH-DNT, but not TNT or 4-ADNT, increased 8-oxodG formation when incubated with calf thymus DNA in the presence of copper (Cu^{2+}). Further studies, in which ^{32}P-5'-end-labeled fragments of the human p16 tumor suppressor gene were incubated with 4-NHOH-DNT, resulted in oxidative DNA damage at sites with consecutive guanine residues. The most common damage sites observed with DNA cleavage occurring at the 5'-most guanine were 5'-GG-3', 5'-GGG-3', and 5'-GGGG-3' sites. DNA damage was enhanced by NADH (nicotinamide adenine dinucleotide, reduced) and inhibited by the addition of catalase or bathocuproine, a Cu^{+} chelator. These studies suggest that hydrogen peroxide and Cu^{+} play an essential role in NADH-mediated redox reactions with TNT and lead to oxidative DNA damage (Homma-Takeda *et al*., 2002).

Sabbioni *et al*. (2007) compared the frequency of chromosomal aberrations in peripheral blood lymphocytes in 54 workers exposed to TNT at a typical ammunition factory in China with seven factory controls (defined as having no current exposure TNT) and 26 laboratory controls. No differences were observed in the rates of the various classes of chromatid-type or chromosome-type aberrations between exposed workers and controls. In further analyses, an effect of age on chromosomal aberration frequency in exposed workers was observed, but no such effect was observed for number of work years or smoking status. An effect of the N-acetyltransferase 1 (NAT1) genotype (rapid vs. slow acetylator) was observed in exposed workers, with increased chromosomal aberrations in TNT-exposed workers with the NAT1 rapid acetylator genotype, as compared to those with the NAT1 slow acetylator genotype (Sabbioni *et al*., 2007). Human polymorphic N-acetyltransferase is involved in the metabolic activation of various therapeutic agents and environmental chemicals with carcinogenic potential (Vatsis *et al*., 1995). When the effect of pairs of genotypes were compared, differences between the NAT1 rapid and slow acetylator genotypes were only seen in TNT-exposed workers that lacked functional glutathione S-transferase M1 (GSTM1 null) or T1 (GSTT1 null) (Sabbioni *et al*., 2007).

Metabolites of TNT

Urine from TNT-exposed workers has been tested in *Salmonella* and shown to have increased mutagenic activity, as compared with unexposed controls (Ahlborg *et al*., 1985; Ahlborg *et al*., 1988, and Sabbioni *et al*., 2007). Sabbioni *et al*. (2007) also looked at the effect of NAT1 polymorphisms on urinary mutagenicity in TNT-exposed workers, and found that mutagenic activity was significantly higher ($p<0.05$) in exposed workers with the NAT1 rapid acetylator genotype (mean revertants/ml-equivalents = 515, n = 7) as compared with those with the NAT1 slow acetylator genotype (124, n = 4). Similarly, the urine of rats administered TNT by intraperitoneal injection was mutagenic in *Salmonella* (Brooks *et al*., 1997).

The mutagenicity of four human metabolites of TNT, namely 2-ADNT, 4-ADNT, 2,4-DANT, and 2,6-DANT, has been tested in *Salmonella* strains TA 98 and TA 100 by several investigators. Although some studies have reported non-positive results, each of the four metabolites has been found to be mutagenic in two or more studies. The mutagenic potencies of these four metabolites were less than that of TNT. Specifically, 2-ADNT was reported to be

mutagenic in TA 98 (in the presence and absence of S9: Tan *et al*., 1992, Lachance *et al*., 1999; in the absence but not the presence of S9: Honeycutt *et al*., 1996; in the presence but not the absence of S9: George *et al*., 2001), and in TA 100 in the presence and absence of S9 (Tan *et al*., 1992, Honeycutt *et al*., 1996, Lachance *et al*., 1999, George *et al*., 2001).

4-ADNT was reported to be mutagenic in TA 98 (in the absence but not the presence of S9: Tan *et al*., 1992, George *et al*., 2001; in the presence but not the absence of S9: Lachance *et al*., 1999), and in TA 100 in three studies (in the presence and absence of S9: Tan *et al*., 1992, Lachance *et al*., 1999, George *et al*., 2001), but not in the studies of Honeycutt *et al*., 1996.

2,4-DANT was reported to be mutagenic in TA 98 (in the presence and absence of S9: George *et al*., 2001; in the presence but not the absence of S9: Lachance *et al*., 1999) and in TA 100 (in the presence and absence of S9: George *et al*., 2001; in the absence but not the presence of S9: Lachance *et al*., 1999), but it was not reported to be mutagenic in either strain in studies by Tan *et al*. (1992) and Honeycutt *et al*. (1996).

2,6-DANT was reported to be mutagenic in TA 98 (in the presence and absence of S9: George *et al*., 2001; in the absence but not the presence of S9: Tan *et al*., 1992, Lachance *et al*., 1999) and in TA 100 (in the presence and absence of S9: Lachance *et al*., 1999, George *et al*., 2001; in the absence of S9: Tan *et al*., 1992)

In mammalian cell assays, Kennel *et al*. (2000) reported that 4-ADNT produced a statistically significant positive response in the CHO-HPRT mutation assay in the presence of rat liver S9, that 2,6-DANT produced a marginally significant positive response ($p<0.087$), and that 2-ADNT and 2,4-DANT were not positive. Lachance *et al*. (1999) tested these four metabolites for mutagenicity in the V79-HGPRT assay, and reported weak mutagenic activity with 4-ADNT (positive in one out of three replicate assays), and no activity with the other three TNT metabolites.

3.3.3 Biomarker Studies

Hemoglobin (Hb) adducts formed as a result of exposure to TNT, Hb-4-ADNT and Hb-2-ADNT, have been evaluated and compared to measurements of 4-ADNT and 2-ADNT (free and conjugated) in the urine for utility as biomarkers of TNT occupational exposure (Liu *et al*., 1995; Sabbioni *et al*., 2005; Sabbioni *et al*., 2007).

In ammunition factory employees in China, Sabbioni *et al*. (2005) detected Hb-4-ADNT in all TNT exposed workers (n = 78), and Hb-2-ADNT in 81% of those workers. A good correlation was observed between levels of Hb-4-ADNT and Hb-2-ADNT (r = 0.81). Urinary metabolites were measured in 71 of these workers; 4-ADNT was detected in 100% and 2-ADNT was detected in 97%. A good correlation was observed between urinary levels of 4-ADNT and 2-ADNT (r = 0.85), however, the levels of urine metabolites and Hb-adducts were weakly correlated (r = 0.34-0.55). This is likely due to the fact that Hb-adduct levels reflect exposure received over the past 120 days, i.e., the lifetime of the red blood cell, while urinary levels of TNT metabolites reflect exposures received over the past day or two. Sabbioni *et al*. (2007) reported Hb-adduct levels or urinary metabolites to correlate weakly with measured levels of TNT on the skin, or obtained from workplace area air sampling. In this worker population, the

level of Hb-4-ADNT was statistically significantly associated with risk of hepatomegaly, splenomegaly and cataract, while urinary levels of 4-ADNT and 2-ADNT were not (Sabbioni *et al.*, 2005; Sabbioni *et al.*, 2007). These findings were in agreement with those of Liu *et al*. (1995), in which Hb-adduct levels were shown to correlate with risk of cataract.

Sabbioni *et al.* (2007) also investigated the effect of genotypes for NAT1, NAT2, GSTT1, GSTM1, and GSTP1 on Hb-adduct levels, urinary levels of 4-ADNT and 2-ADNT, and levels of urinary mutagenic activity as measured in the *Salmonella* mutagenesis assay. The genotypes investigated did not significantly influence Hb-adduct levels, but the NAT1 rapid acetylator genotype was associated with increased urinary mutagenicity. The findings of increased mutagenicity of urine and chromosomal aberrations in NAT1 rapid acetylator individuals suggest that this genotype may result in an increased sensitivity to the genotoxic effects of TNT, and possibly cancer (Sabbioni *et al.,* 2007).

3.3.4 Gene Expression and Other Studies

Tchounwou *et al*. (2001) measured transcriptional activation by TNT in the human liver hepatoma cell line $HepG_2$ to investigate possible molecular mechanisms using the mammalian gene profile (CAT-Tox) assay. In this study, 13 different recombinant constructs were made, each containing a unique stress gene promoter or response element fused to the chloramphenicol acetyltransferase (CAT) reporter gene. The genes included in the test were CYP1A1, GST Ya, HMTIIA, FOS, HSP70, GADD153, GADD45, GRP78, XRE, NFK BRE, CRE, p53RE, and RARE. The authors reported that exposure of $HepG_2$ cells to TNT for 48 hours resulted in significant induction of a number of these stress genes (i.e., CYP1A1, GST Ya, XRE, HMTIIA, c-fos, HSP70, GSDD153, and GADD45). However, the changes in gene expression patterns were reported only at TNT doses that produced significant cytotoxicity (percent cell viability was approximately 64 $\pm$ 6% at 75 µg TNT/ml, 39 $\pm$ 12% at 150 µg TNT/ml, and 10 $\pm$ 5% at 300 µg TNT/ml), rendering interpretation of the gene expression data, with respect to molecular mechanisms related to carcinogenic potential, difficult.

Banerjee *et al*. (2003) treated MCF-7 human breast cancer cells with 2-ADNT, and measured increased levels of p53 protein in treated cells, as compared to controls. Gel-shift assays were also performed, comparing the binding of nuclear extracts from untreated and 2-ADNT treated MCF-7 cells to a 20-basepair double-stranded DNA probe containing a consensus p53 binding sequence. Nuclear extracts from 2-ADNT treated cells bound to the consensus p53 binding sequence, while no binding was detected in the gel-shift assay with nuclear extracts from untreated cells (Banerjee *et al*., 2003). These results indicate that 2-ADNT-treatment increased the level of p53 protein in MCF-7 cells and that p53 proteins present in the nucleus of 2-ADNT treated MCF-7 cells can bind to p53 binding sequences in DNA.

Banerjee *et al*. (2003) concluded from their results that 2-ADNT enhanced expression of the p53 tumor suppressor gene in this cell system, and suggested that increased activation of p53-mediated transcription occurred as a result of 2-ADNT-induced DNA damage. However, Banerjee *et al*. (2003) did not measure and compare p53 mRNA levels in treated and untreated cells, nor did they otherwise demonstrate that 2-ADNT increases p53 gene expression in MCF-7 cells. Indeed, there are alternative explanations other than enhanced gene expression which could account for the observed increase in p53 protein levels, such as for example, a treatment-related increase in the stability of the p53 protein. Moreover, Banerjee *et al*. (2003) did not

demonstrate that 2-ADNT induced DNA damage in this cell system, nor did they investigate whether the p53 gene (or the protein) was altered by 2-ADNT treatment. Thus, it can not be concluded from these data that the increased level of p53 protein observed in 2-ADNT treated MCF-7 cells is indicative of 2-ADNT-induced DNA damage.

3.3.5 Structure Activity Comparisons

2,6-Dinitrotoluene (2,6-DNT), 2,4-dinitrotoluene (2,4-DNT) and 2-nitrotoluene (2-NT) are precursors or byproducts of TNT production (Figure 3), and also share structural similarities with TNT. During production, TNT is synthesized through three separate nitration reactions of toluene to form mononitrotoluenes, dinitrotoluenes, and finally, TNT. IARC has classified both 2,4-DNT and 2,6-DNT as *possibly carcinogenic to humans (Group 2B)*, based on sufficient evidence in experimental animals (IARC, 1996b). 2,4-DNT caused tumors of the renal tubular epithelium in male mice, and hepatocellular carcinomas in male and female rats, fibroadenomas of the mammary gland in female rats, and tumors of the integumentary system (e.g., fibromas and fibrosarcomas of the skin) in male rats (IARC, 1996b). 2,6-DNT caused hepatocellular carcinomas and neoplastic nodules in male rats (IARC, 1996b). The National Toxicology Program tested 2-NT in long-term carcinogenesis studies, and found clear evidence of carcinogenicity in male and female rats and mice (NTP, 2002). 2-NT caused malignant mesothelioma, subcutaneous skin neoplasms, mammary gland fibroadenoma, liver tumors, and lung tumors in male rats; subcutaneous skin neoplasms and mammary gland fibroadenoma, and liver adenoma in female rats; hemangiosarcoma and carcinoma of the large intestine in male and female mice, as well as hepatocellular neoplasms in female mice. All three compounds are listed under Proposition 65 as known to the state to cause cancer. 4-Nitrotoluene has been tested in long-term studies in rats and mice which showed an increase in clitoral gland tumors in female rats fed the compound in their diet for two years (NTP, 2002).

TNT does not share any common tumor sites with 2,6-DNT, 2,4-DNT, or 2-NT, yet all four nitrotoluenes induce tumors in experimental animals. In an analysis of chemical structural alerts, Tennant and Ashby (1991) identified the aromatic nitro group of the nitrotoluenes to be a structural alert to potential DNA reactivity. The four compounds also share commonalities in their metabolic pathways, especially with regard to their aromatic nitro groups. Each of these compounds undergoes nitroreduction to form hydroxylamines and oxidation of the methyl group to form benzyl alcohol and benzoic acid derivatives (IARC, 1996b; NTP, 2002; See also Section 3.3.1). Biliary excretion is also common among the four compounds (IARC, 1996b; NTP, 2002). Enterohepatic circulation has been demonstrated for 2,4-DNT, 2,6-DNT, and 2-NT (IARC, 1996b; NTP, 2002), and for this reason is plausible for TNT. Formation of unstable NO-sulfate conjugates that decompose to form electrophilic nitrenium ions is thought to account for the DNA and protein binding activities of 2,4-DNT, 2,6-DNT, and 2-NT (IARC, 1996b; NTP, 2002). Similarly, formation of reactive nitroso radicals and ROS is thought to account for the DNA damage and protein binding activities of TNT (Leung *et al.,* 1995; Homma-Takeda *et al*., 2002) (See Section 3.4 Mechanisms and Figure 4).

While TNT has not been as extensively tested for genotoxicity as 2,6-DNT, 2,4-DNT, or 2-NT, there are some similarities, and some differences among the compounds (See Section 3.3.2). TNT, 2,6-DNT, and 2,4-DNT were mutagenic in *Salmonella*; 2-NT was not. TNT induced mutations in the mouse lymphoma TK locus assay; 2,4-DNT did not. TNT induced mutations in

the CHO-HPRT locus assay, 2,6-DNT did not. TNT and 2-NT were negative in *in vivo* rat bone marrow micronucleus assays in the rat, and 2,4-DNT was negative in a similar assay in mice. TNT was negative in the *in vivo/in vitro* rat liver UDS assay; 2,4-DNT and 2-NT were positive (IARC, 1996b; NTP, 2002; Section 3.3.2).

2,6-Dinitrotoluene **2,4-Dinitrotoluene** **2-Nitrotoluene**

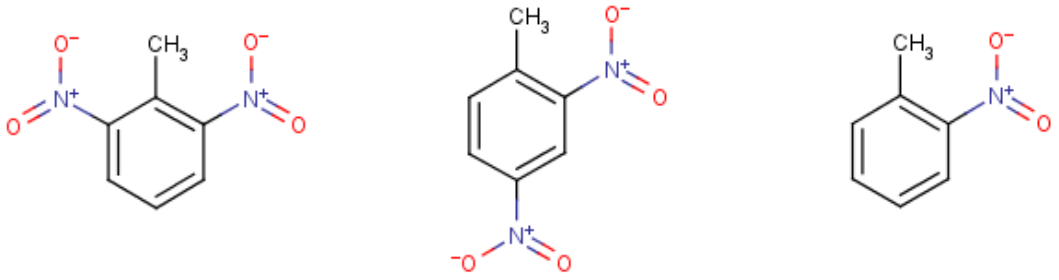

Figure 3. Precursors or Byproducts of TNT Production

3.3.6 Pathology

Liver cancer and leukemia (AML, CML, and MDS) have been reported in humans exposed to TNT. The liver cancer described in the case report of Garfinkel *et al.* (1988), a hepatocellular carcinoma, occurred in an individual with liver cirrhosis. The authors attributed both the liver cirrhosis and the liver cancer to the individual's occupational exposure to TNT over a 35 year period.

In the carcinogenicity studies of TNT in female F344 rats, treatment related increases in urinary bladder papillomas and carcinomas of the transitional epithelium were observed (Table 2) (Furedi *et al.*, 1984a). These types of bladder tumors are rare in this strain. They develop as part of a continuum which begins with transitional cell hyperplasia and progresses to benign papilloma to carcinoma (Boorman *et al.*, 1990). As shown in Table 2, hyperplasia of the transitional epithelium of the urinary bladder and the renal pelvis was also observed in these animals.

A dose-related increase in hepatocellular hyperplasia, associated with peliosis hepatis (angiectasis) and cystic degeneration (spongiosis hepatis) in the liver, was observed in male F344 rats, but not in females (Furedi *et al.*, 1984a). Peliosis hepatis is sometimes associated with hepatocellular neoplasms, and can be induced by nitrosamines. The lesion consists of irregular, dilated sinusoids lined by endothelium (Boorman *et al.*, 1990). Cystic degeneration, a cystic lesion containing finely granular or flocculent eosinophili material, occurs spontaneously in older F344 rats at low incidence, but may be common following exposure to hepatocarcinogens (Boorman *et al.*, 1990). These changes were considered by the cancer bioassay report authors to be naturally occurring lesions exacerbated by TNT (Furedi *et al.*, 1984a). The increase in liver hyperplasia was not accompanied by an increase in liver tumors in male rats.

In the carcinogenicity studies of TNT in $B6C3F_1$ mice, treatment related increases in leukemia and malignant lymphomas of the spleen were observed in females (Table 3) (Furedi *et al*., 1984b). The leukemias were of granulocytic or lymphocytic type, and the malignant lymphomas were of histiocytic, lymphocytic, or mixed type (Furedi *et al*., 1984b). These lesions were systemic reticuloendothelial neoplasias that involved other organs and tissues (Furedi *et al*., 1984b).

3.3.7 Non-Cancer Toxicities

The following effects have been observed in individuals exposed to TNT: liver damage, cyanosis, sneezing, cough, sore throat, peripheral neuritis, muscular pain, kidney damage, cataracts, sensitization dermatitis, leukocytosis, leucopenia, and aplastic anemia (Hathaway *et al.*, 1991; ATSDR, 1995). Toxic effects of TNT, including upper respiratory and gastrointestinal complaints, anemia, liver function abnormalities, and possibly aplastic anemia, have been observed in workers exposed at levels well below the current Occupational Safety and Health Administration (OSHA) Permissible Exposure Level of 1.5 mg/m^3 (OSHA, 1999). Aplastic anemia is caused by other chemicals, in addition to TNT. These include the carcinogens benzene and chlordane, which are also associated with increased risk of lymphohaematopoetic cancers in humans (Hathaway, 1977; U.S. EPA, 1998).

3.4 Mechanisms

The mechanisms of action of TNT with regard to carcinogenicity are unknown. Several lines of evidence suggest that TNT may act through a genotoxic mechanism. These include data indicating that i) TNT and several of its metabolites (i.e., 2-ADNT; 4-ADNT; 2,6-DANT; and 2,4-DANT) are direct mutagens in *Salmonella*, ii) TNT is mutagenic in two mammalian cell assays (i.e., CHO-HPRT mutation assay; P388 mouse lymphoma TK locus mutation assay), one of which is capable of detecting clastogenic events (i.e., P388 mouse lymphoma TK locus mutation assay), iii) one TNT metabolite was clearly mutagenic (4-ADNT), and another weakly mutagenic (2,6-DANT) in the mammalian CHO-HPRT mutation assay, iv) TNT causes oxidative DNA damage in mice *in vivo*, as indicated by increased levels of 8-oxodG in sperm cells, v) *in vitro* studies indicate that the TNT metabolite 4-NHOH-DNT (or reactive intermediates) can oxidize consecutive guanine residues within the DNA, forming DNA breaks and increasing 8-oxodG levels, and vi) TNT (or reactive metabolites) binds covalently to proteins in humans (Hb adducts) and animals (Hb adducts, liver proteins). Studies on TNT metabolism support a genotoxic mechanism of action for TNT, as they indicate that TNT can be metabolized through multiple pathways to form reactive nitroso species and ROS, which may bind covalently with proteins and other macromolecules, induce oxidative stress, and oxidative DNA damage.

Homma-Takeda *et al*. (2002) proposed one mechanism by which TNT induces DNA damage, involving the formation of hydroxylamino dinitrotoluene (i.e., 2-NHOH-DNT, 4-NHOH-DNT), and subsequent metabolism and/or redox cycling reactions to generate nitroso radicals and ROS (Figure 4). This mechanism is based on the studies of Homma-Takeda *et al*. (2002) on TNT-induced oxidative DNA damage, and the studies of Leung *et al*. (1995) on TNT metabolism and protein binding. Leung *et al.* (1995) proposed a similar pathway for TNT bioactivation and covalent binding with cellular proteins, in which reactive intermediates capable of covalent

binding are formed at multiple points on this pathway. Specifically, Leung *et al.* (1995) proposed the formation of reactive intermediates derived from the nitroso dinitrotoluenes, and from the amino nitrotoluenes (i.e., 2-ADNT and 4-ADNT).

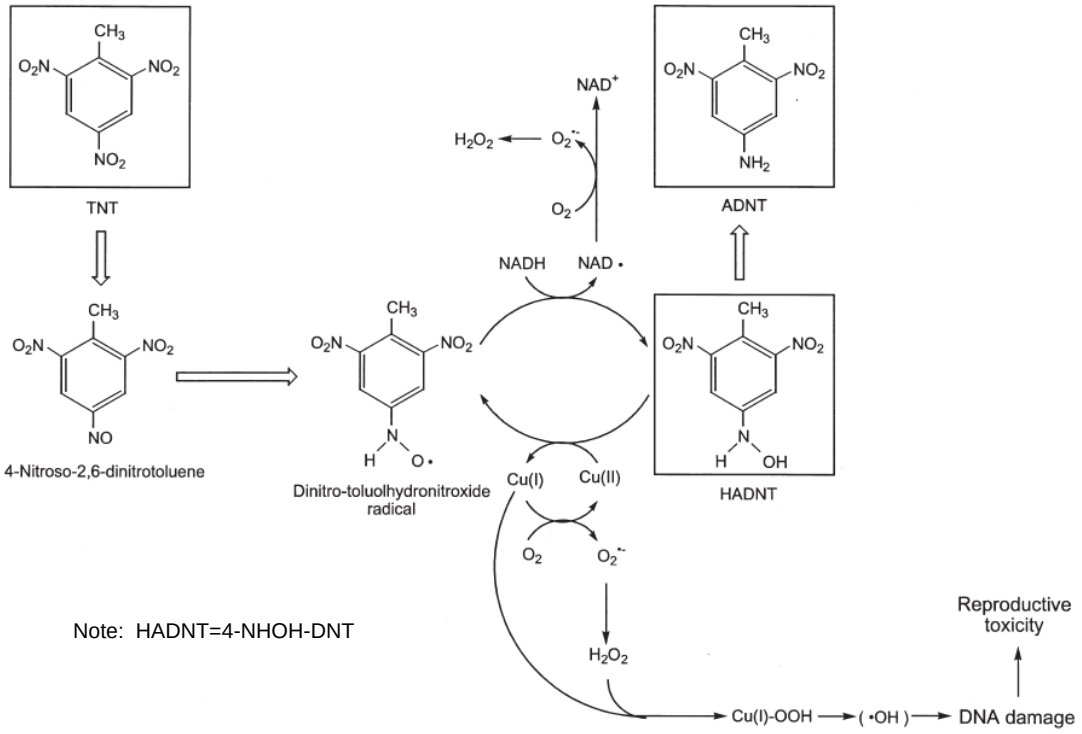

Figure 4. A Proposed Mechanism of DNA Damage by TNT (Homma-Takeda *et al*., 2002)

The findings of Sabbionni *et al.* (2007), with increased mutagenicity of urine and increased chromosomal aberrations in TNT-exposed individuals with the NAT1 rapid acetylator genotype suggest that this genotype may present an increased sensitivity to TNT exposure and result in an increased risk of cancer. These findings further suggest that the role of genetic polymorphisms in susceptibility to TNT merits further investigation.

There is little information available on other possible mechanisms of carcinogenicity of TNT. For example there are no studies on the possible epigenetic actions of TNT with regard to DNA methylation, histone acetylation, etc. The one study that looked at gene expression changes in a human hepatoma cell line was uninformative for this purpose, due to severe cytotoxicity (Tchounwou *et al*., 2001).

4 OTHER REVIEWS

IARC reviewed the carcinogenicity of several nitro compounds, including TNT, in 1996 (IARC, 1996a). IARC found there was *inadequate evidence* in humans and *inadequate evidence* in experimental animals, and concluded that TNT was *not classifiable as to its carcinogenicity to humans (Group 3*) (IARC, 1996a). However, IARC did not review any animal carcinogenicity studies of TNT. The Furedi *et al.* (1984a, 1984b) studies, sponsored by the U.S. Army, were not published in the open scientific literature and at the time IARC as a matter of policy did not review any studies not in the open literature. Many studies have been published on TNT since the 1996 IARC review, including the case-control study of Kilian *et al*. (2001), the cohort study of Yan *et al.* (2002), and multiple studies on TNT metabolism, genotoxicity, and biomarkers of exposure.

U.S. EPA has also reviewed the carcinogenicity of TNT, most recently in 1993. That review included the animal carcinogenicity studies by Furedi *et al.* (1984a, 1984b). U.S. EPA classified TNT as a possible human carcinogen (Group C), based on inadequate evidence in humans, observations of urinary bladder papilloma and carcinoma in female Fischer 344 rats, and mutagenic activity in *Salmonella* in the presence and absence of metabolic activation (U.S. EPA, 1993).

To date, neither the National Toxicology Program, the National Institutes of Occupational Safety and Health, nor the Food and Drug Administration appear to have evaluated the carcinogenic activity of TNT.

5 SUMMARY AND CONCLUSIONS

5.1 Summary of Evidence

An ecological epidemiological study (Kolb *et al*., 1993) found an association between TNT exposure occurring as a result of environmental TNT contamination from munitions plants in Germany operating in the 1940s and leukemia. A follow-up population-based case control study of residents living in the same area found an association for leukemia and TNT exposure in one neighborhood where exposure may have been high (Kilian *et al*., 2001). However, the number of cases was small, and similar findings were not seen for other areas with TNT exposures, or for other measures of TNT exposure. A historical cohort study of munitions workers associated TNT exposure and risk of liver cancer (Yan *et al*., 2002). Case reports of liver cancer and leukemia are consistent with these findings (Garfinkel *et al*., 1988; Yan *et al*., 2002). The findings from each of these studies are tempered by numerous study limitations however.

In long-term carcinogenesis studies, TNT caused rare urinary bladder papillomas and carcinomas in female F344 rats (Furedi *et al*., 1984a), and leukemias and malignant lymphomas of the spleen in female B6C3F_1 mice (Furedi *et al*., 1984b).

While the mechanisms of carcinogenic action of TNT remain unclear, several lines of evidence suggest that TNT may act through a genotoxic mechanism. TNT is genotoxic in bacterial and

mammalian systems *in vivo* and *in vitro*. TNT induced both frameshift and basepair substitution mutations in *Salmonella*, mutations in mammalian cells *in vitro* in the CHO-HPRT locus assay and the mouse lymphoma TK locus assay. TNT induced oxidative DNA damage in rat sperm *in vivo*, as measured by increased formation of 8-oxodG. The TNT metabolite 4-NHOH-DNT damaged DNA, increasing the formation of 8-oxodG and cleaving the DNA at sites with consecutive guanines (Homma-Takeda *et al*., 2002). Several TNT metabolites have also been observed to be genotoxic in the *Salmonella* (2-ADNT; 4-ADNT; 26, DANT; 2,4-DANT) and CHO-HPRT assays (4-ADNT; 2,6-DANT weakly positive). Urine from workers exposed to TNT has increased mutagenic activity in the *Salmonella* assay compared to that from unexposed workers.

TNT binds covalently to proteins in humans (hemoglobin) and animals (hemoglobin, liver proteins), indicating the potential to bind to DNA. TNT can be metabolized through multiple pathways to form reactive nitroso species and ROS, which may bind covalently with proteins and other macromolecules, induce oxidative stress, and oxidative DNA damage.

Structure activity comparisons with the carcinogenic nitrotoluenes 2,4-DNT, 2,6-DNT, and 2-NT suggest that common pathways of metabolism and similarities in the reactivity of metabolic intermediates with proteins and DNA exist for TNT.

5.2 Conclusion

There is evidence for the carcinogenicity of TNT, in the form of data on the development of benign and malignant tumors of the urinary bladder in female rats and hematopoietic tumors in female mice treated for two years by diet. Further evidence of potential carcinogenicity includes genotoxicity of TNT and metabolites in *Salmonella* and mammalian cells, as well as close structural similarity to 2-NT and 2,4- and 2,6- DNT, all three of which are carcinogenic and listed as such under Proposition 65. Human populations exposed to TNT have been inadequately studied with regard to carcinogenicity.

6 REFERENCES

Aaron CS, Bolcsfoldi G, Glatt HR, Moore M, Nishi Y, Stankowski L, Theiss J, Thompson E (1994). Mammalian cell gene mutation assays working group report. *Mutat Res* **312**:235-239.

Ahlborg G Jr, Bergström B, Hogstedt C, Einistö P, Sorsa M (1985). Urinary screening for potentially genotoxic exposures in a chemical industry. *Brit J Ind Med* **42**:691-699.

Ahlborg G Jr, Einistö P, Sorsa M (1988). Mutagenic activity and metabolites in the urine of workers exposed to trinitrotoluene (TNT). *Br J Ind Med* **45**:353-358.

Ashby J, Burlinson B, Lefevre PA, Topham J (1985). Non-genotoxicity of 2,4,6-trinitrotoluene (TNT) to the mouse bone marrow and the rat liver: Implications for its carcinogenicity. *Arch Toxicol* **58**:14-19.

Agency for Toxic Substances and Disease Registry (ATSDR, 1995). Toxicological profile for 2,4,6-trinitrotoluene. ATSDR., U.S. Department Of Health And Human Services. Public Health Service.

Bader M, Göen T, Müller J, Angerer J (1998). Analysis of nitroaromatic compounds in urine by gas chromatography-mass spectrometry for the biological monitoring of explosives. *J Chromatogr B Biomed Sci Appl* **710**:91-99.

Banerjee H, Hawkins Z, Dutta S, Smoot D (2003). Effects of 2-amino-4,6-dinitrotoluene on p53 tumor suppressor gene expression. *Mol Cell Biochem* **252**:387-389.

Bolt HM, Degen GH, Dorn SB, Plöttner S, Harth V (2006). Genotoxicity and potential carcinogenicity of 2,4,6-TNT trinitrotoluene: Structural and toxicological considerations. *Rev Environ Health* **21**:217-228.

Boorman GA, Eustis SL, Elwell MR, Montgomery CA Jr., MacKenzie WF (1990). Pathology of the Fischer Rat. Academic Press, Inc. San Diego, California.

Brooks LR, Jacobson RW, Warren SH, Kohan MJ, Donnelly KC, George SE (1997). Mutagenicity of HPLC-fractionated urinary metabolites from 2,4,6-trinitrotoluene-treated Fischer 344 rats. *Environ Mol Mutagen* **30**:298-302.

Budavari S (1989). The Merck Index, 11th ed., Rahway, NJ, Merck & Co., p1530-1531.

Coombs M, Schillack V (1998). Determination of trinitrotoluene and metabolites in urine by means of gas-chromatography with mass detection. *Int Arch Occup Environ Health* **71(Suppl)**:S22-S25.

Furedi EM, Levine BS, Gordon DE, Rac VS, Lish PM (1984a). Determination of the chronic mammalian toxicological effects of TNT (Twenty-four month chronic toxicity/carcinogenicity

study of trinitrotoluene (TNT) in the Fischer 344 rat). Final Report - Phase III. Volume 1. ITT Research Institute, Project No. L6116, Study No. 9, Chicago, IL. DAMD17-79-C-9120. AD-A168 637.

Furedi EM, Levine BS, Sagartz JW, Rac VS, Lish PM (1984b). Determination of the chronic mammalian toxicological effects of TNT (Twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the B6C3F1 hybrid mouse). Final Report - Phase IV. Volume 1. ITT Research Institute, Project No. L6116, Study No. 11, Chicago, IL. DAMD17-79-C-9120. AD-1168 754.

Garfinkel D, Sidi Y, Steier M, Rother A, Marilus R, Atsmom A, Pinkhas J (1988). Liver cirrhosis and hepatocellular carcinoma after prolonged exposure to TNT: Causal relationship or mere coincidence? *Rev Roum Méd - Méd Int* **26**:287-290.

George SE, Huggins-Clark G, Brooks LR (2001). Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. *Mutat Res* **490**:45-56.

Grummt T, Wunderlich H-G, Chakraborty A, Kundi M, Majer B, Ferk F, Nersesyan AK, Parzefall W, Knasmüller S (2006). Genotoxicity of nitrosulfonic acids, nitrobenzoic acids, and nitrobenzylalcohols, pollutants commonly found in ground water near ammunition facilities. *Environ Mol Mutagen* **47**:95-106.

Hathaway JA (1977). Trinitrotoluene: A review of reported dose-related effects providing documentation for a workplace standard. *J Occup Med* **19**:341-345.

Hathaway G, Proctor N, Hughes J, Fischman M (1991). Proctor and Hughes' Chemical Hazards of the Workplace, 3rd edition, Van Nostrand Reinhold, New York. p570-571.

Hodgson JR, Hwang SW, Dacre JC, Lee CC (1977). Comparative absorption, distribution, excretion and metabolism of 2,4,6-trinitrotoluene (TNT) and isomers of dinitrotoluene (DNT) in rats. *Fed Proc* **36**:996, abstract no. 3826.

Homma-Takeda S, Hiraku Y, Ohkuma Y, Oikawa S, Murata M, Ogawa K, Iwamuro T, Li S, Sun GF, Kumagai Y, Shimojo N, Kawanishi S (2002). 2,4,6-trinitrotoluene-induced reproductive toxicity via oxidative DNA damage by its metabolite. *Free Radical Res* **36**:555-566.

Honeycutt ME, Jarvis AS, McFarland VA (1996). Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. *Ecotoxicol Environ Saf* **35**:282-287.

International Agency for Research on Cancer (IARC, 1996a). 2,4,6-Trinitrotoluene. *IARC Monogr Eval Carcinog Risks Hum* **65**:449-475.

International Agency for Research on Cancer (IARC, 1996b). 2,4-Dinitrotoluene, 2,6-Dinitrotoluene and 3,5-Dinitrotoluene. *IARC Monogr Eval Carcinog Risks Hum* **65**:309-368.

Kaplan DL, Kaplan AM (1982). Mutagenicity of 2,4,6-trinitrotoluene-surfactant complexes. *Bull Environ Contam Toxicol* **28**:33-38.

Karamova NS, Il'inskaia ON, Ivanchenko OB (1994). Mutagenic activity of 2,4,6-trinitrotoluene: the role of metabolizing enzymes. *Genetika* **30**:898-902 (As cited by IARC, 1996a).

Karamova NS, Mynina II, Garaeva GG, Ivanchenko OB, Il'inskaya ON (1995). Compounds 2,4,6-trinitrotoluene and 2,4-diamino-6-nitrotoluene: The absence of recA-dependent mutagenesis. *Russ J Genet* **31**:528-531. (as cited by Lachance *et al*., 1999).

Kennel SJ, Foote LJ, Morris M, Vass AA, Griest WH (2000). Mutation analyses of a series of TNT-related compounds using the CHO-hprt assay. *J Appl Toxicol* **20**:441-448.

Kilian PH, Skrzypek S, Becker N, Havemann K (2001). Exposure to armament wastes and leukemia: a case-control study within a cluster of AML and CML in Germany. *Leuk Res* **25**:839-845.

Kolb G, Becker N, Scheller S, Zugmaier G, Pralle H, Wahrendorf J, Havemann K (1993). Increased risk of acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) in a county of Hesse, Germany. *Soz Präventivmed* **38**:190-195.

Lachance B, Hawari J, Sharmmen N, Dodard S, Robidoux PY, Ampleman G, Thiboutot S, Sunahara G (1996). Cytotoxic and genotoxic effects of some energetic compounds tested using various bacterial and mammalian cell assays. SETAC 17th Annual Meeting, Abstract book, 17-21 November 1996, Washington, DC, SETAC Press, Pensacola, FL, U.S.A. (as cited by Lachance *et al*., 1999).

Lachance B, Robidoux PY, Hawari J, Ampleman G, Thiboutot S, Sunahara GI (1999). Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. *Mutat Res* **444**:25-39.

Leung KH, Yao M, Stearns R, Chiu SH (1995). Mechanism of bioactivation and covalent binding of 2,4,6-trinitrotoluene. *Chem Biol Interact* **97**:37-51.

Lewis RJ Sr (1997). Hawley's condensed chemical dictionary. 13th Edition. John Wiley & Sons, Inc. New York. p. 1140.

Liu Y, Wei W, Wang M, Pu Y, Lin L, Zhang J (1991). Simultaneous determination of the residues of TNT and its metabolites in human urine by thin-layer chromatography. *J Planar Chromatogr* **4**:146-149.

Liu YY, Yao M, Fang JL, Wang YW (1995). Monitoring human risk and exposure to trinitrotoluene (TNT) using haemoglobin adducts as biomarkers. *Toxicol Lett* **77**:281-287.

National Toxicology Program (NTP, 2002). Toxicology and Carcinogenesis studies of o-nitrotoluene (CAS NO. 88-72-2) in F344/N rats and B6C3F$_1$ mice (Feed Studies). NTP Technical Report Series No. 504. NIH Publication No. 02-4438. U.S. Department of Health and Human Services, NTP, Research Triangle Park, NC.

Occupational Safety & Health Administration (OSHA, 1999). 2,4-Dinitrotoluene (DNT) /2,4,6-Trinitrotoluene (TNT) - (Organic Method #44). OSHA, U.S. Department of Labor. Available at URL: http://www.osha.gov/dts/sltc/methods/organic/org044/org044.html.

Peterson MM, Horst GL, Shea PJ, Comfort SD (1998). Germination and seedling development of switchgrass and smooth bromegrass exposed to 2,4,6-trinitrotoluene. *Environ Pollut* **99**:53-59.

Sabbioni G, Liu YY, Yan H, Sepai O (2005). Hemoglobin adducts, urinary metabolites and health effects in 2,4,6-trinitrotoluene exposed workers. *Carcinogenesis* **26**:1272-1279.

Sabbioni G, Sepai O, Norppa H, Yan H, Hirvonen A, Zheng Y, Järventaus H, Bäck B, Brooks LR, Warren SH, Demarini DM, Liu YY (2007). Comparison of biomarkers in workers exposed to 2,4,6-trinitrotoluene. *Biomarkers* **12**:21-37.

Šarlauskas J, Nemeikaite-Čeniene A, Anusevičius Ž, Misevičiene L, Julvez MM, Medina M, Gomez-Moreno C, Čenas N (2004). Flavoenzyme-catalyzed redox cycling of hydroxylamino- and amino metabolites of 2,4,6-trinitrotoluene: implications for their cytotoxicity. *Arch Biochem Biophys* **425**:184-192.

Spanggord RJ, Mortelmans KE, Griffin AF, Simmon VF (1982). Mutagenicity in Salmonella typhimurium and structure-activity relationships of wastewater components emanating from the manufacture of trinitrotoluene. *Environ Mutagen* **4**:163-179.

Styles JA, Cross MF (1983). Activity of 2,4,6-trinitrotoluene in an in vivo mammalian gene mutation assay. *Cancer Lett* **20**:103-108.

Tan EL, Ho CH, Griest WH, Tyndall RL (1992). Mutagenicity of trinitrotoluene and its metabolites formed during composting. *J Toxicol Environ Health* **36**:165-175.

Tchounwou PB, Wilson BA, Ishaque AB, Schneider J (2001). Transcriptional activation of stress genes and cytotoxicity in human liver carcinoma cells ($HepG_2$) exposed to 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. *Environ Toxicol* **16**:209-216.

Tennant RW, Ashby J (1991). Classification according to chemical structure, mutagenicity to *Salmonella* and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. *Mutat Res* **257**:209-227.

U.S. Army (1981). Species differences in the disposition and metabolism of 2,4,6-trinitrotoluene as a function of route of administration. Final report. Contract No. DAMD-17-76-C-6066. Frederick, MD. U.S. Army Medical Research and Development Command, Fort Detrick. Document No. AD-A114025. (As summarized by ATSDR, 1995.)

U.S. Environmental Protection Agency (U.S. EPA, 1998). Integrated Risk Information System (IRIS): Chlordane (Technical) (CASRN 12789-03-6). Section II. Carcinogenicity Assessment last revised February 7, 1998. Available at URL:http://www.epa.gov/NCEA/iris/subst/0142.htm.

U.S. Environmental Protection Agency (U.S. EPA, 1993). Integrated Risk Information System (IRIS): 2,4,6-Trinitrotoluene (TNT) (CASRN 118-96-7). Section II. Carcinogenicity Assessment last revised July 1, 1993. Available at URL:http://www.epa.gov/NCEA-/iris/subst/0269.htm.

Vatsis KP, Weber WW, Bell DA, Dupret JM, Evans DA, Grant DM, Hein DW, Lin HJ, Meyer UA, Relling MV *et al.* (1995). Nomenclature for N-acetyltransferases. *Pharmacogenetics* **5**:1–17.

Whong WZ, Edwards GS (1984). Genotoxic activity of nitroaromatic explosives and related compounds in *Salmonella typhimurium*. *Mutat Res* **136**:209-215.

Won WD, DiSalvo LH, Ng J (1976). Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl Environ Microbiol* **31**:576-580.

Yan C, Wang Y, Xia B, Li L, Zhang Y, Liu Y (2002). [The retrospective survey of malignant tumor in weapon workers exposed to 2,4,6-trinitrotoluene][Article in Chinese] *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* [*Chin J Ind Hyg Occup Dis*] **20**:184-188.

Yinon J, Hwang DG (1985). Identification of urinary metabolites of 2,4,6-trinitrotoluene in rats by liquid chromatography-mass spectometry. *Toxicol Lett* **26**:205-209.

Yinon J, Hwang DG (1986). Metabolic studies of explosives: Part 5. Detection and analysis of 2,4,6-trinitrotoluene and its metabolites in urine of munition workers by micro liquid chromatography/mass spectrometry. *Biomed Chromatogr* **1**:123-125.