TOXICOLOGIC AND PATHOLOGIC EFFECTS OF

1, 3, 5,-TRINITROBENZENE (TNB)

By

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1989

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1995

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PREFACE

The nitroaromatic compound 1,3,5-trinitrobenzene (TNB) has been detected as an environmental contaminant at military installations, test grounds and munition production waste sites. Current information concerning the effects of TNB are based on assumptions of generality that structurally similar compounds behave alike and the toxicity information for TNB is derived by analogy to similar nitroaromatics. This study was designed to evaluate the target organs for toxicity in rats orally exposed to TNB. The dissertation is composed of five manuscripts which are complete and need no supporting material. Chapters II and V have been published in '*The Journal of Toxicology and Environmental Health*' and '*Toxicologic Pathology*' respectively. Chapters III and IV have been formatted for submission to '*The Journal of Toxicology and Environmental Health*' and Chapter VI for '*Veterinary Pathology*'.

ACKNOWLEDGEMENTS

I am greatly indebted to Dr. Charles W. Qualls Jr. my major advisor. Unequivocally, without his guidance and continuous support, this undertaking would not have been possible. I am sure that many times in the future my actions will be a reflection of his mentorship.

I would like to express sincere gratitude to the members of my advisory committee: Drs. Gregory Campbell, Subbiah Sangiah and Robert Lochmiller. They deserve sincere appreciation for their suggestions and guidance. I would like to thank Dr. Gunda Reddy at the U. S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Maryland for financially supporting this project. Additionally, I would like to thank both Dr. Qualls and Dr. Anthony Confer for their trust in me and being instrumental in my development as a pathologist.

I appreciate all the help from Dr. James Lish, Dr. Madhav Paranjpe and fellow graduate students.

Finally, my family deserves my deepest appreciation including my parents, my wife and in-laws for their understanding and encouragement throughout the endeavor.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Nitroaromatic compounds play an extremely important role in the chemical industry. The fundamental importance of nitroaromatics derives from the fact that they are the chemical industry's only commercial source of aromatics with nitrogen bound to the ring. Nitroaromatics are used to make several thousand consumer products, which account collectively for nearly 10 percent of the chemical industry sales (Hartter, 1985).

Nitroaromatic compounds have long been used as intermediates in the preparation of a great number of substances in the chemical industry as well as in the production of high explosives. Explosives and propellants have important military applications, the former are also widely used in mining and construction. In the course of production, handling, loading of military or civilian devices and ultimate dispersal or disposal, explosives and propellants are released to the environment. They are disseminated by natural processes and partially converted to secondary products (Rosenblatt et al., 1991).

The mutagenic and carcinogenic potential of this class of compounds, especially that observed in the nitrated biphenyl's, fluorenes, and naphthalene's, has brought about an increased interest in the risks and hazards that nitroaromatic compounds present to human populations and their environment (Spanggord et al., 1985). The inference that the results of animal experiments can be applied to humans is a fundamental principle of all toxicologic research. For most hazardous chemicals, adequate human data are not available, and risk analyses must rely on information from laboratory studies of rats or mice. Nitroheterocyclic compounds are no exception.

1,3,5-Trinitrobenzene (TNB) is a man-made nitroaromatic compound that is usually associated with the production of munitions and armaments. TNB can enter the environment in wastewater effluent from facilities that synthesize, produce or demilitarize munitions, or from the disposal of solid 2,4,6-trinitrotoluene (TNT) wastes (Ryon et al., 1984; Spalding and Fulton, 1988; Spanggord et al., 1982). In addition to the munition plants, TNB was identified as a by-product in waste-water effluent from the synthesis of p-nitrobenzoic acid, an intermediate used for the synthesis of pharmaceuticals (Wennersten, 1980)

1,3,5-TNB (CAS Number 99-35-4) is known by the synonyms trinitrobenzene, *symmetrical* trinitrobenzene, sym trinitrobenzene (Budavari et al., 1989; Sax and Lewis, 1989; Weast, 1989). TNB is a dimorphic solid with a molecular weight of 213.11. It can be prepared by the decarboxylation of trinitrobenzoic acid or by the oxidation of 2,4,6-trinitrotoluene. TNB is a yellow crystalline solid at room temperature; it is soluble in both polar and nonpolar solvents and sparingly soluble in water (Sax and Lewis, 1987; Windholz et al., 1983).

TNB is a class A explosive that is less sensitive to impact, but more powerful and brisant than TNT (Budavari et al., 1989; Fedoroff et al., 1962). TNB has been classified as a high explosive and has been used in military and commercial explosive compositions (Budavari et al, 1989; Sax and Lewis 1987). TNB has also been used as an explosive for oil wells and mining (USDHHS, 1993). Other uses for TNB include use as a vulcanizing agent in the processing of natural rubber and as an indicator in acid-base reactions in the pH range of 12.0 -14.0 (USDHHS, 1993). TNB is classified as an EPA hazardous waste, and disposal must be carried out according to EPA regulations (USDHHS, 1993). TNB has been identified in 14 of the 1300 hazardous waste sites on the National Priorities List (NPL) (USDHHS, 1993).

An anthropogenic environmental contaminant, exposure to TNB can occur through contact with wastewater effluents released from facilities that synthesize, produce

or demilitarize munitions, or from the disposal of solid TNT wastes (Ryon et al., 1984; U.S. EPA, 1989). Data on oral, dermal, or inhalation exposure of experimental animals or humans to TNB are limited to a few Russian (Korolev et al., 1977; Senczuk et al., 1976) and English abstracts (Kinkead et al., 1994; Qualls et al., 1993). Most of the toxicity data derived for TNB is by analogy to two structurally similar compounds 1,3-dinitrobenzene and 2,4,6-trinitrotoluene.

HEMATOLOGICAL EFFECTS:

Methemoglobinemia

One of the typical signs of nitroaromatic poisoning is cyanosis due to methemoglobinemia. Methemoglobinemia due to nitrobenzene (NB) poisoning in humans has been recognized for more than a century. Nitrobenzene-induced methemoglobinemia has also been reported in a variety of animal species, including cats, rabbits, rats, dogs, and mice, although mice appear to be more resistant to methemoglobinemia than other species (Medinsky and Irons, 1985). A relationship between the metabolism and toxicity of NB has been demonstrated by the absence of NB-induced methemoglobinemia in axenic animals (Reddy et al., 1976), suggesting that intestinal microfloral metabolism of nitrobenzene is essential for methemoglobin production. Cecal microflora sequentially reduce NB to aniline through the intermediates nitrosobenzene and phenylhydroxylamine (Levin and Dent et al. 1982) and these intermediates are known to produce methemoglobinemia.

In common with NB, the three isomers of dinitrobenzene 1,2-DNB, 1,3-DNB and 1,4-DNB have been reported to induce methemoglobinemia both *in vivo and in vitro* (Facchini and Griffiths 1981). Blackburn et al (1988) reported cyanosis (methemoglobinemia) and splenic enlargement with 1,3-DNB and 1,4-DNB but not 1,2-DNB in rats receiving a single oral dose of 50 mg/kg of the isomers contradicting the

results of Facchini and Griffiths (1981). Watanabe et al (1976) concluded that the 1,3-DNB (*m*-DNB) and 1,4-DNB (*p*-DNB) isomers are potent methemoglobin formers *in vivo*, while the third isomer, 1,2-DNB(*o*-DNB) has much less capacity. Among the dinitrobenzene isomers, the commercially important 1,3-DNB is a potent inducer of methemoglobin both *in vivo and in vitro* (Facchini and Griffiiths, 1981; Goldstein and Rickert, 1985). However, Cossum and Rickert (1987) demonstrated an inter-species (and inter-isomer) differences in the *in vitro* production of methemoglobin between rat, monkey and human erythrocytes.

Methemoglobinemia associated with exposure to the widely used military explosive TNT is well known. A significant methemoglobinemia was observed with TNT in rats fed 300 mg/kg/day for 13 weeks (Levine et al. 1984) and also in dogs fed 32 mg/kg/day for 6 months (Levine et al. 1990). In contrast to the above nitroaromatics, information on methemoglobin formation with TNB both *in vivo and in vitro* is rather limited and incomplete. Methemoglobin was not significantly elevated 5 hours after a single intraperitoneal injection of TNB (21.3 mg/kg) in rats (Watanabe et al., 1976). Senczuk et al (1976) (translated from Russian) reported that a single oral dose of TNB at 0.4 micro mole/kg (85 microgram /kg) induced methemoglobin formation in Wistar rats. A recent report also suggests significant methemoglobinemia 5 hours after a single dose (71 mg/kg) and 24 hours after 4 daily doses (Qualls et al., 1993).

Hemolytic Anemia

The most frequently reported consequence of exposure to nitroaromatic compounds is a significant anemia. Poisoning with nitrobenzene, the simplest archetype of the nitroaromatics has been recognized for a long time. Blood dyscrasias have been reported during the stages of acute and chronic nitrobenzene poisoning, including decreases in circulating hemoglobin, and in erythrocyte and platelet counts; hemolytic

anemia; and bone marrow hyperplasia (Shimkin, 1939; Hamblin, 1949; Parkes and Neill, 1953).

In common with NB, the three isomers of dinitrobenzene, 1,2-DNB, 1,3-DNB and 1,4-DNB have been reported to induce anemia on prolonged exposure (Watanabe et al., 1976). As early as 1949, Kiese reported hematological changes in dogs given daily or intermittent subcutaneous injections of 0.1-6.0 mg/kg of 1,3-DNB for as long as 144 days. He noted similar effects in dogs given acute doses of 10 and 20 mg/kg subcutaneously (Kiese 1949a,b). Cody et al (1981) reported that 1,3-dinitrobenzene produced reductions in hematocrit and hemoglobin values in rats after administration in the drinking water for 8 weeks (at 50, 100, 200 mg/L) or 16 weeks (at 3, 8, 20 mg/L).

Among the nitroaromatics, the hematological effects 2,4,6-trinitrotoluene have been extensively documented in humans (Hathway, 1985). The earliest such report of laboratory findings in TNT-exposed workers was by Minot in 1919 (Minot, 1919). In experimental animals adverse effects on standard hematological parameters were observed in rats (Dilley et al. 1982; Levine et al. 1984, 1990), mice (Dilley et al. 1982), and dogs (Dilley et al. 1982; Levine et al. 1990) after intermediate (13 weeks) oral exposure to TNT. Compensatory responses occurring as a result of anemia (including reticulocytosis, macrocytosis, and increased levels of nucleated erythrocytes) were observed in Fischer-344 rats fed 125 mg/kg/day of TNT for 13 weeks (Levine et al. 1990). Similar to methemoglobinemia, reports of anemia after oral, inhalation or dermal exposure to TNB in humans or laboratory animals are confined to tersely reported studies (Qualls et al., 1993; Fogleman et al., 1955).

Fogleman et al (1955) conducted studies using TNB in dogs. They reported derangement of erythrocyte morphology, hemoglobin values within normal limits, and erratic methemoglobin values. The source, isomer or purity of the compound (TNB) was not mentioned in their report. A significant anemia with reductions in PCV, red cell numbers and hemoglobin was reported in rats receiving daily doses of TNB at 71 mg/kg

for 4 days in a report by Qualls et al (1993). In retrospect, the current information on hematological effects of TNB is by analogy to the two structurally similar compounds 1,3-DNB and 2,4,6-TNT. The isomer specificity observed with dinitrobenzenes by Cossum and Rickert (1987) and Blackburn et al (1988) give conclusive evidence that simple extrapolation of toxic effects based on the structure-activity relationship may not be valid with nitroaromatics.

Mechanism of Hemolytic Anemia

Hemolytic anemia (and methemoglobinemia) is a significant toxic side-effect that has been known to accompany treatment with a variety of nitroaromatics. A wide variety of drugs and xenobiotics undergo oxidation-reduction reactions which leads to red cell destruction and hemolytic anemia. Interaction between the xenobiotic and hemoglobin is of prime importance in the process, which is usually characterized by hemoglobin oxidation to methemoglobin, and formation within red cells of Heinz bodies, which represent inclusions of denatured and precipitated hemoglobin called hemichromes. Hemichromes cross-link the major erythrocyte membrane-spanning protein, band 3, into clusters. These clusters provide the recognition site for antibodies directed against senescent cells. These antibodies bind to the red cell and trigger its removal from circulation (Low et al., 1985). The denaturation of hemoglobin to hemichromes, and subsequent destruction of such cells has been conclusively proven with the oxidant drug phenylhydrazine (French et al., 1978, Low et al., 1985, Naughton et al., 1990). Erythrocytes incubated with phenylhydrazine (up to 6 hours) showed no detectable hemolysis (Dornfest et al., 1983) and experiments performed in vivo demonstrated that erythrocytes although not directly lysed by phenylhydrazine, are rapidly removed from the circulation by macrophages after exposure to this drug (Azen and Schilling, 1963, 1964; Jacob and Jandl, 1962). While a great deal of attention has been focused on the methemoglobinemia associated with these compounds, especially with nitrobenzene and to

a lesser extent with 1,3-DNB a mechanistic approach to characterize the hemolytic anemia has never been undertaken.

REPRODUCTIVE EFFECTS

Testicular Toxicity

Nitroaromatic compounds constitute an important class of chemicals known to produce testicular damage in experimental animals. Members of this series which possess such toxicity include, nitrobenzene (Bond et al., 1981), dinitrobenzene (Linder et al., Hess et al), nitrotoluenes (Ciss et al., 1980), dinitrotoluenes (Ellis et al., 1978; Rickert et al., 1984) and trinitrotoluenes (Levine et al., 1984).

Bond et al (1981) described degenerative changes in the germinal epithelium of rats exposed to a single oral dose of 300 mg/kg of nitrobenzene. Similar toxic effects have been observed in rats and mice exposed to nitrobenzene by inhalation (Medinsky and Irons, 1985). Reduced testis weight and testicular atrophy was observed in rats fed TNT in the diet for 13 weeks and the testicular atrophy was not reversible after a 4 week recovery period (Dilley et al., 1982). Similarly testicular atrophy with degeneration of the seminiferous tubular epithelium was observed in rats administered 125 and 300 mg/kg/day of TNT for 13 weeks (Levine et al., 1984).

Among the nitroaromatics, the testicular effects of 1,3-DNB have been the subject of numerous and in-depth investigations in rats and mice. Reproductive toxicity in the form of reduced testis and epididymis weight was consistently observed in rats exposed to a single oral dose (ranging from 32-50 mg/kg) of 1,3-DNB (Blackburn et al. 1988; Linder et al., 1988; Rehnberg et al., 1988). Cody et al. (1981) reported testicular atrophy in the rat after subchronic exposure to m-DNB via the drinking water. In a reproduction study, subchronic gavage exposure (5 days/week) resulted in interruption of spermatogenesis and infertility at a dosage level of 3 mg/kg/day and decreased sperm production at 1.5

mg/kg/day (Linder et al. 1986). A single oral dose of 1,3-DNB can also produce devastating effects on male reproductive system of the rat. In an extensive study of the acute effects and long term sequelae (Linder et al., 1988; Hess et al., 1988) a single oral dose of 1,3-DNB (48mg/kg) produced marked effects on pachytene spermatocytes and older germ cell types within 24 hours. Epididymal sperm quality was diminished by 16 days and fertilizing ability was lost by 5 weeks.

Detailed histological and ultrastructural investigations indicated that the somatic Sertoli cell was the initial target site within the testis for 1,3-DNB toxicity (Foster et al., 1986; Blackburn et al., 1988). These cells showed extensive vacuolation within 24 hours. An initial lesion to this cell, produced by 1,3-DNB, resulted in a complete disruption of the spermatogenic process at later times (Blackburn et al. 1988). The utilization of primary Sertoli-germ cell co-cultures (Foster et al., 1987a) indicated that direct addition of the m-DNB produced an analogous morphological response to that encountered in vivo (i.e. Sertoli cell vacuolation, germ cell exfoliation, and phagocytic vacuoles containing degenerate spermatocytes). The testicular toxicity of dinitrobenzenes is isomer specific. Initial studies indicated that a single oral dose (50 mg/kg) of 1,2-, 1,3-, 1,4-dinitrobenzenes resulted in decreased testicular weight and histopathological changes to the testis only in animals treated with the 1,3 isomer (*m*-DNB) (Blackburn et al., 1988). These findings of an isomer specificity for testicular toxicity with dinitrobenzenes suggests a different mechanism may be involved with TNB. The U. S. Health and Human Services(USDHHS, 1993) and the U. S. Environmental Protection Agency (U. S. EPA, 1989) report no studies regarding reproductive effects in animals after exposure to TNB. The reproductive effects of TNB are, limited to a few brief reports(Kinkead et al., 1994, 1995; Reddy et al., 1993, 1994).

Reversibility of Testicular Toxicity

The reversibility of nitroaromatic compound induced testicular toxicity is varied depending on the compound. The ability of the testis to recover from the toxic insult has been reported by other investigators for nitrobenzene and other nitroaromatic compounds. Bond et al (1981) proposed that testicular changes are reversible in rats receiving single oral doses of nitrobenzene. Later, Levin et al (1988) reported substantial recovery (>90% regeneration of seminiferous epithelium) by 100 days after a single oral dose of nitrobenzene. In a rat fertility study, a five fold increase in fertility index was reported after 9 weeks of recovery from inhalation exposure to nitrobenzene, but reversibility was not studied histologically (Dodd et al., 1987). Rats administered 6 mg/kg of 1,3-DNB (5d/wk for 12 weeks) followed by a 5 month recovery period had partially reversible testicular effects (Linder et al., 1986). Later studies by Linder et al (1988) reported normal fertilizing ability by 13 weeks after a single oral dose (48 mg/kg). In an extensive study of the acute effects and long term sequelae (Linder et al., 1988; Hess et al., 1988) a single oral dose of 1,3-DNB (48 mg/kg) diminished epididymal sperm quality by 16 days and loss of fertilizing ability by 5 weeks. Normal fertilizing ability was recovered in most animals by 5 months post-treatment, in a few animals the effects were not readily reversible, suggesting permanent reproductive damage (Linder et al., 1990). The testicular effects of TNT are not reversible in rats allowed 4 weeks of recovery after exposure to TNT for 13 weeks (Dilley et al., 1982). There is no information on the reversibility of the testicular toxicity after cessation of treatment with TNB.

NEUROLOGICAL EFFECTS:

CNS Effects

Aromatic and heterocyclic nitro compounds cause a variety of toxic effects. Of particular importance is the neurotoxicity associated with these heterocyclic compounds.

Neurotoxic effects have been reported as a consequence of human nitrobenzene exposure since the early 1900's (Hamilton, 1919; Donovan , 1920) Likewise, neurotoxicity has been associated with experimental nitrobenzene poisoning in laboratory animals. Bond et al (1981) and Morgan et al (1985) reported petechial hemorrhages in the brain stem, bilateral malacia and reactive gliosis in the cerebellar peduncles of rats treated with nitrobenzene.

Similarly the dinitrobenzenes, specifically 1,3-DNB (m-DNB) is also implicated as a neurotoxin. Physical signs of neurotoxicity following exposure to 1,3-DNB are manifested as slow movement, loss of equilibrium or ataxia, flaccid paralysis of fore limbs and splaying of hind limbs (Linder et al., 1988; Philbert et al., 1987a, b). Morphologic (microscopic) examination revealed bilaterally symmetrical vacuolated lesions involving cerebellar roof, vestibular and superior olivary nuclei and the inferior colliculi (Philbert et al., 1987a, b). Frequent petechial hemorrhages, with erythrocytes usually being limited to enlarged Virchow-Robin spaces were also reported. Later studies by Ray et al (1992) implicated altered auditory function, with its associated metabolic consequence exercising a significant role in the development of brain stem damage in auditory pathways following 1,3-dinitrobenzene intoxication.

Neurotoxic signs are also reported with the explosive 2,4,6-trinitrotoluene (TNT). Dogs receiving 20 mg/kg/day were inactive (Dilley et al., 1982) and were ataxic when treated at 32 mg/kg/day for 6 months (Levine et al., 1990). Fischer-344 rats receiving 300 mg/kg/day of 2,4,6-trinitrotoluene for 13 weeks had brain lesions which consisted of focal vacuolation and malacia (Levine et al., 1984).

While much has been reported with nitrobenzene, dinitrobenzene (1,3-DNB) and 2,4,6-trinitrotoluene (TNT), information on the neurological effects of TNB are confined to abstracts (Chandra et al., 1994; Kinkead et al., 1994). Physical signs of neurotoxicity reported for TNB include head tilt, loss of equilibrium (Kinkead et al., 1994), walking on toes, hunched back and knuckling of feet (Chandra et al., 1994). Petechial hemorrhages in

the brain stem and bilaterally symmetrical necrosis in the cerebellar peduncles were observed histologically (Chandra et al., 1994).

Etiopathogenesis and Reversibility

The changes observed with TNB, had topographical similarities to those produced by 1,3-dinitrobenzene (Philbert et al., 1987a), nitrobenzene (Morgan et al., 1985), and pyrithiamine (Watanabe, 1978). It has been suggested with 1,3-DNB (Romero et al., 1991), nitrobenzene (Morgan et al., 1985) and pyrithiamine (Watanabe et al., 1981), that the earliest changes probably occur in endothelial/glial cells, neuronal alterations being secondary to the endothelial/glial damage.

The nitroaromatic compound (DNB, NB, TNB) induced encephalopathy is accompanied by widespread vacuolation and necrosis of the neuropil in white matter of the brain. Vacuolation of the white matter (spongy degeneration) has been attributed to both pathological processes and to artifacts of fixation, such as perfusion with hyperosmolar fixatives (Schultz and Karlsson, 1965). In pathological conditions, the vacuolation has been identified ultrastructurally as swollen astrocytic processes, distended extracellular spaces, vacuolated oligodendrocytes, dilated axons, swollen neuronal dendrites and demyelination (Lampert and Schochet, 1968a, b; Tanaka et al., 1977). The vacuolation of the neuropil and the Virchow-Robin spaces expanded with erythrocytes indicates brain edema due to displacement of the denser neuropil components by water (Bothe, Bosch and Hossmann, 1984).

Prior studies with the structurally analogous nitroaromatics DNB or nitrobenzene have not addressed the contribution of blood brain barrier (BBB) breakdown (vasogenic edema) in the evolution of the tissue changes. At present little is known about how vascular changes with TNB lead to brain damage. Increased permeability of the blood brain barrier (BBB) leading to brain damage has been implicated in a variety of unrelated conditions including hypertension (Sokrab et al., 1988), *Clostridium spp* toxin (Finnie and

Hajduk, 1992), infusions of hyperosmolar solutions (Salahuddin et al., 1988), cerebral ischemia and hypoxia (Loberg et al., 1993), infarction (Nordborg et al., 1991) and cryogenic brain lesions (Loberg et al., 1992; Loberg and Torvik 1991). Increased permeability leads to exudation of plasma proteins into the parenchyma and uptake of these proteins causes permanent nerve cell injury (Nordborg et al., 1991; Salahuddin et al., 1988; Sokrab et al., 1988). The extravasation of serum albumin into the neuropil and its uptake by neurons and glial cells has been demonstrated immunohistochemically (Salahuddin et al., 1988; Loberg et al., 1993, 1992). Demonstration of the intimate relationship between vascular disturbance (breakdown of BBB) and secondary tissue damage, would elucidate a possible mechanism for the brain damage.

Astrocytes play a role in the induction and maintenance of the BBB (Janzer and Raff, 1987), homeostasis of water and ion balances (Kimelberg, 1983; Hertz and Schousboe, 1975) and repair after tissue damage (Montogomery, 1994). Astrocytic response to brain damage is evaluated by immunohistochemical staining for the intermediate filament glial fibrillary acidic protein (GFAP) (Norenberg et al., 1994). A good correlation exists between the production of edema and astrocytic response as judged by GFAP immunoreactivity (Schmidt-Kastner et al., 1990). Astrogliosis is the hallmark of injured brain tissue leading to the formation of a glial scar (Montogomery, 1994). Glial scar would act to seal off the injured tissue from the adjacent normal brain (formation of a new glia limitans).

CONCLUSIONS

Nitroaromatic compounds have long been used as intermediates in the preparation of a great number of substances in the chemical industry as well in the production of high explosives. The mutagenic and carcinogenic potential of this class of compounds has brought about an increased interest in the risks and hazards that nitroaromatic compounds

present to human populations and their environment. The nitroheterocyclic compound TNB has been detected as an environmental contaminant at military installations, test grounds and production waste sites. Current information concerning the effects of TNB are based on assumptions of generality that structurally similar compounds behave alike and the toxicity information is derived by analogy to similar nitroaromatics. The incidence of disease end points and health effects criteria in humans resulting from low-dose exposure to TNB is unknown. Attainment of this goal is difficult in large part because extrapolation based on experimental animal studies and detailed mechanisms of action is lacking.

This study was designed to evaluate the target organs for toxicity in rats orally exposed to TNB. Emphasis was largely placed on the three triads, hematological, neurological and reproductive effects, since nitroaromatic compounds consistently affect these systems. An approach to elucidate a possible mechanism and reversibility of the observed toxic effects has been attempted.

REFERENCES

Azen EA and Schilling RF (1963). Role of spleen in acetylphenyhydrazine (APH) anemia in rats. J Lab Clin Med 62:59-66

Azen EA and Schilling RF (1964) Extravascular destruction of acetylphenyldrazine damaged erythrocytes in the rat. J Lab Clin Med 63:122-128

Bond JA, Chism JA, Rickert DE, and Popp JA (1981) Induction of hepatic and testicular lesions in Fischer-344 rats by single oral dose of nitrobenzene. Fundam Applied Toxicol 1:389-394

Bothe HW, BoschW and HossmannKA (1984) Relationship between specific gravity, water content and serum protein extravasation in various types of vasogenic edema. Acta Neuropathol 64:37-42

Blackburn DM, Gray AJ, Lloyd SC, Sheard CM, and Foster PMD (1988) A comparison of the effects of the three isomers of dinitrobenzene on the testis in the rat. Toxicol Appl Pharmacol 63:120-132

Budavari S, O' Neil M.J, and Smith A (1989) In: *The Merck Index*. Merck & Comapny, Inc., Rahway, NJ, pp. 1530

Chandra AMS, Qualls CW Jr. and Reddy G (1994) 1,3,5-Trinitrobenzene induced encephalopathy in male Fischer-344 rats. Am Coll Toxicol, P12

Ciss M, Hwyen N, Dutertre H, Phu-Lich N, Truhart R (1980) Etude toxiciologique des nitrotoluenes: toxicite a long term. Dakar Medical 25:293-302

Cody TE, Witherup S, Hastings L, Stemmer K, Christian RT (1981) 1,3-dinitrobenzene: Toxic effects *in vivo* and *in vitro*. J Toxicol Environ Health 7:829-847

Cossum PA and Rickert DE (1987) Metabolism and toxicity of dinitrobenzene isomers in erythrocytes from Fischer-344 rats, rhesus monkeys and humans. Toxicol Lett 37:157-163

Dilley JV, Tyson CA., Spanggord RJ, Sasmore DP, Newell GW, and Dacre JC (1982) Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats and dogs. J Toxicol Environ Health 9: 565-585

Dodd DE, Fowler EH, Snellings WM, Pritts IM, Tyl RW, Lyon JP, O'Neal FO and Kimmerle G (1987) Reproduction and fertility evaluations in CD rats following nitrobenzene inhalation. Fundam Appl Toxicol. 8:493-499

Donovan WM. The toxicity of nitrobenzene with report of a fatal case (1920) JAMA 74:1647-1650

Dornfest BS, Naughton BA, Johnson R, and Gordon AS (1983) Hepatic production of erythropoietin in a phenylhydrazine-induced compensated hemolytic state in the rat. J Lab Clin Med 102: 274

Dreshbach M, and Chandler WL (1918) The toxic action of nitrobenzene, with special reference to the cerebellum. Proc Soc Exp Biol Med 15:136-137

FacchiniV and Griffiths LA (1981) The involvement of the gastrointestinal microflora in nitro-compound-induced methemoglobinemia in rats and its relationship to nitro group reduction. Biochem Pharmacol 30: 931-935

French JK, Winterbourn CC and Carrell RW (1978) Mechanism of oxyhemoglobin breakdown on reaction with acetylphenylhydrazine. Biochem J 173:19-26

Ellis HV, Hong CB, Dycre JC, Lee CC (1978) Chronic toxicity of 2,4-dinitrotoluene in the rat. Toxicol Appl Pharmacol 45:245-246

Fedoroff BT, Sheffield OE, Reese EF and Clift GD (1962) In: Encyclopedia of Explosives and Related Items, PATR 2700, Volume 2, Picatinny Arsenal, Dover, NJ, pp. B48-B49

Finnie JW and Hajduk P (1992). An immunohistochemical study of plasma albumin extravasation in the brain of mice after the administration of Clostridium perfringens type D epsilon toxin. Australian Veterinary Journal. 69: 261-262.

Fitzgerald GB, De Guilion N, Desai LS, and Reddy G (1992) Acute toxicity evalaution of 1,3,5-Trinitrobenzene. J Amer Coll Toxicol Part B. Acute Toxicity Data. 3:169-170

Fogleman RW, Elsea JR, Paynter OE, and Kundzins W (1955) Toxicity of trinitrobenzene-aniline complex, a rodent repellent. J Agri Food Chem 3:936-939

Foster PMD, Lloyd SC, and Prout MS (1987) Toxicity and metabolism of 1,3-dinitrobenzene in rat testicular cell cultures Toxicol in vitro 1:31-37

Foster PMD (1989) m-dinitrobenzene: Studies on its toxicity to the testicular sertoli cell. Arch Toxicol 13:3-17

Goldstein RS, and Rickert DE (1985) Relationship between red blood cell uptake and methemoglobin production by nitrobenzene and dinitrobenzene in vitro. Life Sci 36:121-125

Hathway JA (1985) Subclinical effects of trinitrotoluene: A review of epidemiology studies. In: Toxicity of Nitroaromatic Compounds, Rickert DE (ed), Hemisphere Publishing Corporation Washington, pp 255-273

Hertz L and Schousboe A (1975) Ion and energy metabolism of the brain at the cellular level. Int Rev Neurobiol 18:141-211

Hess RA, Linder RE, Strader LF, and Perreault SD (1988) Acute effects and long term sequelae of 1,3-Dinitrobenzene on male reproduction in the rat II. Quantitative and qualitative histopathology of the testis. J Androl 9:327-342

Hamblin DO (1949) Nitro and amino compounds of the aromatic series in Industrial Hygiene and Toxicology, (Ed. Patty FA) Vol II, Interscience Publ. New York

Hamilton A (1919) Industrial poisoning by compounds of the aromatic series. J Ind Hyg 1:200-209

Hartter DR (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Toxicity of Nitroaromatic Compounds, Rickert DE (ed), Hemisphere Publishing Corporation Washington, pp 1-13

Jacob HS and Jandl JH (1962) Effects of sulfydryl inhibition on red blood cells. II Studies in vitro. J Clin Invest. 41:1514-1518

Janzer RC and Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. Nature. 325:253-257

Kiese M (1949a) Pharmakologische Untersuchungen uber m-Dinitrobenzol. I Akute Vergiftung mit m-dinitrobenzol. Naun-Schmiedebergs Arch Exp Pathol Pharmakol. 206:361-383

Kiese M (1949b) Pharmakologische Untersuchungen uber m-Dinitrobenzol. III Chronische Vergiftung mit m-dinitrobenzol. Naun-Schmiedebergs Arch Exp Pathol Pharmakol. 206:505-527

Kimelberg HK, Bourke RS, Nelson LR, PoppAJ, Waldman JB, Barron KD, and Cragoe EJ (1984) Anti-edema treatment : Therapeutic approach. In: Bes A, Braquet P, Paoletti R, and Siesjo BK, (Eds) Cerebral Ischemia, Elsevier, New York, pp.343-346

Kinkead ER, Wolfe RE, Salins SA, Miller C, Latendresse JR (1994) Single-generation reproduction study of 1,3,5-trinitrobenzene in the diet of Sprague-Dawley rats. Toxicologist 14:161

Kinkead ER, Wolfe RE, Flemming CD, Caldwell DJ, Miller CR, and Marit GB (1995) Reproductive toxicity screen of 1,3,5-trinitrobenzene administerted in the diet of Sprague-Dawley rats. Toxicologist 15:249

Korolev AA, Voitesekhovskaia T, Bogdanov MV, Arseneva MV, and Azkharova TA (1977) Experimental data on the hygenic standardization of dinitrotoluene and trinitrobenzol in the water reservoirs. Gig Sanit 10:17-20 (Russian)

Lampert PW and Schochet SS (1968a). Electron microscopic observations on experimental spongy degeneration of the cerebellar white matter. J Neuropath Exp Neurol 27:210-220

Lampert PW and Schochet SS (1968b) Demyelination and remyelination in lead neuropathy. Electron microscopic studies. J Neuropath Exp Neurol, 27:527-545

Levin AA and Dent JG (1982) Comparison of the metabolism of nitrobenzene by hepatic microsomes and cecal microflora from Fischer-344 rats in vitro and the relative importance of each in vivo. Drug Metab Disp 10:450-454

Levine BS, Furedi EM, Gordon DE, Lish PM, and Barkley JJ (1984) Subchronic toxicity of trinitrotoluene in Fischer 344 rats. Toxicology 32:253-265

Levin AA, Bosakowski T, Earle LL, Butterworth BE (1988) The reversibility of nitrobenzene-induced testicular toxicity: Continuous monitoring of sperm output from vasocystotomized rats. Toxicology 53:219-230

Levine BS, Rust JH, Barkley JJ, Furedi EM, and Lish PM (1990) Six month oral toxicity study of trinitrotoluene in beagle dogs. Toxicology 63:233-244

Linder RE, Strader LF, Barbee RR, Rehnberg GA, Perreault SD (1990) Reproductive toxicity of a single oral dose of 1,3 -Dinitrobenzene in two ages of young adult male rats. Fund Appl Toxicol 14:284-298

Linder RE, Hess RA, Perreault SD, Strader LF and Barbee RE (1988) Acute effects and long term sequelae of 1,3-Dinitrobenzene on male reproduction in the rat II. Sperm quality, quantity and fertilizing ability. J Androl 9:317-326

Linder RE, Hess RA, Strader LF (1986) Testicular toxicity and infertility in male rats treated with 1,3-dinitrobenzene. J Toxicol and Environ Health 19:477-489

Loberg EM and Torvik A (1991) Uptake of plasma proteins into damaged neurons. An experimental study on cryogenic lesions in rats. Acta Neuropathol 81:479-485

Loberg EM, Brorson SH, Skjorten F, and Torvik (1992) Neuronal uptake of plasma proteins in cryogenic brain lesions. An immunelectron microscopic study. APMIS 100:1033-1040

Loberg EM, Karlsson BR, and Torvik A (1993) Neuronal uptake of plasma proteins after transient cerebral ischemia/hypoxia. Immunohistochemical studies on experimental animals and human brains. APMIS 101:777-783

Low PS, Waugh SM, Zinke K and Drenckhahn D (1985) The role of hemoglobin denaturation and band 3 clustering in red blood cell agin. Science 227:531-533

Minot GR (1919) Blood examinations of trinitrotoluene workers. J Ind Hyg Tox 1:301-318

Morgan KT, Gross EA, Lyght O and Bond JA (1985) Morphologic and biochemical studies of a nitrobenzene-induced encephalopathy in rats. Neurotoxicology 6:105-116

Medinsky MA and Irons RD (1985) Sex, strain, and species differences in the response of rodents to nitrobenzene vapors. In: Toxicity of Nitroaromatic Compounds, Rickert DE (ed), Hemisphere Publishing Corporation Washington, pp 35-51

Naughton BA, Dornfest BS, Bush ME, Carlson CA, and Lapin DM (1990) Immune activation is associated with phenylhydrazine-induced anemia in the rat. J Lab Clin Med 115:498-507

Nordborg C, Sokrab TEO, and Johansson BB (1991). The relationship between plasma protein extravasation and remote tissue changes after experimental brain infarction. Acta Neuropathol 82:118-126

Parkes WE, and Neill DW (1953) Acute nitrobenzene poisoning with transient aminoaciduria. Brit Med J 1:653-655

Philbert MA, Nolan CC, Cremer JE, Tucker D and Brown AW (1987a) 1,3-Dinitrobenzene-induced encephalopathy in rats. Neuropath Appl Neurobiol 13:371-389

Philbert MA, Gray AJ, and Connors TA (1987b) Preliminary investigations into the involvement of the intestinal microflora in CNS toxicity induced by 1,3- Dinitrobenzene in male F-344 rats. Toxicol Lett 38:307-314

Qualls CW Jr., Reddy G, Gunnarson AE, and Rubble DL (1993) Hematologic effects of orally administered 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 13:424

Ray DE, Brown AW, Cavanagh JB, Nolan CC, Richards HK and Wylie SP (1992) Functional/Metabolic modulation of brain stem lesions caused by 1,3-dinitrobenzene in the rat. Neurotoxicology 13:379-388

Reddy TV, Daniel FB, Robinson M, Olson GR, Weichman B, and Reddy G (1993) Fourteen day toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 13:177

Reddy TV, Torsella J, Daniel FB, Olson GR Weichman B, and Reddy G (1994) Subchronic toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 14:117

Reddy BG, Pohl LR, and Krishna G (1976) The requirement of the gut flora in nitrobenzene-induced methemoglobinema in rats. Biochem Pharmacol 25:1119-1122

Rehnberg GL, Linder RE, Goldman JM, Hein JF, McElroy KW, and Cooper RL (1988) Changes in testicular and serum hormone concentrations in male rat following treatment with m-dinitrobenzene. Toxicol Appl Pharmacol 95:255-264

Rickert DE (1987) Metabolism of nitroaromatic compounds. Drug Metb Rev 18:23-53

Rosenblatt DH, Burrows EP, Mitchell WR, and Parmer DL (1991) Organic explosives and related compounds. In: The Handbook of Environmental Chemistry, Hutzinger (Ed) Vol 3 Part G, Springer-Verlag Berlin, pp 195-226

Ryon MG, Pal BC, Talmage SS and Ross RH (1984) Database assessment of the health and environmental effects of munition production waste sites. Final report. ORNL-6018. (NTIS DE84-016512). Oak Ridge National Laboratory, Oak Ridge, TN

Rickert DE, Butterworth BE, Popp JA (1984) Dinitrotoluene: Acute toxicity, oncogenicity, genotoxicity and metabolism. Crit Rev Toxicol 13: 217-234

Salahuddin TS, Kalimo H, Johansson BB and Olsson Y. (1988) Observations on exudation of fibronectin, fibrinogen and albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat indicating longlasting changes in the brain microenvironment and multifocal nerve cell injuries. Acta Neuropathol 76:1-10

Sax NI and Lewis RJ (Eds) (1987) In: Hawley's Condensed Chemical Dictionary , Eleventh Ed., Van Nostrand Reinhold, New York, NY, pp 1190

Sax NI and Lewis RJ (Eds) (1989) In: Dangerous Properties of Industrial Materials, Seventh Ed., Volume III, Van Nostrand Reinhold, New York, NY, pp 3403

Schultz RL and Karlsson U (1965) Fixation of the central nervous system for electron microscopy by aldehyde perfusion. II Effect of osmolality, pH of perfusate, and fixative concentration. J Ultrastru Res 12:187-206

SenczukW, Jodynis J and Roga H (1976) Effect of the chemical structure of some aromatic compounds on their methrmoglobin-inducing properties. Aromatic nitro compounds. Bromat Chem Toksykol 9:289-294 (Russian translated)

Shimkin MB (1939) Acute toxicity of mononitrobenzene in mice. Proc Soc Exp Biol Med 42:844-846

Sokrab TEO, Johansson BB, Kalimo H, and Olsson Y (1988) A transient hypertensive opening of the blood brain barrier can lead to brain damage. Extravasation of serum proteins and cellular changes in rats subjected to aortic compression. Acta Neuropathologica. 75: 557-565

Spalding RF and Fulton JW (1988) Groundwater munition residues and nitrate near Grand Island, Nebraska. J Contam Hydrol 2:139-153

Spanggord RJ, Mabey WR, Chou TW, and Smith JH (1985) Environmental fate of selected nitroaromatic compounds in the aquatic environment In: Toxicity of Nitroaromatic Compounds, Rickert DE (ed), Hemisphere Publishing Corporation Washington, pp 15-33

Spanggord RJ, Mill T, Chou TW, Mabey WR, Smith JH and Lee S (1982) Environmental fate studies on certain muitions wastewater constituents. Final Report, Phase I - Literature Review. SRI Project No. LSU-7934. Contract No. DAMD 17-78-C-8081. U.S. Army Medical Res and Devlop. Command, Fort Detrick, MD

Tanaka R, Tanimura K, and Ueki K (1977) Ultrastructural and bochemical studies on oubain-induced oedematous brain. Acta Neuropathol 37:95-100

U.S. Department of Health and Human Services (USDHHS) (1993) Public Health Service. Agency for Toxic Substances and Disease Registry. Toxicological Profile for 1,3-Dinitrobenzene and 1,3,5-Trinitrobenzene

U.S. EPA (1989) Health and Environmental Effects Profile for 1,3,5-trinitrobenzene. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Cincinnati, OH, ECAO-CIN-G071

Watanabe I (1978) Pyrithiamine-induced acute thiamine-deficient encephalopathy in the mouse. Exp Mol Pathol 28:381-394

Watanabe I, Iwasaki Y, Aikawa H, Satoyoshi E, and Davis JW (1981) Hemorrhage of thiamine deficient encephalopathy. J Neuropathol Exp Neurol 40:566-580, 1981

Watanabe T, Ishihara N, and Ikeda M (1976) Toxicity of and biological monitoring for 1,3 diamino-2,4,6 TNB and other nitro-amino derivatives of benzene and chlorobenzene. Intl Arch Occup Environ Health 37:157-168

Weast RC (Ed) (1989) In: CRC (Chemical Rubber Company) Handbook for Chemistry and Physics, 69th ed., Astle MJ and Beyer WH, Assoc. Ed., CRC Press, Inc., Boca Raton, FL., p. C-119

Wennersten R (1980) Extraction of organic pollutants from an effluent stream in the manufacture of p-nitrobenzoic acid. In: Proc Int Solvent Extr Conf Assoc, Ing Univ, Leige, Leige, Belgium 2:1-6

Windholz MS, Budavari RF, Blumetti RF, and Otterbein ES (1983) The Merck Index. Merck and Co., Inc., Rahway, NJ, p. 1389

CHAPTER II

HEMATOLOGICAL EFFECTS OF 1,3,5-TRINITROBENZENE (TNB) IN RATS *IN VIVO* AND *IN VITRO*

INTRODUCTION

1,3,5-Trinitrobenzene is a nitroaromatic compound detected as an environmental contaminant of surface water, ground water and soil. The waste waters discharged from 2,4,6-trinitrotoluene (TNT) manufacturing processes contain a large number of aromatic compounds, including TNB. TNB is found in aquatic systems as a byproduct of biotransformation and photolysis of TNT. TNB is not easily biodegradable, it persists in the environment and can eventually leach out and contaminate ground water near production waste disposal sites and military test grounds (Chudoba and Pitter, 1976; Garman et. al., 1987; Layton et. al., 1987). TNB is an anthropogenic environmental contaminant and exposure can occur through contact with waste water effluents released from facilities that synthesize, produce or demilitarize munitions or from the disposal of solid TNT wastes (Ryon et. al., 1984; US EPA 1989)

The U.S. Department of Health and Human Services (1993) reported finding no studies of the respiratory, hematologic, hepatic, neurologic, reproductive or systemic effects of TNB. Therefore most of the toxicity information derived for TNB is by analogy to the structurally similar compounds 1,3-dinitrobenzene (DNB) and 2,4,6-trinitrotoluene (TNT). Toxicity data on TNB are limited. TNB has been found to have an oral LD_{50} of 284 mg/kg for combined sexes in rats (Fitzgerald et. al., 1992). In a tersely reported study with TNB, toxic effects were reported to include formation of methemoglobin,

derangement of erythrocyte morphology and histopathologic changes in spleen and kidneys in dogs (Fogleman et al., 1955). Our recent acute studies showed that TNB at 71 mg/kg for 10 days produced encephalopathy in male F-344 rats (Chandra et al., 1995). In subchronic studies we have observed hematological and histopathological changes in the liver and testis (Reddy et. al., 1994). The purpose of this study is to evaluate the hematologic effects of TNB under different acute exposure periods. This experiment is one of a series of toxicological studies on TNB currently being conducted.

TNB is structurally related to nitrobenzene (NB), DNB and TNT, hence we hypothesized the mechanism of toxicity to be similar. A major component of toxicity with NB (Beauchamp et al., 1983; Bond et al., 1981) and DNB (Cody et al., 1981; Watanabe et al., 1976) and TNT (Dilley et al., 1982; Levine et al., 1984, 1990; Morton et al., 1976) is the development of anemia and methemoglobinemia. DNB is a potent inducer of methemoglobin (MHB) both *in vivo* and *in vitro* (Cossum and Rickert, 1987; Facchini and Griffiths, 1981; Goldstein and Rickert, 1985). On the contrary, NB requires metabolism by intestinal microflora before producing methemoglobin (Reddy et al., 1976). In our pilot experiments rats treated with TNB developed MHB, but it is not known whether metabolism by intestinal bacteria was involved. In order to determine whether TNB is an inducer of methemoglobin, experiments utilizing rat blood were conducted *in vitro.*

A second component of this study was to elucidate the mechanism of the hemolytic anemia which we observed with TNB, which is common to a number of other nitroaromatics. The work of Rachmilewitz and co-workers (1969, 1971, 1974) clearly demonstrated that the hemichromes formed are common intermediate molecular species within the hemoglobin denaturation pathway. Hemichromes are low spin derivatives of ferrihemoglobin (high spin form, i.e. methemoglobin) with unique spectral properties in

the visible region as well as by electron spin resonance measurements. Since methemoglobin is the intermediate product before transition to hemichrome, we speculated that TNB is an inducer of hemichrome.

MATERIALS AND METHODS

In Vivo

TNB (99.83% purity) was obtained from Naval Surface Warfare Center (Silver Springs, MD) and the purity of the compound was confirmed by HPLC at U.S. Army Biomedical Research and Development Laboratory. TNB was mixed with corn oil in a Potter-Elvehjem tissue grinder to form a suspension. The TNB corn oil mixture was prepared daily just prior to dosing.

Male Fischer-344 rats after one week of acclimation (initial body weight 220 g) were used in all the experiments. The oral LD_{50} values for TNB in rats has been reported to be 284 mg/kg for combined sexes (Fitzgerald et al., 1992). The one-fourth LD_{50} value (71 mg/kg) and the one-eighth LD_{50} value (35.5 mg/kg) were selected for these studies. The rats were randomly assigned by body weight to four exposure periods of 5 hours, 1 day, (single dose) or 4 days, and 10 days (multiple daily doses). Within each exposure period, four rats were assigned to each of three TNB dose groups of 0, 35.5, and 71 mg/kg. The rats were gavaged with TNB in corn oil by a feeding needle (1.35 ml/kg). Control rats received the same volume of corn oil. Rats had free access to water and standard commercial diet (Purina Rat Chow). Rats receiving daily oral doses of TNB for 4 and 10 days were fasted for 12 hours prior to euthanasia. At the end of each exposure period, rats were anaesthetized by CO_2 and blood was collected via cardiac puncture in EDTA tubes. Euthanasia was by the resulting exsanguination.

Hematologic evaluation included complete blood counts using a Coulter counter. Manual differential counts of 100 white blood cell were performed. Blood (wet mount) was stained with New Methylene Blue for the examination of Heinz bodies. Additionally an aliquot of whole blood (0.1 ml) was placed in 10 ml of chilled (4^{0}C) phosphate buffer (1/60M) with saponin for methemoglobin assay according to the method of Buetler and Gelbart (1990). Methemoglobin levels were assayed within 24 hours of blood collection (Sleight and Sinha, 1968). Serum was submitted for an automated chemistry profile (Kodak® Ektachem ® 500, Eastman Kodak, Rochester, NY). Assays included glucose, serum urea nitrogen, creatine, sodium, potassium, chloride, total CO_2, amylase, calcium, phosphorus, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine phosphokinase, alkaline phosphatase, gamma glutamyltransferase, bilirubin, cholesterol and triglyceride.

In vitro

Male Sprague-Dawley rats (300 g) were deeply anaesthetized with Metofane® (Pittman-Moore, Mandelein, IL) and blood was collected via cardiac puncture in heparinized tubes. The pooled blood samples were centrifuged at 2000 x G for 5 minutes to separate the plasma and the buffy coat. The erythrocytes were washed with 0.9% saline and suspended at the original hematocrit in phosphate buffered saline (110 mM sodium chloride, 20 mM disodium hydrogen phosphate and 4 mM potassium dihydrogen phosphate, with 10 mM glucose, pH 7.4) based on the method of Grossman and Jollow (1988). After preincubation for 5 minutes at 37^{0}C, 100 microliters of 1 mM TNB (dissolved in methanol) was added to 3 ml of the RBC suspension. Control tubes were incubated with methanol (100 microliters) alone. The blood tubes were incubated at 37^{0}C under air in a shaking incubator. Samples (0.1 ml) of the red cell suspension were removed at different intervals (upto 9 hours) for the assay of methemoglobin. Methemoglobin levels were measured as previously mentioned in the in vivo experiments.

At the end of the incubation period the samples were centrifuged and the absorbance of the supernatant was measured at 414 nm to detect hemolysis against erythrocytes lysed with distilled water (100 % hemolysis) based on the method of Breslin et al. (1991).

For spectrophotometric examination of the interaction of TNB with hemoglobin, 1 ml of 0.5% suspension of erythrocytes in phosphate buffered saline (pH 7.4) was incubated at 37°C for 3 hours with 100 microliters of sodium nitrite (1mM) or TNB (1mM) in a quartz cuvette. Spectral recordings were made at 0, 30, 60, 90, 120 and 180 minutes. The reaction (spectra) of the erythrocytes with the test chemicals was followed as described by Winterbourn (1990). This method makes it possible to distinguish hemoglobin, methemoglobin, hemichromes and choleglobin. Hemichromes are ferric hemoglobin derivatives in which the heme iron is coordinated either to the distal histidine or to an endogenous ligand, while the term choleglobin is used to describe denatured hemoglobin in which the porphyrin ring is hydroxylated or broken open. Choleglobin does not have any absorption spectra in the visible region and its formation is suggested by an increase in absorption at 700 nm. Sodium nitrite was used as a negative and positive control for hemichromes and methemoglobin respectively. All spectral measurements were performed on a Shimadzu MPS 2000 recording spectrophotometer with a built-in incubator (Shimadzu Corp. Japan).

Statistical differences were determined using PC-SAS (SAS Institute Inc. Cary, NC). All data were tested for homogeneity of variances prior to analysis of variances (Proc. GLM, SAS). When significant F-values were obtained, the method of Least Square Means (LSM) was used to determine significant differences between treatment means. P-values of ≤ 0.05 were considered to be significant, unless otherwise mentioned.

RESULTS

Clinical Signs

Rats which received 71 mg/kg of TNB exhibited depression, rapid breathing, pale ears and eyes and dark feet (cyanotic) within 30 minutes of dosing. The same clinical signs were exhibited an hour later by rats dosed with 35.5 mg/kg of TNB. The clinical signs lasted for approximately 6 hours after dosing. In addition, three rats (dosed at 71 mg/kg) in the ten day exposure period developed neurologic signs on days 5 to 7. Neurologic signs included walking on toes, hunched back, partial disuse of rear legs and knuckling of the feet (1 rat). In two of these rats, the signs disappeared by the eighth day. One rat was euthanatized on day 7 prior to the termination of the experiment due to the severity of the neurologic signs.

In Vivo

Hematological Parameters

There was no change in the hemogram at 5 hours and 24 hours after a single oral dose of TNB. A highly significant dose dependent anemia was observed in rats killed 24 hours after 4 and 10 daily doses of TNB at 35.5 and 71 mg/kg (Table 1). The anemia was characterized by a pronounced decrease in red cell numbers, hemoglobin, and hematocrit. Other red cell indices, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Crpuscular Hemoglobin Concentration (MCHC) were altered both in the 4 day and 10 day exposure periods, but did not correlate with the dose or exposure period. In the 4 day study, the MCHC was significantly elevated at both doses of TNB, while the MCH was significant only at the high dose. In the 10 day study, both the MCV and MCH, but not the MCHC, were significantly elevated over the corresponding controls. In the same exposure period (10 day), the reticulocyte count was statistically significant.

Other alterations in the 10-day study were a dose dependent thrombocytosis and elevated nucleated RBC's.

Blood samples collected 5 hours after a single dose of TNB had a dose dependent leukopenia (data not shown). There was an absolute decrease in all cell types, but lymphocytes were predominantly decreased. Differential leukocyte count revealed a significant lymphopenia. In the rats killed 24 hours (1 day) after a single dose or after 4 daily doses, a similar but less pronounced effect was observed and the leukopenia was significant only in the high dose group. On the contrary, a dose dependent leukocytosis ($P<0.07$) was present in the 10-day exposure periods. In all the exposure periods (5h or 24h after a single dose and 24 hours after 4 or 10 daily doses of TNB), the absolute and percentage counts of immature neutrophils (bands), monocytes, eosinophils and basophils were not significantly affected by treatment with TNB (data not shown). There was no evidence of Heinz body formation in blood smears examined from all four exposure periods.

Clinical Chemistry

A definitive trend in the serum chemistry values was not observed (Table 2). Elevated CO_2 and total bilirubin was observed at the low dose of TNB in the 5 hour study(data not shown). Rats killed 24 hours after a single oral dose had significantly decreased serum Ca^{++} at both dose levels of TNB (35.5 and 71 mg). The serum Ca^{++} was also decreased in rats receiving oral doses of 71 mg/kg of TNB for 4 days, but not in the 10-day exposure period. The serum triglyceride was the predominant chemistry parameter consistently affected by TNB in both the 4-day and 10-day studies. This effect was dose dependent. Other alterations included an elevated blood glucose and a decrease in BUN at 71 mg/kg of TNB for 10 days (data not shown).

Methemoglobin

Significant MHB was present only in rats killed 5 hours after a single dose of TNB (Table 3). The effect at this time showed a positve dose response. The mildly elevated methemoglobin observed 24 hours after a single dose was not significant ($P<0.06$). Rats receiving daily oral doses of TNB for 4 or 10 days had minimal methemoglobin 24 hours after the last dose.

In Vitro

When suspensions of red blood cells were incubated with sodium nitrite or TNB (1mM), the color of the solution changed to a light brown tint within 30 minutes. As hypothesized, there was a progressive increase in methemoglobin levels with time (Fig 1). The formation of methemoglobin in vitro was also confirmed by the spectroscopic changes with a peak absorption at 630 nm. At the end of the incubation period, erythrocytes incubated with sodium nitrite, control (methanol) and TNB had negligible hemolysis.

Figures 2 and 3 show the spectroscopic changes induced in hemoglobin by addition of sodium nitrite and TNB respectively in the visible region (500-700 nm). This range was chosen because it demonstrates all the oxidation products of hemoglobin. The hemoglobin absorption spectrum before the addition of TNB (0 time) has two absorption peaks at 540 nm and 577 nm. Progressively the hemoglobin absorption peak changes so that by 30 min. there is an increase in the absorption at 630 nm indicating the formation of methemoglobin, whereas the intensity of hemoglobin peaks at 540 nm and 577 nm have decreased. Repetitive scanning of the TNB spectra at 60 and 90 minutes of the reaction demonstrated two isosbestic points at 523 and 589 nm indicating the presence of two reaction products (hemoglobin and methemoglobin), which were also characteristic of the sodium nitrite spectra (Rachmilewitz, 1969, 1971). However, with time at 120 and 180

minutes the TNB spectra (Figure 3) no longer passed through the isosbestic points, indicating the presence of a third component (hemichrome). On the contrary, the sodium nitrite spectra (Figure 2) always passed through the isosbestic points (523 and 589 nm). In RBC's incubated with TNB, the absorption at the longer wavelength (577 nm) became less intense than the absorption at the shorter wavelength (540 nm), with a concomitant shallowing of the trough at 560 nm, a change that is characteristic of the development of a hemichrome. A similar pattern of hemoglobin oxidation spectra has been observed with other oxidant drugs (acetylphenylhydrazine - French et al., 1978; Peisach et al., 1975: menadione - Winterbourn et al., 1979; Phosphine - Chin et al., 1992; Potter et al., 1991). The absorption at 700 nm also marginally increased indicating the formation of choleglobin. An identical spectra to that of TNB was also obtained with acetylphenylhydrazine used as a positive control.

DISCUSSION

This initial experiment was designed to test the hypothesis that oral exposure to TNB will result in hematologic alterations, which have been reported with other nitroaromatics *viz.* nitrobenzene (Beauchamp et al., 1983; Shimkin, 1939; Hamblin, 1949; Parkes and Neill, 1953), dinitrobenzene (Cody et al., 1981; Watanabe et al., 1976) and TNT (Dilley et al., 1982; Hathway, 1977; Levine et al., 1984). Results reported here indicate that there are considerable similarities in the mechanism of toxicity of TNB with NB, DNB and TNT.

TNB caused a dose and time dependent anemia with reductions in hematocrit, hemoglobin and red cell count. The significant anemia was present in rats receiving TNB for 4 and 10 days, but not in rats killed 5 or 24 hours after a single dose. Similar results were also observed in rats fed diet containing TNB (50 to 100mg) for 14 days (Reddy et

al., 1994). Elevated reticulocyte count and increased circulating nucleated red cells were also present on day 10 in anemic animals. The increased reticulocyte count reflects accelerated erythroid production in the bone marrow in response to the anemia. Responding anemias are also commonly associated with increased numbers of circulating nucleated red cells; however bone marrow endothelial insult, possibly as a direct effect of the compound or a result of methemoglobin induced hypoxia, could also contribute to this finding. The significant methemoglobinemia produced by TNB could also have contributed to the accelerated bone marrow erythropoiesis, which is hypoxia driven

The splenomegaly observed at necropsy and lack of hemoglobinuria suggest hemolytic anemia of extravascular origin. TNB was a potent inducer of methemoglobin, but methemoglobin *per se* does not lead to hemolysis (Beutler, 1969). Results from the *in vitro* study confirm this hypothesis. There was no apparent hemolysis when erythrocytes were incubated with TNB *in vitro* even though there was significant production of methemoglobin. The hemolytic anemia observed with TNB is analogous to that reported with phenylhydrazine (PHZ). PHZ, like TNB, is a potent oxidant drug but does not cause hemolysis *in vitro* (Dornfest et. al., 1983). It has been reported that PHZ can cross link red cell band 3 protein (senescent antigen) resulting in the binding of autologus immunoglobulin G (IgG) and complement deposition. Recognition of this complex by macrophage Fc receptor mechanism triggers rapid erythrophagocytosis of the opsonized RBC's in the spleen and liver (Naughton et al., 1990). Results from the in vitro study reported here confirms this hypothesis.

The most prominent finding in the leukogram was present in the 5 hour study. There was a significant leukopenia, entirely due to a lymphopenia. The lymphopenia with a mature neutrophilia observed in the 5 hour study indicates a stress leukogram, even though there was an overall leukopenia, instead of a leukocytosis traditionally observed

with glucocorticoids. Stress induced lymphopenia is maximal in most species at around 4-8 hours and is resolved or diminished by 24 hours. In rats killed 24 hours after a single dose, the stress leukogram was significant only at 71 mg/kg of TNB. Rats treated with TNB for 4 days did not have significant changes in the leukogram. A non-significant dose-dependent leukocytosis was present in the 10 day study. This leukocytosis, with the concurrent anemia may be due to the stimulation of the immune system, as has been observed with PHZ (Dornfest et al., 1986; Naughton et al., 1990). A significant dose-dependent thrombocytosis was observed in the 10 day study. Reactive thrombocytosis commonly occurs secondary to splenic contraction in responding anemias, and inflammatory conditions since high numbers are sequestered in the spleen (Bithell, 1993).

Perhaps the most significant serum chemistry abnormality was the decrease in serum triglyceride observed both in the 4 day and 10 day study. This response is consistent and dose dependent. A decrease in food intake and altered hepatic metabolism might have contributed to decreases in triglyceride levels, even though body weight changes were minimal. Many hypolipidemic agents are known to cause peroxisomal proliferation in the liver and TNB is possibly a peroxisomal proliferator. A decrease in BUN was observed in the 10-day study. This is attributable to a metabolite of TNB having a diuretic effect (A similar result was obtained in pilot experiments with TNB). The mild decrease in the serum calcium observed in the 24-hour and 4-day studies is not clinically significant.

As hypothesized TNB was a potent inducer of MHB *in vivo*. The methemoglobin was markedly elevated in rats 5 hours after a single dose. The methemoglobin values 24 hours after a single dose (1 day) after 4 or 10 daily doses had returned to control levels. This is probably due to the high levels of the enzyme methemoglobin reductase present in rodents (Smith, 1991) and/or rapid metabolism of TNB.

Results of the *in vitro* study indicate that, unlike NB (Reddy et al., 1976), TNB does not require metabolism by intestinal bacteria to produce methemoglobin. There was a progressive increase in the methemoglobin levels with time when rat erythrocytes were incubated with TNB (Fig 1). Similar results have been reported by Watanabe et. al., (1976) when the hemolysate was incubated with TNB. At the end of the incubation, samples were centrifuged to detect the presence of hemolysis. An apparent lack of hemolysis in the samples treated *in vitro* treated further confirms that TNB is not directly hemolytic even with significant oxidative damage with high methemoglobin levels.

The interaction of erythrocytes with TNB led to a slow but progressive loss of hemoglobin with concomitant formation of a hemichrome. The increase in absorption at 630 nm (isosbestic points at 523 and 589) indicates that methemoglobin was the first product of the interaction and the hemichrome formed later. In TNB toxicity the methemoglobin reduction mechanism is overwhelmed because of the continuous and constant presence of the oxidant; therefore, instead of being reduced methemoglobin is slowly transformed into hemichromes. The methemoglobin was only a transient intermediate before the formation of hemichrome in erythrocytes incubated with TNB. There was no evidence of hemichrome formation with sodium nitrite.

Further, band 3 clustering, an event which triggers IgG opsonization (mentioned above), can be caused by hemichrome binding (Low, 1991). Thus hemichrome formation and band 3 clustering can trigger red cell removal. This correlation has been rigorously tested by Low and co-workers (Low et al., 1991). In ultrastructural studies, Rifkind (1965) showed that the site of recognition of phenylhydrazine-treated RBC's by macrophages is directly over the site where hemichromes cluster on the membrane. Thus, these *in vitro* studies all point to the denaturation of hemoglobin (formation of

hemichromes) by TNB which in turn leads to antigen clustering (band 3) and mediating red cell removal. The proposed sequence is schematically shown in Fig 4.

It has long been known that administration of nitrite to animals induces MHB but not hemolytic anemia (Beutler and Mikus, 1961). Even though nitrite can induce very high levels of methemoglobin, there is little evidence for hemoglobin denaturation leading to hemichrome formation (Winterbourn et al, 1985). Peisach et al., (1975) suggest that the presence of an aromatic nucleus of acetylphenylhydrazine (also TNB) endows this molecule with denaturing properties. Other hydrazines with a large bulk which lack the aromatic nucleus act only as oxido-reductants. Similar to acetylphenylhydrazine the mechanism of action of nitro compounds is one in which the unlike hemoglobin chains of the $\alpha 2\beta 2$ tetramers are separated during incubation. These isolated chains are unstable in the ferric state and spontaneously form hemichromes (Rachmilewitz et al., 1971).

Hemichromes are the main constituents of Heinz bodies, but there was no evidence of Heinz body formation in rats with TNB either *in vivo* or *in vitro* in our studies. A similar discrepancy was also observed with phosphine, where human erythrocytes developed Heinz bodies but not rat erythrocytes, even though both human and rat cells showed evidence of hemichrome formation (Potter et al , 1991 and Chin et al., 1992).

Similarly we observed the formation of hemichromes with DNB and TNT (1 mM) (data not shown). Based on these results it is reasonable to extrapolate the findings to other nitroaromatics like DNB and TNT where a significant hemolytic anemia is observed. In conclusion, our results indicate the blood to be a prime target for TNB toxicity. Detailed studies on the interaction of the hematopoietic system on the overall toxicity are required.

REFERENCES

Beauchamp RO Jr, Irons RD, Rickert DE, Couch DB, and Hamm TE Jr (1983) A critical review of the literature on nitrobenzene toxicity. CRC Crit Rev Toxicol 11:33-96

Beutler E (1969) Drug-induced hemolytic anemia. Pharmacol Rev 21:73-103

Beutler E, and Gelbart T (1990) Carboxyhemoglobin, methemoglobin and sulfhemoglobin determinations. In 'Hematology', 4th ed. William WJ(Ed.). McGraw-Hill. 1732-1734

Beutler E, and Mikus BJ (1961) The effect of sodium nitrite and paraaminopropriophenone administration on blood methemoglobin levels and red blood cell survival. Blood 18:455-476

Bithell TC (1993) 'Thrombocytosis' in Wintrobes's Clinical Hematology, 9th edition. Lee GR, et al., (Eds). Leaifebiger, Philadelphia

Bond JA, Chism JA, Rickert DE, and Popp JA (1981) Induction of hepatic and testicular lesions in Fischer-344 rats by single oral dose of nitrobenzene. Fundam Appl Toxicol 1:389-394

Breslin WJ, Phillips J E, Lomax G, Bartles M J, Dittenber DA, Calhoun LL, and Miller RR (1991) Hemolytic activity of ethylene glycol phenyl ether (EGPE) in rabbits. Fundam Appl Toxicol 17:466-481.

Chandra AMS, Qualls, CW Jr, and Reddy G (1995) 1,3,5-Trinitrobenzene induced encephalopathy in male Fischer 344 rats. Toxicol Pathol 23(4)

Chin KL, Mai X, Meaklim J, Scollary GR, and Leaver DD (1992) The interaction of phosphine with haemoglobin and erythrocytes. Xenobiotica 22:599-607

Chudoba J, and Pitter D (1976) Biological Purification of wastewaters form nitrobenzene production. Chem. Prum. 26:541-544 (Chem Abstr. 86: 95461p)

Cody TE, Witherup S, Hastings K, Stemmer K, and Christian RT (1981) 1,3-dinitrobenzene: Toxic effects in vivo and in vitro. J. Toxicol Environ. Health 7:829-847

Cossum PA, and Rickert DE (1987) Metabolism and toxicity of dinitrobenzene isomers in erythrocytes from Fischer-344 rats, rhesus monkeys and humans. Toxicol Lett 37:157-163.

Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, and Dacre JC (1982) Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats and dogs. J Toxicol Environ Health 9:565-585

Dornfest BS, Naughton BA, Johnson R, and Gordon AS (1983) Hepatic production of erythropoietin in a phenylhydrazine-induced compensated hemolytic state in the rat. J Lab Clin Med 102: 274-281

Dornfest BS, Lapin DM, Naughton BA, Adu S, Korn L, and Gordon AS (1986) Phenylhydrazine-induced leukocytosis in the rat. J Leu Biol 39:37-48

Facchini V, and Griffiths LA (1981) The involvement of the gastrointestinal microflora in nitro-compound-induced methemoglobinemia in rats and its relationship to nitro group reduction, Biochem Pharmacol 30:931-935

Fitzgerald GB, De Guilion N, Desai LS, and Reddy G (1992) Acute toxicity evalaution of 1,3,5-Trinitrobenzene. J Am Coll Toxicol Part B. Acute Toxicity Data. 3:169-170

Fogleman RW, Elsea JR, Paynter OE, and Kundzins W (1955) Toxicity of trinitrobenzene-aniline complex, a rodent repellent. J Agri Food Chem 3:936-939

French JK, Winterbourn CC, and Carrell RW (1978) Mechanism of oxyhemoglobin breakdown on reaction with acetylphenylhydrazine. Biochem J 173:19-26

Garman JR, Freund T, and Lawless EW (1987) Testing for ground water contamination at hazardous waste sites. J Chromat Sci 25:328-337

Goldstein RS, and Rickert DE (1985) Relationship between red blood cell uptake and methemoglobin production by nitrobenzene and dinitrobenzene in vitro. Life Sci 36:21-125

Grossman SJ, and Jollow DJ (1988) Role of dapsone hydroxylamines in dapsone-induced hemolytic hemolytic anemia. J Pharmacol Exp Ther 244:118-125

Hamblin DO (1949) Nitro and amino compounds of the aromatic series in Industrial Hygiene and Toxicology, Patty FA (Ed) Vol II, Interscience Publ. New York

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

Levine B S, Furedi EM, Gordon DE, Lish PM, and Barkley JJ (1984) Subchronic toxicity of trinitrotoluene in Fischer 344 rats. Toxicology 32:253-265

Levine BS, Rust JH, Barkley JJ, Furedi EM, and Lish PM (1990) Six month oral toxicity study of trinitrotoluene in beagle dogs. Toxicology 63:233-244

Layton D, Mallon B, Mitchell W, Hall L, Fish R, Perry L, Snyder G, Bogen K, Malloch W, Ham C, and Dowd P (1987) Data-base assessment of the health and environmental effects of conventional weapons demilitarization: Explosive and their co-contaminants.

Draft Report, Project order 83PP3818. Livermore, CA. Lawrence Livermore National Laboratory

Low PS, Waugh SM, Zinke K, and Drenckhahn D (1985) The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science 227:531-533

Morton AR, Ranadive MV, and Hathaway JA (1976) Biological effects of trinitrotoluene from exposure below the threshold limit value. Am Ind Hyg Assoc J 37: 56-60

Naughton BA, Dornfest BS, Bush ME, Carlson CA, and Lapin DM (1990) Immune activation is associated with phenylhydrazine-induced anemia in the rat. J Lab Clin Med 115:498-507

Parkes WE, and Neill DW (1953) Acute nitrobenzene poisoning with transient aminoaciduria. Brit Med J 1:653-655

Peisach J, Blumberg WE, and Rachmilewitz EA (1975) The demonstration of ferrihemochrome intermediates in Heinz body formation following the reduction of oxyhemoglobin A by acetylphenylhydrazine. Biochim Biophys Acta 393:404-418

Potter WT, Rong S, Griffith J, White J, and Garry VF (1991) Phosphine-mediated Heinz body formation and hemoglobin oxidation in human erythrocytes. Toxicol Lett 57:37-45

Rachmilewitz EA (1969) Formation of hemichromes from oxidized hemoglobin subunits. Ann N Y Acad Sci 165:171-184

Rachmilewitz EA (1974) Denaturation of the normal and abnormal hemoglobin molecule. Semin Haematol 11:441-462

Rachmilewitz EA, Peisach J, and Blumberg WE (1971) Studies on the stability of oxyhemoglobin A and its constituent chains and their dervatives. J Biol Chem 246:3356-3366

Reddy BG, Pohl LR, and Krishna G (1976) The requirement of the gut flora in nitrobenzene-induced methemoglobinema in rats. Biochem Pharmacol 25:1119-1122

Reddy TV, Daniel FB, Robinson M, Olson GR, Wiechman G, and Reddy G (1994) Fourteen day toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 13:177

Ryon MG, Pal BC, Talmage SS, and Ross RH (1984) Database assessment of the health and environmental effects of munition production waste sites. Final report. ORNL-6018. (NTIS DE84-016512). Oak Ridge National Laboratory, Oak Ridge, TN.

Shimkin MB (1939) Acute toxicity of mononitrobenzene in mice. Proc Soc Exp Biol Med 42:844-846

Sleight SD and Sinha DP (1968) Prevention of methemoglobin reduction in blood samples. J Am Vet Med Assoc 152:1521-1525

Smith RP (1991) Toxic response of the blood. In: Casarett and Doull's Toxicology, The basic science of poisons, Amdur MO, Doull J, Klaassen CD (Eds). Pergamon Press, New York

U.S. Department of Health and Human Services (1993). Toxicological Profile for 1,3-Dinitrobenzene and 1,3,5-Trinitrobenzene. Public Health Service. Agency for Toxic Substances and Disease Registry, Atlanta, Georgia

U. S. EPA. (1989) Health and environmental effects profile for 1,3,5-Trinitrobenzene. Environmental criteria and assessment office, Office of health and environmental assessment, Cincinnati, OH, ECAO-CIN-G071

Watanabe T, Ishigara N, and Ikeda M (1976) Toxicity and biological monitoring for 1,3-diamino-,2-4-6-trinitrobenzene and other nitroamino derivatives of benzene and chlorobenzene. Intl Arch Occup Environ Health 37:157-168

Winterbourn CC, French JK, and Claridge RF (1979) The reaction of menadione with hemoglobin. Mechanism and effect of superoxide dismutase. Biochem J 179:665-673

Winterbourn CC (1985) Free-radical production and oxidative reactions of hemoglobin. Environ Health Perspectives 64:321-330

Winterbourn CC (1990) Oxidative reactions of hemoglobin. Methods in enzymology. 186:265-272

Table 1: Effects of oral administration of TNB to male rats on hematology parameters

	Single dose, 5 hrs after			Single dose, 24 hrs after			4 daily doses, 24 hrs after			10 daily doses, 24 hrs after		
	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg
WBC (10^3)	8.8 (0.92)	6.3 (1.42)*	4.95 (1.05)**	8.63 (1.0)	7.58 (1.5)	5.5 (1.50)*	11 (1.67)	10.6 (2)	7.5 (2.6)	6.47 (2.11)	10.6 (2.9)	10.7 (1.3)
CWBC (10^3)	8.8 (0.5)	6.3 (1.1)*	4.9 (0.92)**	8.63 (1.15)	7.53 (0.8)	5.5 (1.50)*	10.3 (1.33)	10.5 (1.9)	7.3 (2.4)	7.66 (0.52)	9.5 (1.3)	8.5 (2.3)
RBC (10^6)	8.8 (0.3)	8.7 (0.2)	8.72 (0.32)	8.50 (0.24)	8.89 (0.2)	8.8 (0.1)	8.87 (0.27)	8.4 (0.1)*	8.1 (0.0)**	8.66 (0.45)	7.7 (0.19)**	6.8 (0.1)**
HGB (g/dl)	15.7 (0.5)	15.6 (0.5)	15.83 (0.61)	15.2 (0.37)	15.9 (0.3)	15.5 (0.3)	15.52 (0.32)	15 (0.2)*	14.7 (0.2)**	15.7 (0.96)	13.9 (0.21)*	13.5 (0.3)**
HCT (%)	44.2 (1.0)	43.4 (1.3)	43.98 (1.31)	43 (1.20)	45.03 (1.1)	44.3 (0.6)	45.73 (1.04)	42.8 (1.0)**	41.8 (0.4)**	44.25 (2.06)	39.7 (0.87)*	37.5 (0.6)**
MCV (fl)	50.4 (0.4)	49.7 (1)	50.48 (0.42)	50.6 (0.14)	50.65 (0.4)	50.3 (0.1)	51.55 (0.65)	51.1 (0.5)	51.5 (0.6)	51.05 (0.32)	51.4 (0.39)	55.2 (0.9)**
MCH (pg)	17.8 (0.1)	17.9 (0.3)	18.15 (0.11)	17.9 (0.08)	17.88 (0.1)	17.6 (0.1)	17.52 (0.24)	17.9 (0.1)	18.1 (0.2)	18.12 (0.32)	18 (0.49)	19.9 (0.4)**
MCHC (gm/dl)	35.4 (0.4)	35.9 (0.1)	35.98 (0.32)	35.33 (0.12)	35.35 (0.5)	35.1 (0.1)	33.97 (0.48)	35 (0.5)*	35.2 (0.1)**	35.5 (0.77)	34.9 (0.8)	36.1 (0.3)
PLT (10^3)	800 (52)	726 (58)	811 (31.57)	641 (177)	834.5 (31.9)	818 (49)	762.2 (98.6)	880 (73.5)	945 (0.0)	632.75 (136.75)	911.3 (35)*	968 (56)**
RETIC (%)	1.2 (0.7)	1.2 (0.5)	2.05 (1.13)	1.8 (1.09)	1.5 (0.6)	1.8 (0.8)	1.65 (0.72)	1.5 (0.4)	2.5 (0.6)	1.35 (0.58)	4.1 (1.73)*	4.5 (2.0)*

Data expressed as Mean (S.D.). Significantly different from control mean within same exposure period * $P<0.05$; ** $P<0.005$.

Table 2: Effects of oral administration of TNB to male rats on serum calcium and triglyceride levels.

	Single dose, 5 hrs after			Single dose, 24 hrs after			4 daily doses, 24 hrs			10 daily doses, 24 hrs		
	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg	0 mg/kg	35.5 mg/kg	71 mg/kg
Ca mg/dl	11.5 (0.5)	11.4 (0.2)	11.3 (0.25)	12.1 (0.32)	11.5 (0.3)*	11.5 (0.2)*	12.1 (0.49)	11.7 (0.2)	11.4 (0.2)*	11.35 (0.11)	11.4 (0.3)	11.7 (0.1)
Trig mg/dl	189 (108)	249 (58)	264 (98)	95 (16.4)	94.8 (11.6)	93 (13)	93.7 (15.3)	59.8 (14.8)*	51 (16)*	82.75 (11.29)	61 (3.9)*	48.3 (11.1)**

Data exprssed as Mean (S.D.). Significantly different from control mean within same exposure period *P<0.05; **P<0.005.

Table 3: Methemoglobin (as % total hemoglobin) in F-344 treated with TNB

	TNB mg/kg		
Duration of TNB exposure	**0**	**35.5**	**71**
Single dose, 5 hours after	0.51 (0.44)	20.56 (3.54)*	35.64 (10.6)**
Single dose, 24 hours after	0.75 (0.45)	0.32 (1.42)	1.39 (0.58)
4 daily doses, 24 hours after	0.54 (0.40)	0.40 (0.47)	1.14 (0.77)
10 daily doses, 24 hours after	0.93 (0.67)	0.91 (0.24)	1.61 (0.78)

Data expressed as Mean (S.D.). Significantly different from control mean within same exposure period *P<0.005; **P<0.0005

Figure 1. Methemoglobin (%) in rat blood incubated with TNB (1mM) in methanol. Each value represents the mean (SD) of 3 values. Control samples were incubated with methanol.

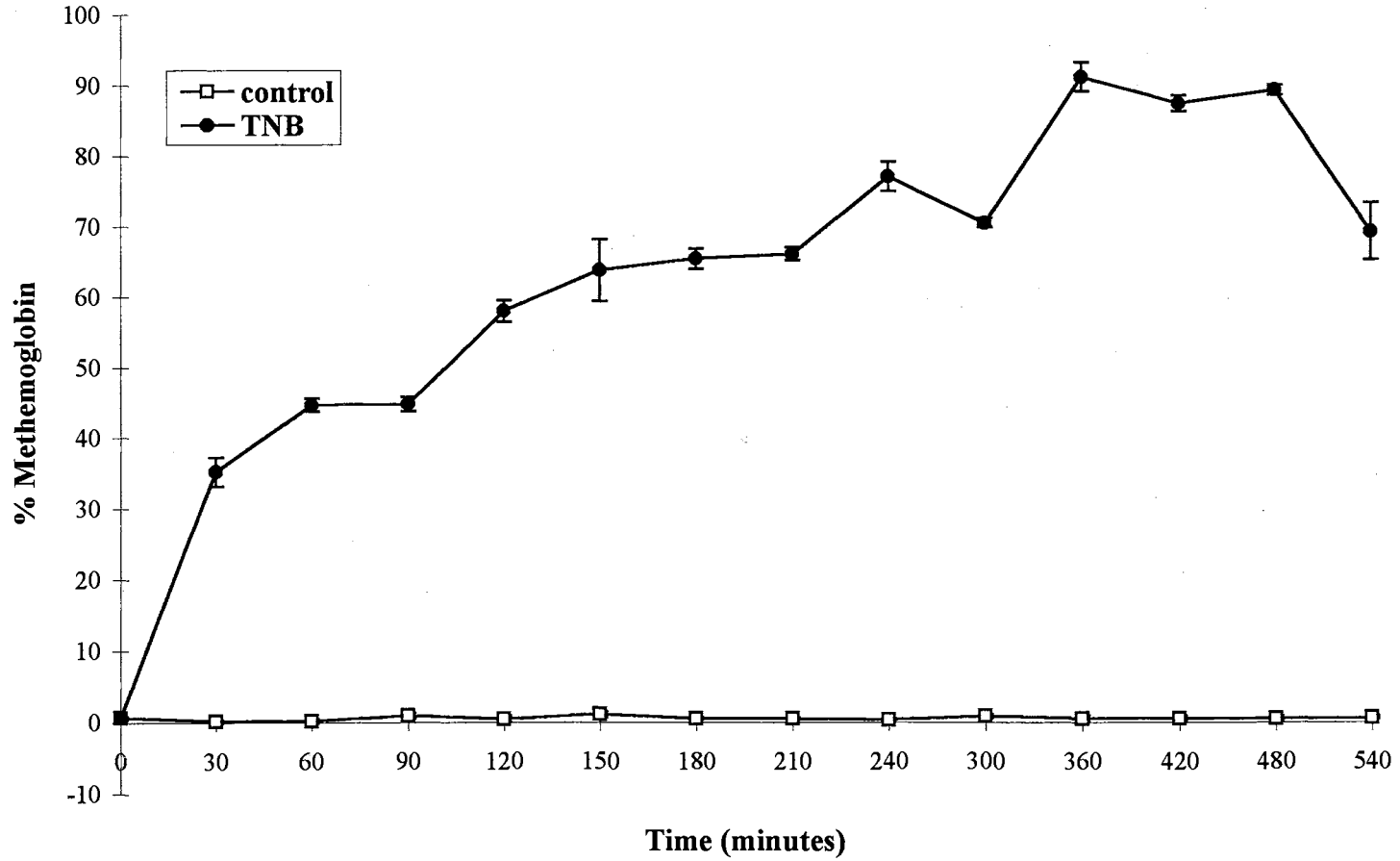

control
TNB
% Methemoglobin
Time (minutes)
100
90
80
70
60
50
40
30
20
10
0
-10
0
30
60
90
120
150
180
210
240
300
360
420
480
540

Figure 2. Progressive changes in the optical absorption spectra recorded during the reaction of erythrocytes (hemoglobin) with sodium nitrite (1mM) dissolved in methanol. The arrowheads indicate the isosbestic points at 523 and 589 nm. See text for explanation.

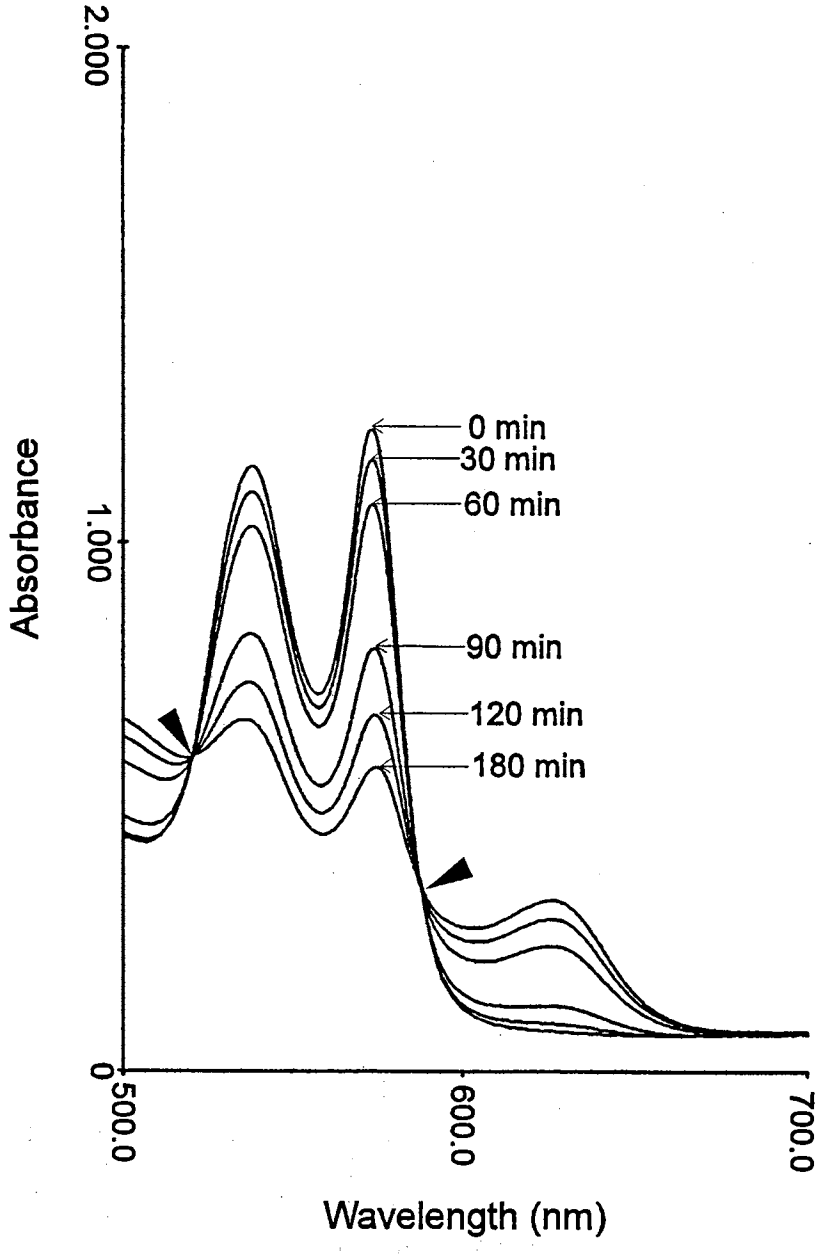
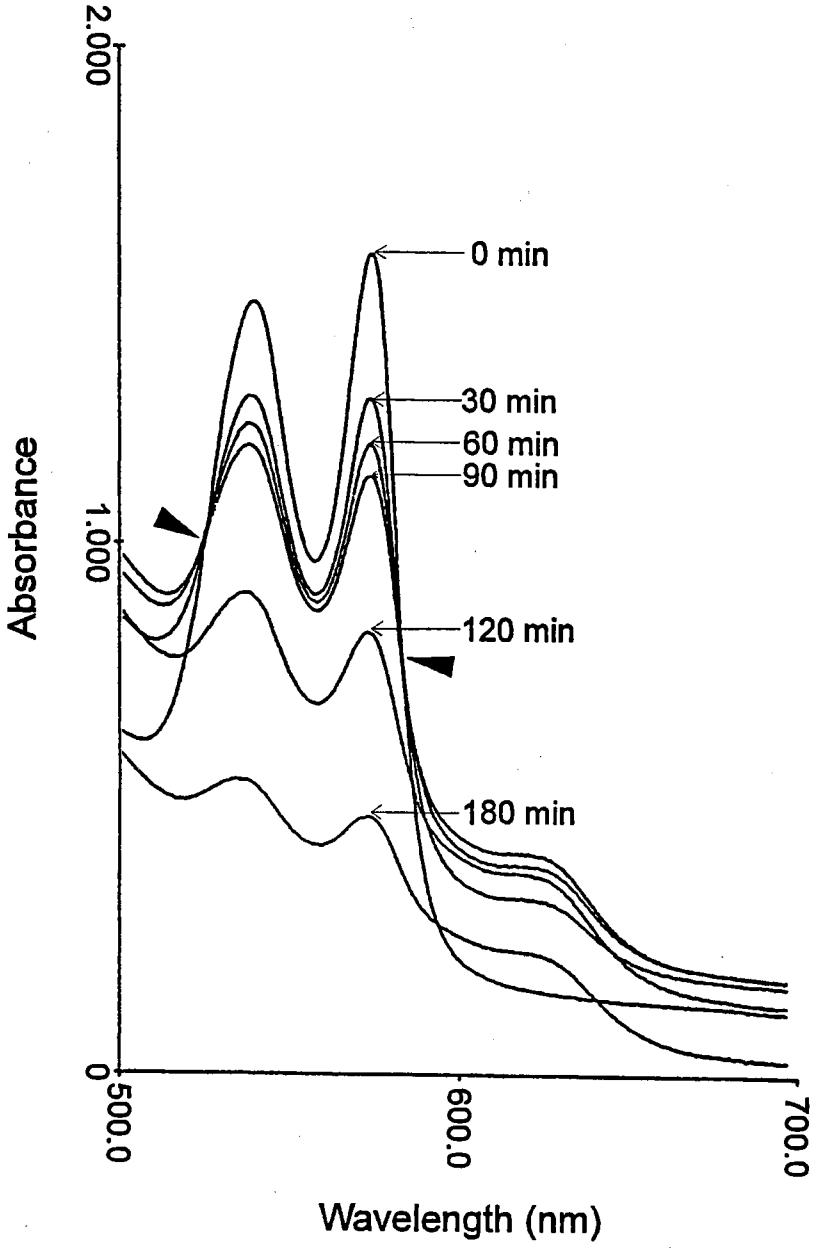

2.000
1.000
0
Absorbance
0 min
30 min
60 min
90 min
120 min
180 min
500.0
600.0
700.0
Wavelength (nm)

Figure 3. Progressive changes in the optical absorption spectra recorded during the reaction of erythrocytes (hemoglobin) with TNB (1mM) dissolved in methanol. The arrowheads indicate the isosbestic points at 523 and 589 nm. See text for explanation.


2.000
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700.0
Wavelength (nm)

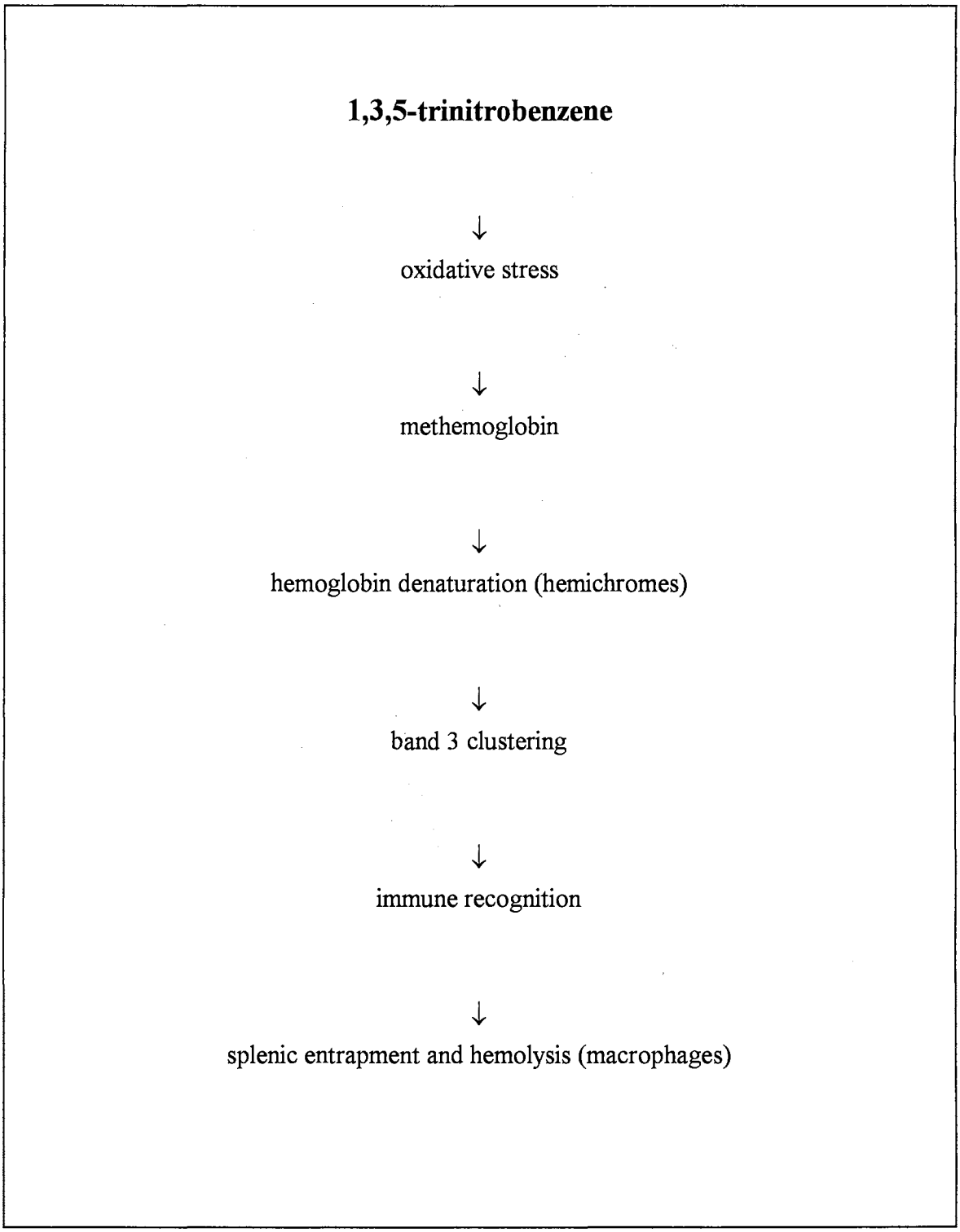


Fig 4. Hypothesis for the mechanism of TNB-induced hemolytic anemia

CHAPTER III

RAT TESTIS DURING EXPOSURE AND RECOVERY FROM 1,3,5-TRINITROBENZENE (TNB) INTOXICATION.

I. DOSE RESPONSE AND REVERSIBILITY STUDIES

INTRODUCTION

Nitroaromatic compounds constitute an important class of chemical intermediates known to produce testicular damage in experimental animals. Members of this series which possess such toxicity include, nitrobenzene (Bond et al., 1981; Levin et al., 1988), 1,3-dinitrobenzene (Linder et al., 1986, 1988, 1990; Hess et al., 1988; Cody et al., 1981), nitrotoluene (Ciss et. al., 1980), and dinitrotoluene (Ellis et. al., 1978; Rickert et. al., 1984).

Among the nitroaromatics the testicular toxicity of DNB has been the subject of several recent investigations in laboratory animals (Blackburn et. al., 1988; Ellis and Foster 1992; Foster et. al., 1987, 1989; Hess et. al., 1988; Linder et. al., 1986, 1988, 1990; Obasaju et al., 1991; Reader et. al., 1991). Effects of TNT on the testis, though not extensively investigated like DNB nevertheless have been documented (Levine et al., 1984, 1990; Dilley et al., 1982).

1,3,5-Trinitrobenzene (TNB) is a nitroaromatic compound, detected as an environmental contaminant of surface water, ground water and soil near trinitrotoluene (TNT) production sites and military test grounds. TNB has been classified as hazardous

waste by the EPA and it has been identified in 14 sites on the National Priorities List (NPL) (USDHHS, 1993; U. S. EPA, 1989). Exposure to TNB can occur through contact with wastewater effluents released from facilities that synthesize, produce or demilitarize munitions, or from the disposal of solid TNT wastes (Ryon et al., 1984; U.S. EPA, 1989). Due to limited studies with TNB, testicular toxicity information derived for TNB is by analogy to the structurally similar compounds 1,3-dinitrobenzene (DNB) and 2,4,6-trinitrotoluene (TNT).

Testicular toxicity of TNB is confined to a few tersely reported abstracts. Kinkead et al (1994) reported decreased testicular and epididymal weights, testicular degeneration and sperm depletion in Sprague-Dawley rats receiving 800 or 400 mg of TNB for 14 days. Sperm depletion and degeneration of the seminiferous tubules were observed histopathologically in similar experiments (Kinkead et al., 1995). Reddy et al (1993, 1994) reported decreased testicular weight and degeneration of the seminiferous tubules in rats receiving TNB in the diet for 14 or 90 days.

This report is a detailed histopathologic evaluation of the testicular effects of TNB. The purpose of this study was to document 1) the morphologic progression of degenerative changes after single and multiple doses of TNB and 2) the extent of reversibility after cessation of treatment.

MATERIALS AND METHODS:

Chemicals

1,3,5-trinitrobenzene (99.83 % purity) was obtained from Naval Surface Warfare Center (Silver Springs, MD) and the purity of the compound was confirmed by high performance liquid chromatography (HPLC). Just prior to use, TNB was ground to a fine powder and mixed with corn oil in a Potter-Elvehjem grinder. The oral LD_{50} value for TNB in rats has been previously determined to be 284 mg/kg for combined sexes (Fitzgerald et al., 1992). The one eighth (35.5 mg/kg) and one fourth (71 mg/kg) LD_{50} doses were selected for use in these studies. The rats were gavaged with TNB in corn oil by feeding needle. Control rats received only the vehicle (corn oil).

Animals

After a 2-week acclimatization to housing conditions (12 h light/12 h dark cycle, $72^0 \pm 2^0$F, 50±10% relative humidity), F-344 (initial body weight 220 g) rats were ranked by body weight and randomly assigned to one of three experimental groups. Animals were housed singly and provided with Purina Laboratory Chow and tap water *ad libitum*.

Histopathology

At the end of each exposure period, rats were deeply anesthetized using carbon dioxide, and euthanatized by exsanguination. At necropsy, the testis was excised and weighed. The testicles were fixed whole by immersion in Bouin's fixative for histopathologic examination. The epdidymis was collected in 10% neutral buffered formalin. Tissues were routinely processed, embedded in paraffin wax, sectioned at 5-6 microns, and stained with hematoxylin and eosin (H&E). Sections of testis were also stained with periodic acid-Schiff-hematoxylin (PAS-H) to aid in the discrimination of the stages of spermatogenesis according to Leblond and Clermont (1952). Since accurate staging was

difficult in paraffin embedded sections, frequently the stage-dependent testicular damage was evaluated by grouping of tubules into the following stages: stages I-IV, stages V-VIII, stages IX-XIII and stage XIV (Leblond and Clermont, 1952).

Experimental

I *Dose Response and Reversibility Study*:

A histopathologic evaluation was done after a single dose or multiple oral doses of TNB at 35.5 and 71 mg/kg. Forty six male Fischer-344 rats were randomly assigned by body weight to three exposure periods (12 rats per group) of 1 day, 4 days and 10 days. Within each exposure period, four rats were further separated into three TNB dose groups of 0, 35.5 and 71 mg/kg. This resulted in four rats/group/dose within each exposure period.

A reversibility study was conducted with an additional 2 groups consisting of 5 animals per group (4 treated and 1 control) Rats in group 1 and 2 were killed after 10 consecutive daily oral doses of TNB (at 71 mg/kg) with a recovery period of 10 and 30 days respectively. Limited control rats (1 per group) were used in the reversibility study since morphological (histopathological) changes were evaluated.

Statistical differences in testicular weights between exposure periods were determined using PC-SAS (SAS Institute Inc. Cary, NC). All data were tested for homogeneity of variances prior to analysis of variances (Proc. GLM, SAS). When significant F-values were obtained, the method of Least Square Means (LSM) was used to determine significant differences between treatment means. P-values of ≤ 0.05 were considered to be significant, unless otherwise mentioned.

II *Sequential (Histopathological) Changes:*

The objective in this study was to observe sequential changes in the seminiferous tubules, since rats in the first experiment had subtle changes at 4 days or had severe seminiferous tubular atrophy at 10 days. Twenty five male F-344 rats were randomly assigned by body weight to one of five different exposure periods. Within each exposure period, four rats were treated with 71 mg/kg of TNB and one rat which served as the control received corn oil (vehicle). Rats in this experiment were killed after 4, 5, 6, 8, or 10 doses of TNB. Histopathologic evaluation of the testicles was done on Bouin's fixed, paraffin embedded sections stained with either H&E or PAS-H.

RESULTS

Dose Response and Reversibility:

Single dose of TNB

The testicular weights were not significantly different between control and treated (35.5 or 71 mg of TNB) rats sacrificed 24 hours after a single dose (Table 1). Histopathologic examination did not reveal any treatment-related changes in the testis and epididymis.

Four daily doses of TNB

There was no treatment-related change in testicular weights or histopathology between rats receiving 0 or 35.5 mg/kg/day for 4 days. Testicular weights were significantly ($P<0.05$) decreased in rats receiving 71 mg/kg/day of TNB for 4 days compared to controls (Table 1). Histopathologic examination revealed degenerative changes in the seminiferous tubules of all four rats. There was necrosis (and degeneration) of pachytene spermatocytes in stages VIII-XIII, though stage VIII and IX were consistently affected (Fig 2). Seminiferous tubular lumina had one or more multinucleate

(syncytial) spermatidic giant cells. A few tubules had gaping holes between the spermatids and Sertoli cells indicating prior loss of pachytene spermatocytes. In addition to the necrosis of pachytene spermatocytes, round spermatids had marginated chromatin (halo appearance). The residual bodies had been phagocytzied, but the step 19 spermatids were still in the lumina. This change was rarely present in the control rats, but was very frequently observed in the treated rats. Seminiferous tubules in stages I-IV were spared from the toxic effects observed in other stages.

10 daily doses of TNB

Testicular weights were significantly ($P<0.05$) decreased in rats receiving 35.5 or 71 mg/kg/day of TNB for 10 days compared to controls (Table 1). The testicular lesions in rats receiving 35.5 mg of TNB for 10 days were similar to the effects observed in rats given 71 mg/kg/day for 4 days. Other histopathologic changes included, a severe Sertoli cell vacuolation and degeneration of round spermatids having a marginated chromatin (halo spermatids) were observed in treated rats. These halo spermatids coalesced to form multinucleate syncytial cells in stages I-IV of the cycle (Fig 3). Necrotic round spermatids appeared as eosinophilic spheroids. The oval and oblong spermatids in stages IX-XIII had a kinked or a knobbed head (acrosomal cap). The changes in the epididymis reflect the changes in the testis. The caput epidiymis was completely devoid of sperm, but instead contained numerous exfoliated syncytial (multinucleate) spermatids which extend also into the corpus of the epididymis (Fig 4). The cauda epididymis was normal and the ducts were filled with sperm.

The testicular changes observed in rats administered 71 mg/kg/day of TNB for 10 days was markedly exacerbated compared to rat given 35.5 mg/kg of TNB for 10 days. Virtually all the tubules were atrophic with a complete cessation of spermatogenesis in this group (Fig 5). The interstitial space was also increased due to this atrophy. The most

marked change observed was the complete absence of all round and elongate spermatids which were present at the lower dose (35.5 mg). The multinucleate cells were also missing and the tubules were lined by Sertoli cell nuclei and rarely a few spermatogonia. Binucleate and multinucleate cells formed by the fusion of spermatocytes were present (Fig 6). Examination of the epididymis revealed a pattern consistent with the observed testicular damage. The caput was completely devoid of sperm and contained numerous exfoliated germ cells. The shed germ cells were present in the caput, corpus and also in the cauda.

Rats administered TNB at 71 mg/kg for 10 days also had a lesion in the ventral prostate. The epithelium of the ventral prostate had numerous scattered apoptic cells (Fig 7) lining the acini. The apoptic cells either had pyknotic or karyorrhetic nuclei. This change was present in all the four rats given 71 mg/kg for 10 days. The dorsal prostate, lateral prostate, anterior prostate (coagulating gland) and the seminal vesicles did not have any treatment related changes.

Ten day recovery

The testicular weights in rats given a ten day recovery after 10 daily oral doses was significantly ($P<0.005$) decreased compared to the control rats and rats killed after 10 doses. The decreased testicular weight, however did not correlate with the histopathologic change since a significant regenerative attempt with numerous proliferating cells were present in the tubules. Seminiferous tubules were lined by Sertoli cell nuclei and spermatogonia in rats receiving 71 mg/kg for 10 days. In comparison the testiscles now had actively dividing spermatocytes. Numerous tubules had 3-4 generations of spermatocytes, but these had not matured into round or elongate spermatids (Fig 8). A few (probably less affected) tubules had round spermatids. Not all the tubules showed regeneration, since some were completely atrophic and were lined only by Sertoli cell

nuclei and spermatogonia. The tubules were widely separated by an interstitial edema. The absence of normal active spermatogenesis was evident as empty epididymal ducts.

Thirty day recovery

The testicular weights in rats given a thirty day recovery after 10 daily doses was significantly increased compared to the rats killed after 10 doses ($P<0.005$) and rats killed after a 10-day recovery ($P<0.005$). Round and elongate spermatids which were absent in the 10-day recovery group were now present in a majority (90%) of the tubules (Fig 9). The multinucleate (syncytial) cells were not present in the tubules. Staging of the cycle was possible in some tubules, eventhough all the fourteen stages were not present. A large percentage of tubules were in stages XI-XIII or either in XIV, with cells in meiosis. It appeared that elongate spermatids had not progressed to step 19, since stage VII or VIII was not present in all the four rats given the 30 day recovery. The absence of step 19 spermatids, was also evident in the caput epididymis which was completely empty and lacked spermatozoa. The cauda still contained degenerate cells and necrotic debris. Similar to the 10-day recovery period, a small percentage (approximately 5-10% of tubules) had markedly altered tubular architecture that were lined only by Sertoli cell nuclei and spermatogonia. Treatment related changes were not present in the accessory sex glands during the recovery.

Sequential (histopathological) Changes

Rats given daily oral doses for 5 consecutive days had more advanced changes than those dosed for 4 days. Vacuolation and rarefaction of the Sertoli cells was now clearly evident. A large number (60-80%) of tubules had necrotic round spermatids which had the appearance of eosinophilic spheroids along with the syncytial cells present at 4 days. Large gaping holes with loss of synchrony indicated loss of pachytene spermatocytes. Seminiferous tubules in the early stages (I-V) are apparently normal and

unaffected at this time frame. Rats killed after 6 doses had a continuum of changes observed at 5 days. In this group all the tubules (100%) in the testis were consistently affected and the rarefaction of the Sertoli cell cytoplasm was more extensive (Fig 10). Another change observed aafter 6 doses was the appearance of degenerate elongate spermatids in stages XI-XIII having a kinked or knobbed appearance. Rats killed after 8 consecutive doses of TNB had tubules lined by Sertoli cell nuclei and few spermatocytes. Two distinct changes at this time frame were the complete absence of round and elongate spermatids and the appearance of binucleate spermatocytes. The changes in rats given 8 doses, to a large extent resembled the changes observed at 10 days.

DISCUSSION

An initiative was undertaken by this laboratory to understand more about the pathogenesis of the testicular lesion and its reversibility, with a view to the possible usefulness of this information in contributing towards the comprehensive toxicity of TNB. We have previously reported the hematological effects and neurotoxicity (encephalopathy) of TNB (Chandra et al 1995 a, b).

Testicular damage with other structurally analogous nitroaromatic compounds like nitrobenzene (Bond et al., 1981; Levin et al., 1988), TNT (Levine et al., 1984, 1990; Dilley et al., 1982) and 1,3-dinitrobenzene (DNB) (Cody et al., 1981; Hess et al., 1988; Linder et al., 1990, 1986) is well known. Studies by Blackburn et al (1988) indicated that a single oral dose (50 mg/kg) of *o*-, *p*-, or *m*-dinitrobenzenes resulted in decreased testicular weight and histopathologic changes in the testis only in animals treated with the *meta* isomer, although both the *m*- and *p*-dinitrobenzenes produced hematological effects.

These findings of an isomer specificity for testicular toxicity with DNB have practical implications. Previously toxicity (testicular) data derived for TNB was by extrapolation based on the studies conducted with DNB. Isomer specificity observed with DNB indicate simple extrapolation based on the chemical structure may not be true. There is wealth of literature on the testicular toxicity of 1,3-DNB which is being studied to this day.

The morphological (histopathological) pattern we observed with TNB are remarkably similar to what has been reported with DNB (Hess et al., 1988; Blackburn et al., 1988). A longer duration and higher dose levels were required to produce the same histopathologic lesion with TNB. For example, a single oral dose of 1,3-DNB (48 mg/kg) caused severe damage by 24 hours with degenerating pachytene spermatocytes, chromatin margination in round spermatids, formation of giant cells and deformed spermatid heads. These regressive effects continued until 24 days, after which the tubules either recovered or became atrophic (Hess et al., 1988). Similarly Blackburn et al (1988) reported morphological changes with DNB in the testis at much lower doses than that reported by Hess et al (1988). Vacuolation of the Sertoli cell, degenerative changes in the spermatocytes were present as early as 12 hours at 25 mg/kg of 1,3-DNB. An identical change was observed at 48 hours after a single dose of 15 mg/kg of 1,3-DNB (Blackburn et al., 1988). In contrast, the changes observed with a single dose of DNB on days 1, 2, or 4, were present only after 4 daily doses of TNB (at 71 mg). Vacuolation of Sertoli cells and the kinked or knobbed spermatids observed with 1,3-DNB on the second day (Hess et al., 1988), were apparent after 6 daily doses of TNB. Based on the morphological findings it appears that 1,3-DNB is a more potent testicular toxicant than TNB.

Testicular lesions have also been reported with another structurally similar nitroaromatic compound 2,4,6-trinitrotoluene in rats (Levine et al., 1984; Dilley et al., 1982; Levine et al., 1990). Reduced testis weight, testicular atrophy and hyperplasia of the interstitial cells were observed in rats receiving 0.25% of TNT in their diet and the testicular changes were not reversible after a 4 week recovery (Dilley et al., 1982). Levine et al (1984) have reported diminution of spermatozoa, spermatids and spermatocytes as a result of degeneration and necrosis in rats receiving 300 mg of TNT per day in the diet for 13 weeks. Atrophy of the seminiferous tubules, spermatocytic and spermatidic giant cells were also observed in their study. The Sertoli cells and spermtogonia appeared to be unaffected with TNT (Levine et al., 1984). In a more recent study by Levine et al (1990), rats receiving 125 mg of TNT for 13 weeks had degeneration of the germinal epithelium and the changes were characterized as minimal to mild (Levine et al., 1990). Although acute testicular changes with TNT are not reported, it is reasonable to conclude that TNB appears to be a more potent testicular toxicant than TNT, since a prolonged exposure (13 weeks) at much higher doses (125 or 300 mg) is necessary to induce degenerative testicular changes with TNT.

The ability of the testis to recover from toxic insult has been reported by other investigators for nitroaromatic compounds. Rats allowed to recover after exposure to TNB had a decrease in the testicular weight, although histologic examination revealed partial restoration of germ cell production. Tubular architecture was relatively normal, except that they were lacking the mature spermatozoa. Hess et al (1988) have reported regeneration of seminiferous tubules with identifiable stages as early as day 16 with DNB, compared to 30 days with TNB in this study. However comparison between DNB and TNB is rather not appropriate, since rats in our study received 10 doses of TNB, whereas rats in their study received a single dose of DNB. On the contrary, recovery with TNB appears to be rapid, since repopulation with new germ cells was observed after a 10 day

recovery and elongate spermatids were present after a 30 day recovery. A small percentage of tubules remained atrophic in the DNB study by Hess et al (1988). A similar scenario (i.e. tubular atrophy) was observed with TNB which may indicate irreversible damage or perhaps a longer time period to repair the damage. Reversibility with TNB should be interpreted cautiously since rats were given only a 30 day recovery period. A recovery period of 154 days (12 cycles of the seminiferous epithelium) is recommended by Amman (1982) for evaluation of reversibility of germ cell damage. The presence of the regenerating germ cells at 10 and 30 days post-treatment indicate that effects are partially reversible.

The mechanism(s) of action of TNB on the testis is not known. The closely-related compound 1,3-DNB exerts its principle effect on the testis with the Sertoli cell as the primary target (Rehnberg et al., 1988; Blackburn et al., 1988; Foster et al., 1987). Foster et al (1987) reported Sertoli cell damage following *in vitro* treatment which was comparable to the *in vivo* response reported by Blackburn et al (1988). Hess et al (1988) suggested that damage to the developing germ cells was an indirect effect of Sertoli cell dysfunction.

The apparent initial sensitivity of pachytene spermatocytes to TNB toxicity (like DNB) suggests that Sertoli cell is also the target for TNB, since Sertoli cell functional changes might be manifested by loss of germ cells. The Sertoli cell is known to provide a 'nurse' function for the developing germ cells and has been shown to be intimately involved in the control of spermatogenesis (Rich and De Krester, 1983). If the Sertoli cell is the target for the toxic action of TNB in the testis then damage to these cells could precipitate the range of effects seen in the germ cells. Further support for this view comes from the observation that the testis is able to recover from the toxic insult after cessation of treatment. Hess et al (1988) suggest, if spermatogonia were the target cells, recovery

following cessation of the treatment would not have been possible. In the accompanying adjunct study, we have utilized proliferating cell nuclear antigen as an endogenous marker to identify spermatogonia and pachytene spermatocytes. Observations from that study indicate that the spermatogonia proliferate even in completely atrophic tubules to form spermatocytes, but due to a lack of a 'nurse' function by the Sertoli cell these cells do not mature into spermatozoa. Russell et al (1990) pointed out that the Sertoli cells are resistant to many treatments that affect germ cells. These cells are often present when all germ cell types are missing.

The presence of apoptic cells in the ventral prostate observed with TNB has not been reported earlier with DNB or TNT. Even though a direct toxic effect cannot be ruled out, this appears unlikely. Since castration induces apoptosis in the rat ventral prostate (Brandstrom et al., 1994), the apoptosis observed with TNB is suggestive of a secondary effect probably related to the severe atrophy of the seminiferous tubules.

In conclusion, the present study confirms that acute exposure to relatively low levels of TNB has an adverse effect on the testis. Recovery studies indicate the effects are partially reversible, but long term recovery studies are required. Further, the sequelae of recovery including fertilizing ability would be a better measure of the reversibility. Detailed studies are needed to determine if the mechanism of testicular toxicity proposed for DNB is also applicable to TNB.

REFERENCES

Amman RP (1982) Use of animal models for detecting specific alterations in reproduction. Fundam Appl Toxicol 2:13-26

Blackburn DM, Gray AJ, Lloyd SC, Sheard CM, and Foster PMD (1988) A comparison of the effects of the three isomers of dinitrobenzene on the testis in the rat. Toxicol Appl Pharmacol 63:120-132

Bond JA, Chism JP, Rickert DE, Popp JA (1981) Induction of hepatic and testicular lesions in Fischer-344 rats by single oral doses of nitrobenzene. Fund Appl Toxicol 1:389-394

Brandstrom A, Westin P, Bergh A, Cajander S, and Damber JE (1994) Castration induces apoptosis in the ventral prostate but not in an androgen-sensitive prostatic adenocarcinoma in the rat. Cancer Res 54:3594-3601

Chandra AMS, Qualls CW Jr, Reddy G, and Meinkoth JH (1995a) Hematological effects of 1,3,5-trinitrobenzene in rats. In vivo and In vitro. J Toxicol Environ Health (in press)

Chandra AMS, Qualls CW Jr, and Reddy G (1995b) 1,3,5-trinitrobenzene induced encephalopathy in male F-344 rats. Toxicol Pathol (in press)

Ciss M, Hwyen N, Dutertre H, Phu-Lich N, Truhart R (1980) Etude toxiciologique des nitrotoluenes: toxicite a long term. Dakar Medical 25:293-302

Cody TE, Witherup S, Hastings L, Stemmer K, Christian RT (1981) 1,3-dinitrobenzene: toxic effects in vivo and in vitro. J Toxicol Environ Health 7:829-847

Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, and Dacre JC (1982) Short term oral toxicity of 2,4,6-trinitrotoluene in mice, rats and dogs. J Toxicol Environ Health. 9:565-585

Ellis MK, and Foster PMD (1992) The metabolism of 1,3-dinitrobenzene by rat testicular subcellular fractions. Toxicol Lett 62:201-208

Ellis HV, Hong CB, Dycre JC, Lee CC (1978) Chronic toxicity of 2,4-dinitrotoluene in the rat. Toxicol Appl Pharmacol 45:245-246

Fitzgerald GB, De Guilion N, Desai L S, and Reddy G (1992) Acute toxicity evalaution of 1,3,5-Trinitrobenzene. J Amer Coll Toxicol Part B. Acute Toxicity Data. 3:169-170

Foster PMD, Lloyd SC, and Prout MS (1987) Toxicity and metabolism of 1,3-dinitrobenzene in rat testicular cell cultures Toxicol in vitro 1:31-37

Foster PMD (1989) m-dinitrobenzene: Studies on its toxicity to the testicular sertoli cell. Arch Toxicol 13:3-17

Hess RA, Linder RE, Strader LF, and Perreault SD (1988) Acute effects and long term sequelae of 1,3-Dinitrobenzene on male reproduction in the rat II. Quantitative and Qualitative histopathology of the testis. J Androl 9:327-342

Kinkead ER, Wolfe RE, Salins SA, Miller C, Latendresse JR (1994) Single-generation reproduction study of 1,3,5-trinitrobenzene in the diet of Sprague-Dawley rats. Toxicologist 14:161

Kinkead ER, Wolfe RE, Flemming CD, Caldwell DJ, Miller CR, and Marit GB (1995) Reproductive toxicity screen of 1,3,5-trinitrobenzene administerted in the diet of Sprague-Dawley rats. Toxicologist 15:249

Leblond CP, Clermont Y (1952) Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann NY Acad Sci 55:548-573

Levine BS, Furedi EM, Gordon DE, Lish PM, Barkley JJ (1984) Subchronic toxicity of trinitrotoluene in Fischer-344 rats. Toxicology 32:253-265

Levine BS, Furedi EM, Gordon DE, Barkley JJ, Lish PM (1990) Toxic interactions of the munitions compounds TNT and RDX in F344 rats. Fundam Appl Toxicol 15:373-380

Levin AA, Bosakowski T, Earle LL, Butterworth BE (1988) The reversibility of nitrobenzene-induced testicular toxicity: Continuous monitoring of sperm output from vasocystotomized rats. Toxicology 53:219-230

Linder RE, Hess RA, Strader LF (1986) Testicular toxicity and infertility in male rats treated with 1,3-dinitrobenzene. J Toxicol and Environ Health 19:477-489

Linder RE, Strader LF, Barbee RR, Rehnberg GA, Perreault SD (1990) Reproductive toxicity of a single oral dose of 1,3 -Dinitrobenzene in two ages of young adult male rats. Fund Appl Toxicol 14:284-298

Linder RE, Hess RA, Perreault SD, Strader LF, Barbee RE (1988) Acute effects and long term sequelae of 1,3-Dinitrobenzene on male reproduction in the rat I. Sperm quality, quantity and fertilizing ability. J Androl 9:317-326

Obasaju MF, Katz DF, Miller MG (1991) Species differences in susceptibility to 1,3-dinitrobenzene-induced testicular toxicity and metheoglobinemia. Fund Appl Toxicol 16:257-266

Reader SCJ, Shingles C, Stonard MD (1991) Acute testicular toxicity of 1,3-dinitrobenzene and ethylene glycol monomethyl ether in the rat: Evalaution of biochemical effect markers and hormonal responses. Fund Appl Toxicol 16:61-70

Reddy TV, Daniel FB, Robinson M, Olson GR, Weichman B, and Reddy G (1993) Fourteen day toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 13:177

Reddy TV, Torsella J, Daniel FB, Olson GR Weichman B, and Reddy G (1994) Subchronic toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in Fischer-344 rats. Toxicologist 14:117

Rehnberg GL, Linder RE, Goldman JM, Hein JF, McElroy KW, and Cooper RL (1988) Changes in testicular and serum hormone concentrations in the male rat following treatment with m-dinitrobenzene. Toxicol Appl Pharmacol 95:255-264

Rich KA and De Krester DM (1983) Spermatogenesis and the Sertoli cell. In: The Pituitary and the testis. Clinical and experimental studies, De Krester DM, Burger HG, Hudson B (Eds), Springer, Berlin. pp 84-105.

Rickert DE, Butterworth BE, Popp JA (1984) Dinitrotoluene: Acute toxicity, oncogenicity, genotoxicity and metabolism. Crit Rev Toxicol 13:217-234

Russell LD, Ettlin RA, Sinha Hikim AP, and Clegg ED (1990) In: Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL

Ryon MG, Pal BC, Talmage SS, Ross RH (1984) Database assessment of the health and environmental effects of munition production waste products. Final Report. ORNL-6018 (NTIS DE84-016512). Oak Ridge National Laboratory, Oak Ridge, TN

U.S. Department of Health and Human Services (USDHHS). Public Health Service. Agency for Toxic Substances and Disease Registry (1993). Toxicological Profile for 1,3-Dinitrobenzene and 1,3,5-Trinitrobenzene.

U.S. EPA (1989) Health and Environmental Effects Profile for 1,3,5-trinitrobenzene. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Cincinnati, OH, ECAO-CIN-G071

Table 1. Testicular Weights (g) in F-344 rats treated with TNB

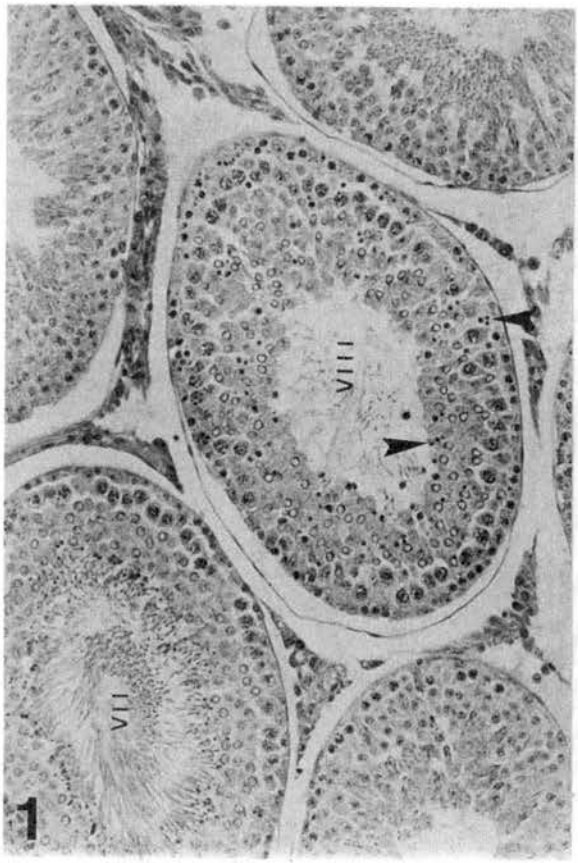
TNB mg/kg	Single dose	4 daily doses	10 daily doses
0	2.65 (0.05)	2.63 (0.02)	2.76 (0.17)
35.5	2.72 (0.13)	2.69 (0.11)	2.53 (0.06)*
71	2.69 (0.09)	2.59 (0.13)*	1.38 (0.11)*

Data expressed as Mean (SD). * $P < 0.05$ Significantly different from control mean within the same exposure period.

Figure 1. Seminiferous tubules from a rat administered corn oil (vehicle treated control). Note the continuity and the organized arrangement of the germ cells. Stages VII and VIII are present. Arrowheads indicate residual body. H&E. X280

Figure 2. Seminiferous tubules from a rat administered TNB at 71 mg/kg for 4 days. Only pachytene spermatocytes are affected (arrowhead), with condensed cytoplasm and nuclear pyknosis. Note the unaffected healthy tubule at a non susceptible stage (asterisk). H&E. X280

Figure 3. Multinucleate (syncytial) cells from a rat administered 35.5 mg of TNB for 10 days. H&E. X80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VII
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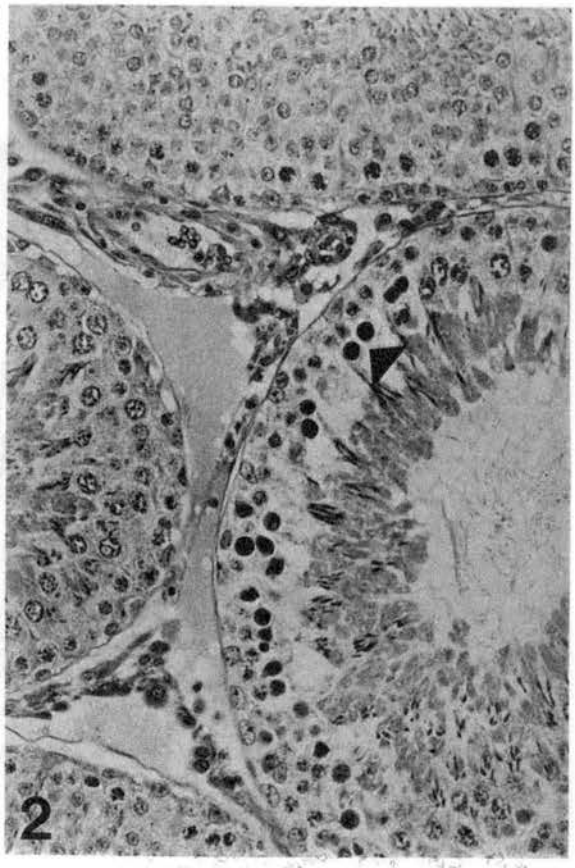
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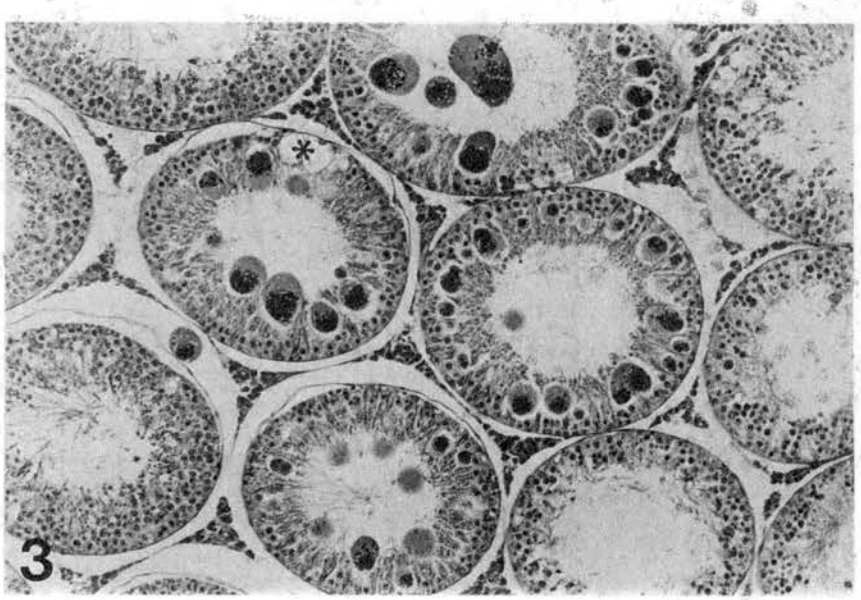
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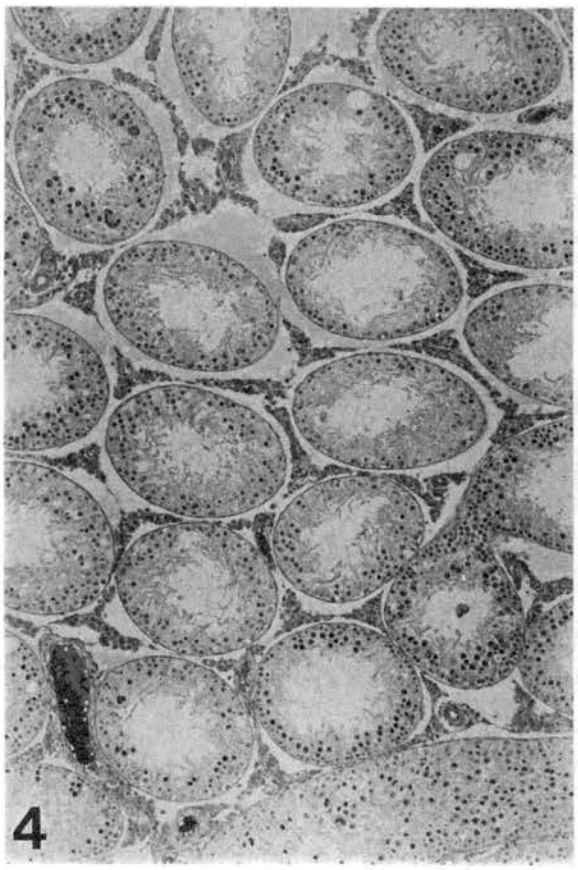
Figure 4. Seminiferous tubules from a rat administered TNB at 71 mg/kg for 10 days. More number of tubules are present due to decreased tubular diameter. H&E. X110

Figure 5. Higher magnification of fig 4. Note the prominence of Leydig cells due to tubular atrophy. Spermatocytic giant cells are also present. Note absence of round and elongate spermatids. Compare with fig 1. H&E. X280

Figure 6. Caput epididymis from a rat administered TNB at 35.5 mg for 10 days. Ducts are completely devoid of spermatozoa (asterisk), instead contain multinucleate spermatids arrested in meiosis. H&E. X280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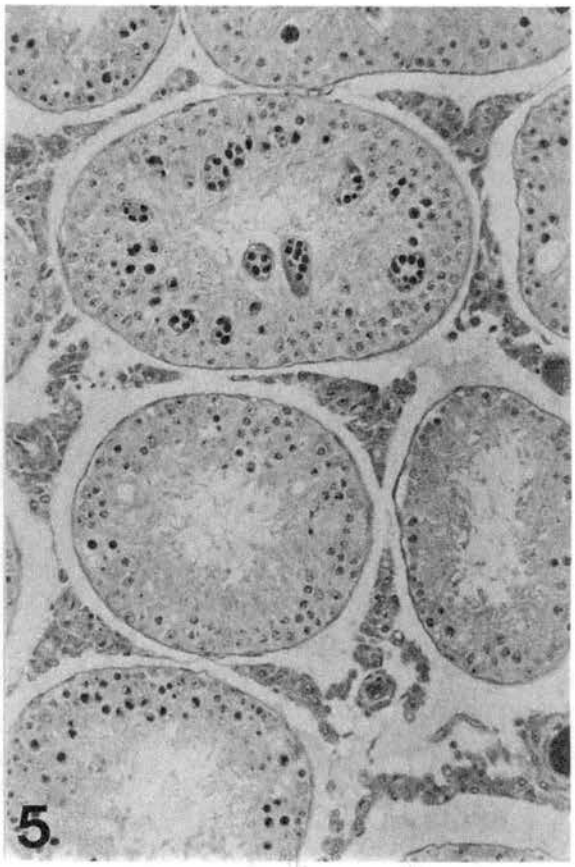
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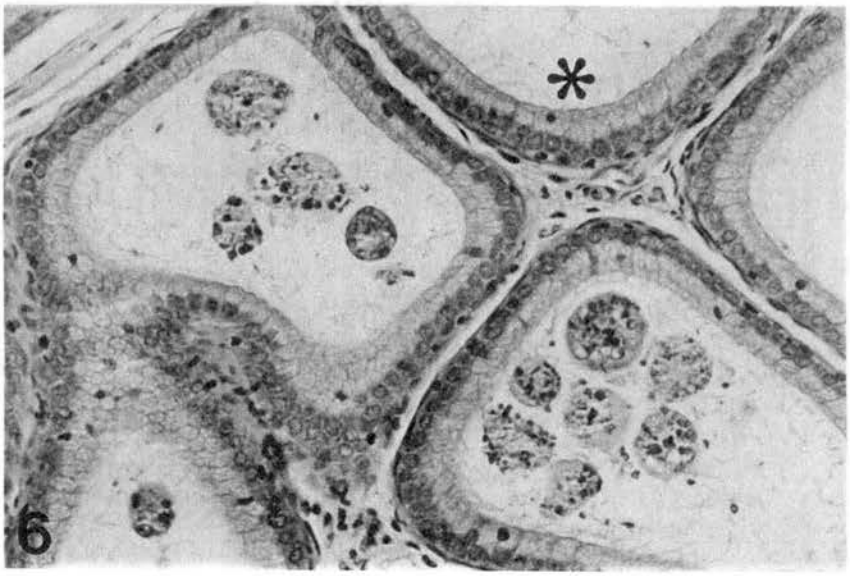
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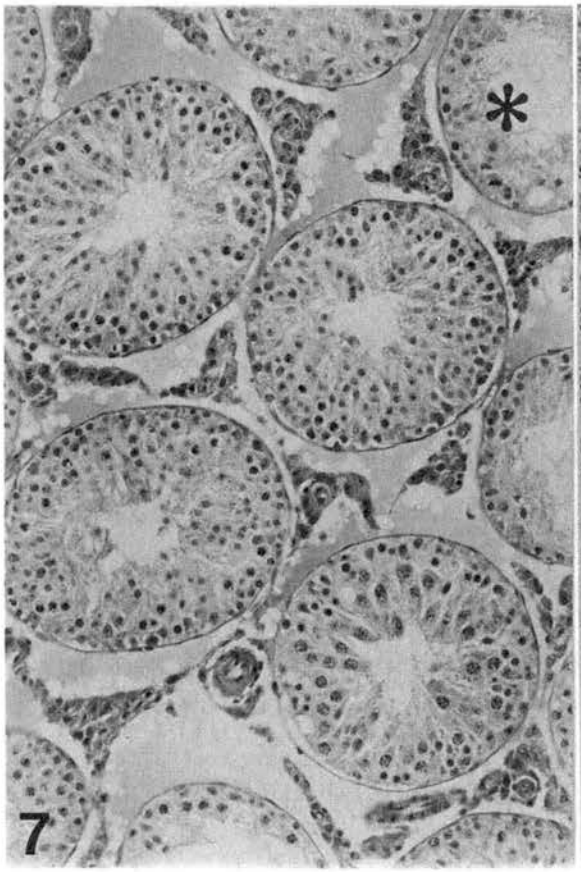
Figure7. Seminiferous tubules from a rat given a 10 day recovery. Only round spermatids are evident. Asterisk indicates an atrophic tubule. Tubular size is still less compared to the control. H&E. X110

Figure 8. Seminiferous tubules from a rat given a 30 day recovery. Germ cells have progressed to the elongate spermatid stage. An atrophic tubule is shown (asterisk). Compare with figs 1 and 5. H&E. X110

Figure 9. Ventral prostate from a rat administered TNB at 71 mg/kg for 10 days. Prostatic epithelium undergoing apoptosis (arrowheads). H&E. X300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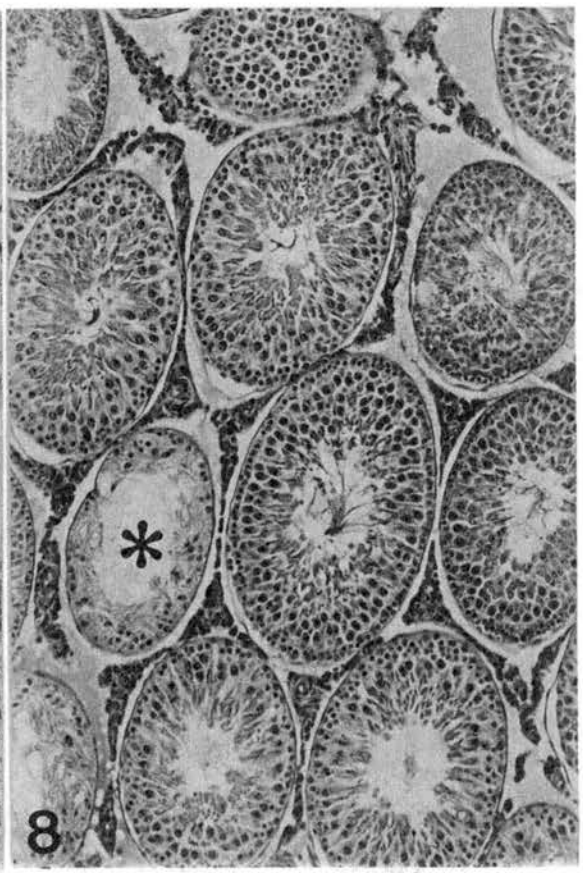
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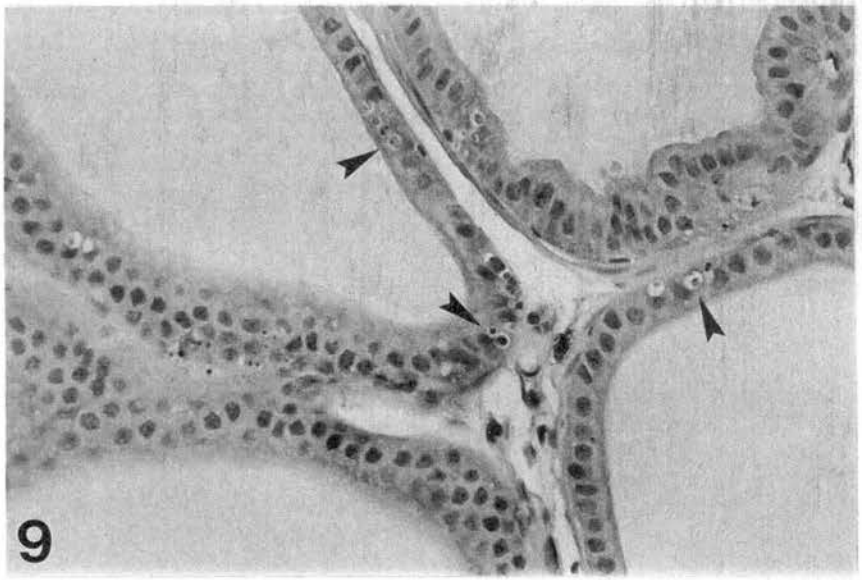
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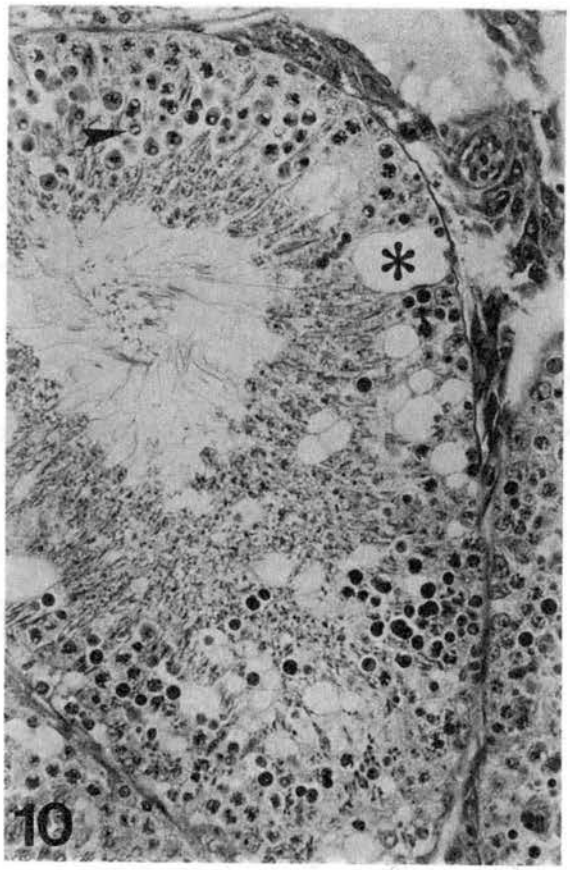
Figure 10. Seminiferous tubules from a rat administered TNB at 71 mg/kg for 6 days. Necrosis of pachytene spermatocytes, halo spermatids (arrowhead) and marked vacuolation of Sertoli cell cytoplasm (asterisk). H&E. X250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CHAPTER IV

RAT TESTIS DURING EXPOSURE AND RECOVERY FROM 1,3,5-TRINITROBENZENE (TNB) INTOXICATION. II. IMMUNOLOCALIZATION OF GERM CELLS USING PROLIFERATING CELL NUCLEAR ANTIGEN AS AN ENDOGENOUS MARKER

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a 36KD auxiliary protein to DNA polymerase-δ (Mathews et al., 1984; Bravo et al., 1987), which is found in various concentrations within the cell throughout the cell cycle and in greatest quantities during S-phase (Celis and Celis, 1985). The use of monoclonal antibody to PCNA for examining cell proliferation in fixed embedded tissues has been recommended as an alternative to DNA-incorporated tritiated thymidine (Galand and Degraef, 1989; Foley et al., 1991) and 5-bromo-2'-deoxyuridine (BrdU) (Foley et al., 1991). In particular, the introduction of a commercially available microwave-based system for retrieving antigens in formalin-fixed tissues (Shi et al., 1991), was shown to be effective with respect to PCNA (Greenwell et al., 1991).

Potential applications of PCNA are wide-ranging in the field of diagnostic oncology. The use of PCNA in toxicology has been employed to assess cell proliferation to understand chemical carcinogenesis in rodent liver (Connolly and Bogdanffy, 1993; Jones et al., 1993; Greenwell et al., 1991; Foley et al., 1993) and other organs (Stefanski

et al.,1994; Takahashi et al., 1991). The use of PCNA as a tool to study cell proliferation after toxicant-induced testicular injury has received little attention.

1,3,5-trinitrobenzene (TNB) is a soil and groundwater contaminant at certain military installations. In a companion article, we have reported germ cell necrosis and reversibility of testicular changes in F-344 rats treated with TNB. In this study we have utilized PCNA as an endogenous germ cell marker. Immunohistochemical localization of regenerating germ cells was evaluated using PCNA performed on Bouin's-fixed, paraffin embedded testis. Testis from rats allowed to recover from the TNB exposure were also evaluated.

MATERIALS AND METHODS

Experimental

Male Fischer-344 rats were assigned, grouped, and dosed as described in the companion report. Selected archival rat tissues from three experimental groups were used in this study. Briefly, rats received TNB (71 mg/kg) or corn oil (control) by gavage. For this study, testis from rats that received a) corn oil (vehicle control) for 10 days, b) 10 daily doses (group I), c) 10 daily doses of TNB followed by a 10-day recovery (group II), d) 10 daily doses of TNB followed by a 30-day recovery (group III), were utilized. At necropsy, testicles were excised, weighed, and immersion-fixed in Bouin's fixative. The next day testicles were cut into 3-mm thick slices using a razor blade and rinsed with 5% sodium thiousulfate to remove excess picric acid. Tissue slices were routinely processed and embedded in paraffin. PCNA staining was performed on 4-6 micron thick paraffin sections.

PCNA staining procedure

The biotin-streptavidin (ABC Staining Procedure) method for immunohistochemical localization of PCNA was performed as described by Foley et al (1993) with minor changes, including an enhancing technique for antigen retrieval (Shi et al., 1991). For immunohistochemisry 4-6 μm thick paraffin-embedded sections containing both the testicles from each individual animal including both the caput and cauda epididymis were utilized. Tissue sections were placed onto poly-L-lysine-coated slides and dried overnight. The following day the sections were de-waxed in xylene and hydrated via a graded ethanol series. Endogenous peroxidase activity was blocked with a 30 minute incubation (room temperature) in methanol containing 3% hydrogen peroxide. Slides were washed with distilled water for 5 min. Slides were then placed in plastic Coplin jars containing antigen retrieval solution (Biogenex Laboratories, San Ramon CA) and microwaved at high power for 10 min (as per manufactures specification). The sections were allowed to cool in the same jars and later washed with isotonic phosphate buffered saline (pH=7.4). Sections were pre-incubated for 30 minutes in normal horse serum (from which the secondary antibody was derived) to decrease nonspecific binding. Excess serum was blotted before primary antibody application. For the determination of PCNA sections were incubated with mouse anti-human PCNA (1:100) which cross reacts with rat cells (product information for PC 10, Dako Corp., Carpinteria, CA). The incubation with the primary antibody was carried out at 37^0C for 60 minutes. The bound antibodies were visualized with a commercially available avidin-biotin-peroxidase complex (Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA). Immunolabelled peroxidase was visualized by using the commercially available chromogen Vector VIP (Vector Laboratories, Burlingame, CA), which yields an intense purple color; no counterstain was used. After a brief rinse with tap water, sections were dehydrated and coverslipped.

The specificity of the immunohistochemical reaction was evaluated by the omission of individual steps. Normal mouse serum was substituted instead of the primary antibody (i.e. anti-PCNA). Other control procedures included omission of biotinylated secondary antibody and avidin-biotin peroxidase complex. Sections from all the rats were immunohistochemically stained (on the same day) using the same diluted antibody solution throughout to avoid variation in staining between sections.

RESULTS

Nuclear staining was observed on all sections except for those run as negative controls. The PCNA positive nuclei ranged in color from dark purple to purple-brown. The staining varied between different cells, with a speckled to uniform staining pattern.

Testis from control rats

Immunoreactvity was sharply defined, although the staining pattern differed between different germ cells. PCNA-positive cells were present in the basal part of all the seminiferous tubules. In individual tubules the staining was confined only to one germ cell type (spermatocytes) or two types of germ cells (spermatocytes and spermatogonia). The majority of the tubules had a single (circumferential) row of pachytene spermatocytes (Fig 1). Occasionally certain tubules had a second somewhat irregular row of speckled leptotene spermatocytes. Spermatogonial staining was not present in all stages of the cycle. Spermatogonia appeared as oval, intensely stained cells adherent to the basement membrane in stages IX-XII and also in stages I-IV. The precise identification of the stages was not possible since sections were not counter-stained. In addition to spermatocytes and spermatogonia, only the nuclei of elongate spermatids in stages XI-XIII and rarely interstitial cells were also stained positively with PCNA. The nuclei of round spermatids, Sertoli cells, and elongate spermatids (except XI-XIII) did not stain

positively with PCNA. Other negatively stained cells were the endothelium and the myoid cells within the basement membrane of the tubules.

Testis from rats treated with TNB for 10 days

A severe testicular atrophy (together with decreased weight) with complete cessation of spermatogenesis was observed on hematoxylin and eosin stained sections. Nevertheless, the tubules still contained PCNA-positive cells (Fig 2). In contrast to the control rats, the number of positive cells were reduced in all the seminiferous tubules. Both spermatogonial and spermatocytic cells were positive. There was a wide variation among the tubules, some were completely devoid of staining, whereas immediately adjacent tubules had positive cells. Unlike the controls, the positive cells were haphazardly arranged, with loss of synchrony and the presence of 3-4 generations of spermatocytes in the same seminiferous tubule. Similar to the control testicles, both speckled and diffuse staining pattern was observed.

Testis from rats allowed a 10 day recovery

Seminiferous tubules contained 3-4 generations of PCNA-positive spermatocytes in well organized (synchronous) fashion (Fig 3). PCNA-positive cells identifiable as spermatogonia were also present adjacent to the basement membrane of the tubules.

Testis from rats allowed a 30 day recovery

The staining pattern observed in these rats was identical to that observed in the control rats (Fig 4). The majority (98%) of the tubules contained a single (circumferential) row of spermatocytes and spermatogonial cells. The remaining few tubules (1-2%) had randomly scattered PCNA positive cells, which were probably in the process of recovering from the toxic insult.

DISCUSSION

Assessment of cell proliferation both in vivo and in vitro has involved incorporation of tritiated thymidine into cells during the DNA synthesis phase (S-phase) of the cell cycle, followed by autoradiography (Hall and Levison, 1990; Loury et al., 1987). This assessment can also be performed by use of 5-bromo-2'-deoxyuridine (BrdU), a non-radioactive analogue of thymidine which is readily incorporated into cells during S-phase and evaluated by standard immunocytochemical methods (Fredericks et al., 1990; Hanazono et al., 1990; Lanier et al., 1989). Owing to the requirement for invasive delivery procedures, physical stress of surgery (implantation of osmotic pumps), and the inability to utilize archival tissues for analysis, use of BrdU or tritiated thymidine is limited. In view of these limitations another useful marker of S-phase cells is an endogenous nuclear protein which is variably expressed at different phases of the cell cycle, the amount binding to chromatin increasing to a maximum at S-phase (Morris and Matthews, 1989). Matthews et al (1984) designated it as proliferating cell nuclear antigen (PCNA)

In this study, identification of immunostained populations of germ cells was accomplished in the seminiferous tubules. Using PCNA (PC10), specific immunostaining of spermatogonia (although it was not possible to identify the specific differentiation stage) and pachytene spermatocytes was observed. In contrast, neither round spermatids or Sertoli cell nuclei were immunostained with this antibody. Identical results were reported by Schlatt and Weinbauer (1994) in Sprague-Dawley rats using the same antibody (PC10). Immuno-positive elongating spermatids were also reported by Schlatt and Weinbauer (1994). This was considered as non-specific staining due to the binding of the secondary biotinylated antiserum to the acrosome of elongating spermatids (Schlatt and Weinbauer, 1994).

The value of PCNA staining for the evaluation of spermatogenesis was evaluated in testicles of rats treated with TNB for 10 days and showing complete atrophy of seminiferous tubules. Hematoxylin and eosin-stained tissue sections from rats administered TNB for 10 days revealed complete atrophy and cessation of spermatogenesis. In these rats, PCNA immunostaining was still present in a number of spermatogonia and spermatocytes, showing that the mitotic activity of spermatogonia is not totally inhibited, and mitoses occurs even after pronounced inhibition of spermatogenesis. Similar to the toxicant induced atrophy in this study, Schlatt and Weinbauer (1994) induced maximal testicular regression (with decreased testicular weight and seminiferous tubular diameter) with a GnRH antagonist. Nevertheless, the seminiferous tubules still contained a number of PCNA-positive cells (Schlatt and Weinbauer, 1994) .

During the recovery phase, numerous positive cells were observed after 10 days of recovery. After 30 days of recovery, a staining pattern identical to the control rats was evident indicating normalization of changes. The kinetics of germ cell repopulation in rats allowed a 10 and 30 day recovery period indicate that spermatogonia (stem cell) are probably not a target for TNB toxicity. Therefore, failure of the few (2%) tubules to recover at 30 days may be related to factors other than a direct toxicity to the stem cell. Another possibility, is the short recovery period, since a recovery period of 154 days (12 cycles of the seminiferous epithelium) is recommended for evaluation of testicular damage (Amman, 1982).

In addition to PCNA (PC10), other researchers have immunostained germ cells in testis using different antibodies. Using a monoclonal antibody (JC1) raised against a nuclear antigen present in proliferating cells, undifferentiated spermatogonia, some spermatocytes and spermatids were labeled in the human testis (Garrido et al., 1992). Oke

and Suarez-Quian (1993) specifically immunostained pachytene spermatocytes and spermatogonia using a monoclonal antibody 37B3 that recognizes a nuclear lamin.

This study demonstrates that toxicological effects on the testis may be assessed by demonstrating alterations in PCNA staining, a good specific marker of germ cells. The commercial availability of antibodies to PCNA and the established protocols for staining (both in fresh and archival tissues) support its applicability.

REFERENCES

Amman RP (1982) Use of animal models for detecting specific alterations in reproduction. Fundam Appl Toxicol 2:13-26

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-d. Nature 326:515-517

Celis JE and Celis A (1985) Cell cycle dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc Natl Acad Sci USA 82:3262-3266

Connolloy KM and Bogdanffy MS (1993) Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: A dual-stain comparison with 5-bromo-2'-deoxyuridine. J Histochem Cytochem 41:1-6 41:1-6

Foley JF, Dietrich DR, Swenberg JA, Maronport RR (1991) Detection and evaluation of proliferating cell nuclear antigen (PCNA) in rat tissue by an improved immunohistochemical procedure. J Histotech 14:237-245

Foley JF, Ton T, Maronport RR, Butterworth B and Goldsworthy TL (1993) Comaprison of proliferating cell nuclear antigen to tritiated thymidine as a marker of proliferating hepatocytes in rats. Environ Health Perspect 101(5):199-206

Frederiks WM, Chamuleau RAFM, James J, Marx FM, van Noorden CJF (1990) Immunocytochemical determination of ploidy class-dependent bromodeoxyuridine incorporation in rat liver parenchymal cells after partial hepatectomy Histochemistry 93:627-637

Galand P, and Degraef C (1989) Cyclin/PCNA immunostaining as an alternative to tritiated thymidine pulse labeling for marking S phase cells in paraffin sections from animal and human tissues. Cell Tissue Kinet 22:283-292

Garrido MC, Cordell JL, Becker MHG, Key G, Gerdes J, Jones M, Gatter KC, and Mason DY (1992) Monoclonal antibody JC1: new reagent for studying cell proliferation. J Clin Pathol 45:860-865

Greenwell A, Foley JF, Maronport RR (1991) An enhancement method for immunohistochemical staining of proliferating cell nuclear antigen in archival rodent tissues. cancer Lett 59:251-256

Hall PA and Levinson DA (1990) Assessment of cell proliferation in histological material. J Clin Pathol 43:184-191

Hanozono M, Kitoh J, Kusakabe M, Ota K, Yoshiki (1990) Immunohistochemical detection of DNA replication in mouse uterine cells by bromodeoxyuridine labelling of wax- and resin-embedded sections. Stain Tech 65:139-150

Jones HW, Clarke NAB and Barrass NC (1993) Phenobarbital-induced hepatocellular proliferation: Anti-bromodeoxyuridine and anti-proliferating cell nuclear antigen immunocytochemistry. J Histochem Cytochem 41:21-27

Lanier TL, Berger EK, and Eacho PI (1989) Comparison of 5-bromo-2'-deoxyuridine and tritiated thymidine for studies of hepatocellular proliferation in rodents. Carcinogenesis 10:1341-1353

Loury DJ, Butterworth BE, and Smith-Oliver T (1987) Asssessment of unscheduled and replicative DNA synthesis in rat kidney cells exposed in vitro or in vivo to unleaded gasoline. Toxicol Appl Pharmacol 87:127-137

Matthews MB, Bernstein RM, Franza BR Jr., Garrels JI (1984) Identity of the proliferating cell nulear antigen. Nature 309:374-376

Morris GF and Matthews MB (1989) Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 264:13856-13902

Oke BO and Suarez-Quian CA (1993) Localization of secretory, membrane-associated and cytoskeletal proteins in rat testis using an improved immunocytochemical protocol that employs polyester wax. Biol Reprod 48:621-631

Schlatt S and Weinbauer GF (1994) Immunohistochemical localization of proliferating cell nuclear antigen as a tool to study cell proliferation in rodent and primate testes. Int J Androl 17:214-222

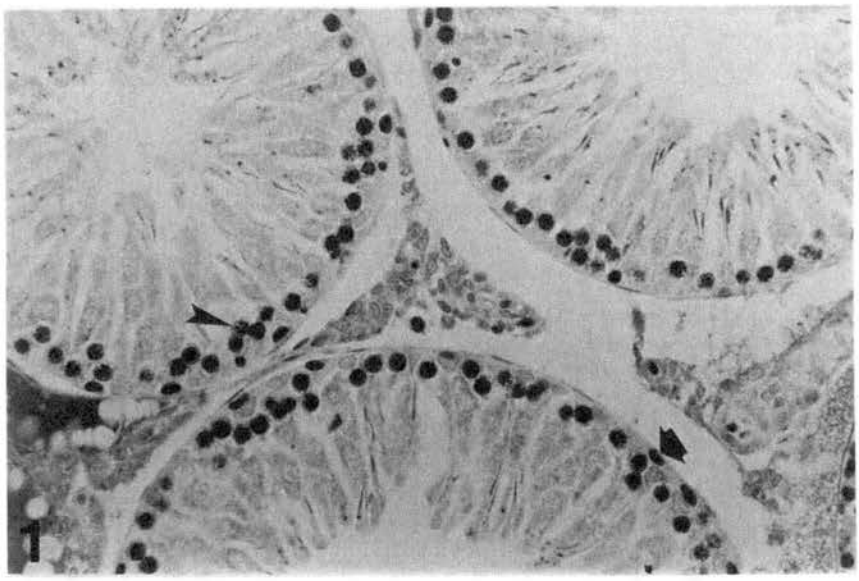
Shi SR, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed paraffin embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections J Histochem Cytochem 39:741-748

Takahashi H, Strutton GM, and Parsons PG (1991) Determination of proliferating fractions in malignant melanomas by anti-PCNA/cyclin monoclonal antibody. Histopathology 18:221-227

Stefanski SA, Greenwell A, Merrick BA, Brown TT, and Reynolds SH (1995) Proliferating cell nuclear antigen staining of Fischer-344/N rat spleens affected by large granular lymphocytic leukemia Toxicol Pathol 23:1-6

Figure 1. Immunohistochemical (ABC method) staining of testis from a control rat. PCNA immunostaining is confined to the nuclei of spermatogonia (arrow) and spermatocytes (arrowhead). No counterstain. X280

Figure 2. Immunohistochemical staining of testis from a rat administered TNB for 10 days. The tubules still contain positive cells. Note the haphazard organization and the complete absence of round and elongate spermatids. No counterstain. X185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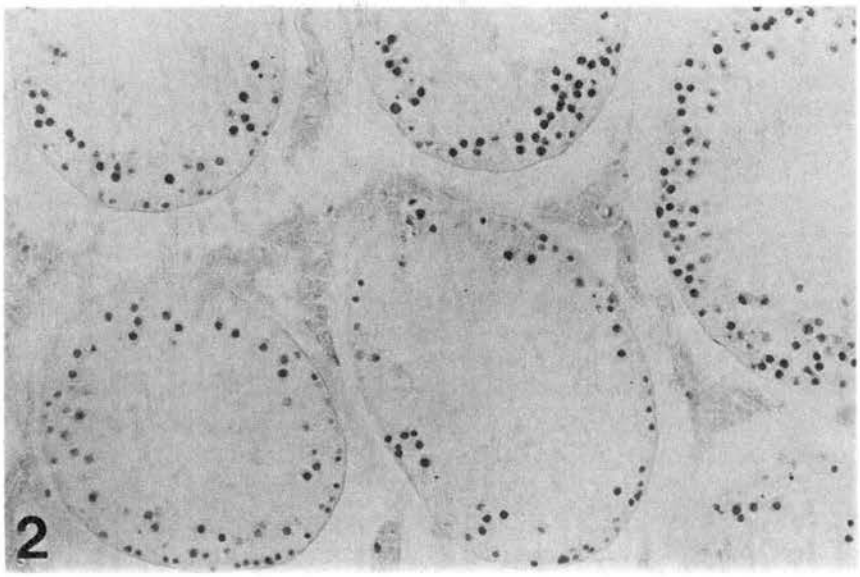
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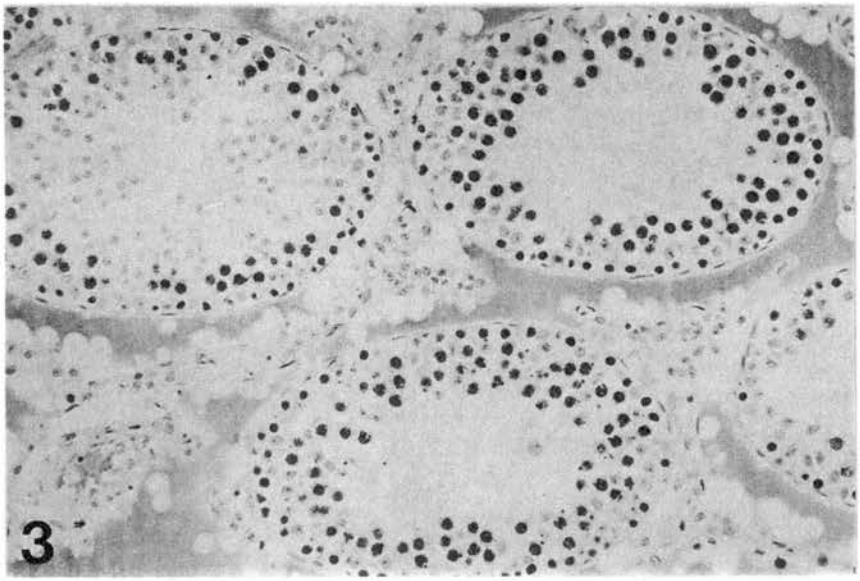
Figure 3. Immunohistochemical staining of testis from a rat given a recovery period of 10 days. There is active proliferation of germ cells in a synchronous fashion. Note the appearence of round spermatids. No counterstain. X170

Figure 4. Immunohistochemical staining of testis from a rat given a recovery period of 30 days. Staining pattern is identical to the control rat. Compare with Fig1. No counterstain. X170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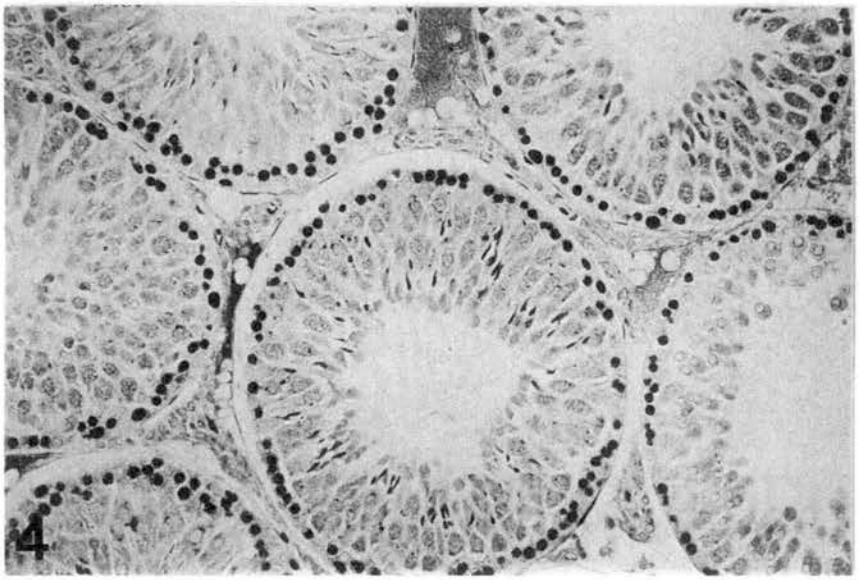
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CHAPTER V

1,3,5-TRINITROBENZENE-INDUCED ENCEPHALOPATHY IN MALE FISCHER-344 RATS

INTRODUCTION

1,3,5-trinitrobenzene (TNB) is a nitroaromatic compound and a class A explosive that is less sensitive to impact, but more powerful than 2,4,6-trinitrotoluene (TNT) (Budavari et al., 1989; Fedoroff et al., 1962). The waste waters discharged from trinitrotoluene (TNT) manufacturing processes contain large numbers of aromatic compounds, including TNB. TNB has been detected as an environmental contaminant of surface water, ground water and soil near production waste sites and at military test grounds. It is also found in aquatic systems as a by-product of biotransformation and photolysis of TNT. TNB is not easily biodegradable; it persists in the environment and can eventually leach out of soil and contaminate ground water near production waste disposal sites (Chudoba and Pitter, 1976; Garman et al., 1987; Layton et al., 1987). It is an anthropogenic environmental contaminant, exposure to TNB can occur through contact with waste-water effluents released from facilities that synthesize, produce or demilitarize munitions or from the disposal of solid TNT wastes (Ryon et al., 1984; US EPA, 1989).

Toxicity data on TNB are limited. Skin irritation, liver damage and anemia have been observed in munition workers exposed to TNB (Hathaway, 1977; Morton et al., 1976), but encephalopathy in humans or experimental animals has not been reported.

Encephalopathy induced by nitrobenzene (NB) (Bond et al., 1981; Dreshbach and Chandler, 1918; Morton et al., 1985) and 1,3 dinitrobenzene (DNB) (Philbert et al., 1987a, b) has been well documented. A single oral dose of NB causes bilaterally symmetric degeneration (malacia) and petechial hemorrhages in the cerebellum and cerebellar peduncles (Bond et al., 1981; Morgan et al., 1985). Later studies by Philbert et al (Philbert et al., 1987a) with DNB revealed no histologic alterations in conventional rats given a single oral dose (20 mg/kg), however germ free rats given the same dose had lesions in the brain sacrificed at 24 hours. There were bilaterally symmetrical vacuolated lesions in the hind brain and cerebellum. DNB and NB have been studied extensively. References to TNB-induced encephalopathy were not found in an extensive review of the literature. The aim of this report is to document the neuropathologic effects of TNB in rats.

MATERIALS AND METHODS

1,3,5-trinitrobenzene (99.83 % purity) was obtained from Naval Surface Warfare Center and the purity of the compound was confirmed by HPLC. TNB was ground to a fine powder and then mixed with corn oil to form a solution in a Potter-Elvehjem grinder. The TNB corn oil mixture was prepared daily just prior to dosing. The oral LD_{50} value for TNB in rats has been previously determined to be 284 mg/kg for combined sexes (Fitzgerald et al., 1992). The one eighth (35.5 mg/kg) and one fourth (71 mg/kg) doses were selected for these studies. Twelve male Fischer-344 rats (initial body weight 220 g) per group were randomly assigned by body weight to three exposure periods of 1 day, 4 days and 10 days. Within each exposure period, four rats were further separated into three TNB dose groups of 0, 35.5 and 71 mg/kg. The rats were gavaged with TNB in corn oil by feeding needle. Controls received the same volume of corn oil. The rats were allowed free access to water and standard commercial diet (Purina Rat Chow). All rats

were fasted for 15 hours prior to termination. At the end of each exposure period, rats were anesthetized by carbon dioxide, and whole blood was collected via cardiac puncture. The rats were euthanatized by exsanguination. Immediately after euthanasia, a complete necropsy was performed to determine weights of liver, spleen, kidneys, adrenals, testicle and brain. At necropsy, brain and the major parenchymal organs (liver, spleen, kidneys, adrenals, testicle, pancreas, lung, heart, stomach, duodenum, jejunum, ileum, cecum and colon) were collected in 10% neutral buffered formalin for histopathologic examination. All tissues were routinely processed, embedded in paraffin wax, sectioned at 5-6 microns, and stained with H & E. Sections of the brain were also stained with Luxol Fast Blue-periodic acid-Schiff Hematoxylin stain.

RESULTS

One day study

Rats which received 71 mg/kg exhibited depression, rapid breathing, pale ears, eyes and dark feet (cyanotic) within 30 minutes of dosing. The same clinical signs were exhibited an hour later by rats dosed with 35.5 mg/kg of TNB. Rats sacrificed 24 hours after single dose (35.5 or 71 mg/kg) had no gross or histologic lesions in the brain.

Four day study

Rats dosed with 71 mg/kg for 4 days showed depression, rapid breathing, pale ears, eyes and dark feet (cyanotic) within 30 minutes of dosing. Rats on 35.5 mg/kg also exhibited the same clinical signs after an hour of dosing on each day. There were no gross or microscopic lesions of the brain in either dose group.

Ten day study

Similar clinical signs (observed with rats from 24 hour and 4 day study) were exhibited soon after oral doses of 35.5 and 71 mg/kg of TNB was administered. In addition neurologic signs were also observed in three rats (3/4) dosed with 71 mg/kg of TNB. Three of the four rats exposed to 71 mg/kg of TNB developed clinical neurologic signs on days 5 to 7. Neurologic signs included walking on toes, hunched back, partial disuse of rear legs and knuckling of the feet (1 rat). In 2 of these rats, the signs disappeared by the eighth day. One rat was euthanatized on day 7 prior to the termination of the experiment due to the severity of the neurologic signs of the central nervous system. Rats orally given 35.5 mg of TNB for ten days, had neither gross or microscopic lesions in the brain nor exhibited neurologic signs.

Macroscopically all the four rats dosed with 71 mg/kg had petechial hemorrhages around the cerebellar peduncles and brain stem. On histologic examination, the cerebellar peduncles, inferior colliculi and brain stem were the only affected areas of the brain. The lesions were bilaterally symmetrical and well demarcated from the normal neuropil. The lesion is characterized by extensive vacuolation (malacia) of the white matter with neuronal necrosis (often with terminal synaptic boutons). Within this vacuolated lesion there were dense aggregates of large macrophages (gitter cells) with severe gliosis (fig 1). Along the edge of this necrotic focus, astrocytes with prominent glial filaments were present. Additionally within the vicinity (cerebellar peduncle and brain stem), there was extensive rod-shaped glial (microglial) cell proliferation. Oligodendroglial changes were not apparent. Within the vacuolated focus, mitotic figures were rarely observed. Those seen were interpreted to be of macrophageal/astrocytic origin. Frequently hemorrhage accompanied the malacic lesions. Neurons adjacent to the areas of malacia had pyknotic and hyperchromatic nuclei. Eosinophilic axonal spheroids were also observed. Expanded Virchow-Robin spaces were present around many arterioles and to a lesser extent around

venules. Few of these vessels (arterioles) had well demarcated cuffs of erythrocytes with plump endothelial cells (fig 2).

The lesions (listed in descending order of severity) were observed in medial cerebellar nuclei (nucleus fastigius), both anterior and posterior interposed cerebellar nuclei (nucleus interpositus), lateral cerebellar nuclei (dentate nucleus), vestibular nuclei (medial, lateral, superior, and spinal vestibular nuclei), dorsal cortex of inferior colliculi, olivary nuclei (lateral superior, superior paraolivary nuclei, medioventral periolivary nuclei), ventral cochlear, dorsal cochlear, pontine nuclei, paramedian reticular nuclei. Not all of these nuclei were uniformly involved in all the animals. The lesion was always present in the cerebellar nuclei, vestibular nuclei (medial and lateral), olivary nucleus (lateral superior) and inferior colliculi (3/4 rats). The severe demyelinating lesion was confined to the cerebellar nuclei and vestibular nuclei. The mild lesion was present in the olivary nuclei and inferior colliculi. In these two locations, the gliosis was very prominent, but the demyelination was minimal. Erythrocytic cuffs were also present around blood vessels with leakage of serum. Interestingly the dorsal cochlear nuclei had only perivascular hemorrhage, unlike the ventral cochlear which had the vacuolated lesion. On examining the sections stained with Luxol Fast Blue-periodic acid-Schiff-Hematoxylin the lesion was confirmed as malacia (rarefaction) with loss of myelin and intracytoplasmic periodic acid-Schiff positive material in the macrophages. Three of the four animals with the brain lesions had neurologic signs.

DISCUSSION

The present study shows that repeated administration of TNB at 71 mg/kg is neurotoxic in the rat. In this study only rats dosed with 71 mg/kg of TNB for 10 days had brain lesions (4/4). There was no evidence of brain lesions in rats receiving 35.5 mg/kg of

TNB over the same period. Similarly rats administered the same doses (71 and 35.5 mg/kg) for 1 or 4 days were free of brain lesions. The affected areas exhibited severe gliosis and numerous vacuoles, most of which could be identified as malacia (rarefaction) with loss of myelin. Petechial hemorrhages in the cerebellar peduncles and brain stem were also present. Additionally periarteriolar edema and sleeve-like arteriolar hemorrhages were observed in these regions.

The brain lesions seen after giving TNB in this study have morphologic (histologic) and topographical similarities to those produced by DNB (Philbert et al., 1987a, b; Romero et al., 1991) and NB (Bond et al., 1981; Morgan et al., 1985) which share similar chemical structure. A single oral dose of NB (550 mg/kg) induced ataxia in the rat within 24 hr, petechial hemorrhages in the brain stem and cerebellum, and bilaterally symmetrical degeneration (malacia) lateral and dorsal to the fourth ventricle involving the vestibular nuclei at 48 hours. The malacia was attributed to the edematous swelling of a membrane bound tissue compartment (Morgan et al., 1985). Similarly bilateral malacia and reactive gliosis was found in the brain of a rat which received a single dose of NB (450 mg/kg) and was sacrificed five days later (Bond et al., 1981). Histologic lesions were present in the brains of germ free rats within 24 hours after a single oral dose (20 mg/kg) of DNB, but not in conventional rats sacrificed after 24-72 hours. Multiple doses of DNB by oral or ip routes produced histologic lesions in conventional rats after 48-72 hours of dosing. Those authors observed bilaterally symmetrical vacuolated lesions involving the cerebellar roof nuclei, vestibular nuclei, inferior colliculi, superior olivary nuclei, paramedian reticular formation, ventral cochlear nuclei and nuclei of the spinal tracts of the trigeminal nerves (Philbert et al., 1987a, b). More recently Romero et al (1991) induced symmetrical brain stem lesions using a 3 x 10 mg/kg dose (oral) schedule of DNB over two days in Fischer 344 rats. They observed petechial hemorrhages and vacuolated neuropil as early as 12 hrs after the final dose. The brain damage in our experiment was evident only after 10

consecutive oral doses with TNB (71 mg/kg). The symmetrical brain stem lesions produced by TNB are analogus to the morphological changes in the rat brain caused by other similarly toxic nitroheterocyclic compounds (Bond et al., 1981; Philbert et al., 1987a). But the time and dose required for the genesis of the lesion are highly variable.

Eventhough NB, DNB and TNB share a similar chemical structure, they differ in their metabolism. Intestinal bacteria are the most quantitatively important site for NB reduction, on the contrary intestinal microflora is not essential for the reduction of DNB (Rickert, 1987). This might explain the variability in the onset of the brain lesions. Studies by Blackburn et al (1988) also give credence to this hypothesis. They reported testicular damage resulting from dosing with DNB is isomer specific. They found that 1,2-DNB and 1,4-DNB were without effect on the testis but that 1,3-DNB is a testicular toxicant. Studies with NB indicate that approximately 0.02% of the total NB administered was present in the cerebellum as the parent compound at 12 hours after administration (Morgan et al., 1985). This suggests that dose also may play a role, since rats given 35.5 mg of TNB for 10 days did not have any brain lesions. Rats dosed at 35.5 and 71 mg for 4 days also did not have any brain lesions. Since brain damage is not evident after acute exposure (4 days), a longer duration of exposure may be necessary. Perhaps a massive dose is needed for the production of these lesions. The toxicokinetic studies revealed that [^{14} C] TNB residues in the brain of rats were about 0.001% of dose (52 mg/kg) after 4 days (Reddy and Gunnarson, 1993). This suggests that multiple dose (71 mg/kg) for 10 days might have accumulated to induce neurotoxicity. NB, DNB and TNB are analogus chemical compounds producing the same morphologic picture, perhaps with a common pathogenic pathway. But the final outcome should be treated as a separate disease entity.

The formation of large vacuoles within myelin sheaths has been reported with the thiamine antagonist pyrithiamine which also induces petechial haemorrhages in the brain

stem. The changes produced by TNB resemble those described in mice treated with the thiamine antagonist pyrithiamine (Watanabe 1978; Watanabe and Kanabe, 1978; Watanabe et al., 1981a, 1981b). A similar spongy degeneration of the brain has been associated with liver disease (Hooper 1972). The reactive gliosis was more prominent with TNB. The serum enzymes alanine aminotransferase (AL), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) was normal in TNB treated rats (data not shown). The selectivity of the damage to these particular brain stem centers is less clear. It may merely be an indicator of the neuronal-glial-vascular relantionship and interdependency or to an indirect mechanism of toxicity which has yet to be determined. Little is as yet known about the biochemical events associated with the development of lesions induced by TNB. Nitroaromatic compounds can undergo redox cycling in the presence of diatomic oxygen to give the superoxide free radical (Mason and Josephy, 1985) with consequent depletion of GSH and NADPH.

The primary target for neurotoxicity of DNB are the macroglia, with swollen astrocytes and oligodendrocytes (Philbert et al., 1987a), interestingly astrocytes and oligodendrocytes were unaffected by NB (Morgan et al., 1985). In our experiment rats treated with TNB were also free from oligodendroglial changes. The reactive gliosis (and malacia) observed with TNB has been described with nitrobenzene (Bond et al., 1981), but not with dinitrobenzene by Philbert et al (1987a). The time frame might explain this variability, since rats were sacrificed at 72 hours after dosing by Philbert et al (1987a), and sufficient time had not elapsed for the tissue to respond adequately. We hypothesize that the earliest lesion with TNB would be identical to the changes observed with DNB, since one rat sacrificed 3 days prior to the termination of the experiment had minimal gliosis and a hemorrhagic lesion. Also the earliest lesion with DNB is the inferior colliculi (Romero et al., 1991), but in our experiment the lesion in the inferior colliculi was mild compared to the lesions observed in the cerebellar nuclei. Interestingly one rat sacrificed prior to the

termination of the experiment (i.e. on day 11) did not have any morphological changes in the inferior colliculi.

The cyanosis resulting from TNB administration in the rat has been found to be due to the formation of methemoglobin. TNB is a less potent inducer of methemoglobinemia than 1,3 DNB and 1,4 DNB (Watanabe et al., 1976). Tissue anoxia due to prolonged methemoglobinemia is unlikely to be directly involved in the genesis of the present lesions (Bond et al., 1981; Philbert et al., 1987a). Sodium nitrite, at dosages which produced methemoglobinemia equilvalent to that of nitrobenzene did not produce any histopathologic changes in the brain observed with NB (Bond et al., 1981). In conclusion, detailed studies on the toxicity of TNB require further investigation and chronic experimental studies with TNB should focus on the neurological damage.

REFERENCES

Bond JA, Chism JP, Rickert DE and Popp JA (1981) Induction of hepatic and testicular lesions in Fischer-344 rats by single oral doses of nitrobenzene. Fundam Appl Toxicol 1:389-394

Blackburn DM, Gray AJ, Lloyd SC, Sheard CM, and Foster PMD (1988) A comparison of the effects of the three isomers of dinitrobenzene on the testis in the rat. Toxicol Appl Pharmacol. 63:120-132

Budavari S, O' Neil M.J, and Smith A (1989) The Merck Index. Merck & Comapny, Inc., Rahway, NJ, pp. 1530

Chudoba J, and Pitter D (1976) Biological purification of wastewaters from nitrobenzene production. Chem Prum 26:541-544

Dreshbach M, and Chandler WL (1918) The toxic action of nitrobenzene, with special reference to the cerebellum. Proc Soc Exp Biol Med 15:136-137

Fedoroff BT, Sheffield OE, Reese EF, and Clift GD (1962). Encyclopedia of Explosives and Related Items, PATR 2700. Vol 2, Picatinny Arsenal, Dover, NJ, pp. B48-B49

Fitzgerald G B, DiGuilio N, Desai LS, Reddy G (1992) Acute toxicity evaluation of 1,3,5-trinitrobenzene.Part B. Acute Toxicity Data. J Amer Coll Toxicol. 1:169-170

Garman JR, Freund T and Lawless EW (1987) Testing for ground water contamination at hazardous waste sites. J Chromat Sci 25:328-337

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

Hooper PT (1972) Spongy degeneration in the brain in relation to hepatic disease and ammonia toxicity in domestic animals. Vet Res 90:37-38

Layton D, Mallon B, Mitchell W, Hall L, Fish R, Perry L, Snyder G, Bogen K, Malloch W, Ham C, and Dowd P (1987) Data-base Assessment of the Health and Environmental. Effects of Conventional Weapons Demilitarization: Explosive and Their Co-contaminants. Draft Report, Project Order 83PP3818. Livermore, CA: Lawrence Livermore National Laboratory

Mason RP and Josephy PD (1985) Free radical mechanism of nitroreductase. In: Toxicity of Nitroaromatic Compounds, Rickert DE (ed) (CIIT), Hemisphere, NY, pp 121-140

Morgan KT, Gross EA, Lyght O and Bond JA (1985) Morphologic and biochemical studies of a nitrobenzene-induced encephalopathy in rats. Neurotoxicology 6:105-116

Morton AR, Ranadive MV and Hathaway JA (1976) Biological effects of trinitrotoluene from exposure below thw threshold limit value. Am Ind Hyg Assoc J 37:56-60

Philbert MA, Nolan CC, Cremer JE, Tucker D and Brown AW (1987a) 1,3-Dinitrobenzene-induced encephalopathy in rats. Neuropath Appl Neurobio 13:371-389

Philbert MA, Gray AJ and Connors TA (1987b) Preliminary investigations into the involvement of the intestinal microflora in CNS toxicity induced by 1,3- Dinitrobenzene in male F-344 rats. Toxicol Lett 38:307-314

Reddy G and Gunnarson AE (1993) Toxicokinetics of ^{14}C-1,3,5-trinitrobenzene in F-344 rats after oral administration. Toxicologist 13: 179

Rickert DE (1987) Metabolism of nitroaromatic compounds. Drug Metb Rev 18:23-53

Romero I, Brown AW, Cavanagh JB, Nolan CC, Ray DE, and Seville MP (1991) Vascular factors in the neurotoxic damage caused by 1,3,-Dinitrobenzene in the rat. Neuropathol Appl Neurobiol 17:495-508

Ryon MG, Pal BC, Talmage SS and Ross RH (1984) Database assessment of the health and environmental effects of munition production waste sites. Final report. ORNL-6018. (NTIS DE84-016512). Oak Ridge National Laboratory, Oak Ridge, TN

US EPA (1989) Health and environmental effects profile for 1,3,5-Trinitrobenzene. Environmental criteria and assessment office, Office of health and environmental assessment, Cincinnati, OH, ECAO-CIN-G071

Watanabe I (1978a) Pyrithiamine-induced acute thiamine-deficient encephalopathy in the mouse. Exp Mol Pathol 28:381-394

Watanabe I and Kanabe S (1978b). Early edematous lesion of pyrithiamine induced acute thiamine deficient encephalopathy in the mouse. J Neuropathol Exper Neurol 37: 401-413

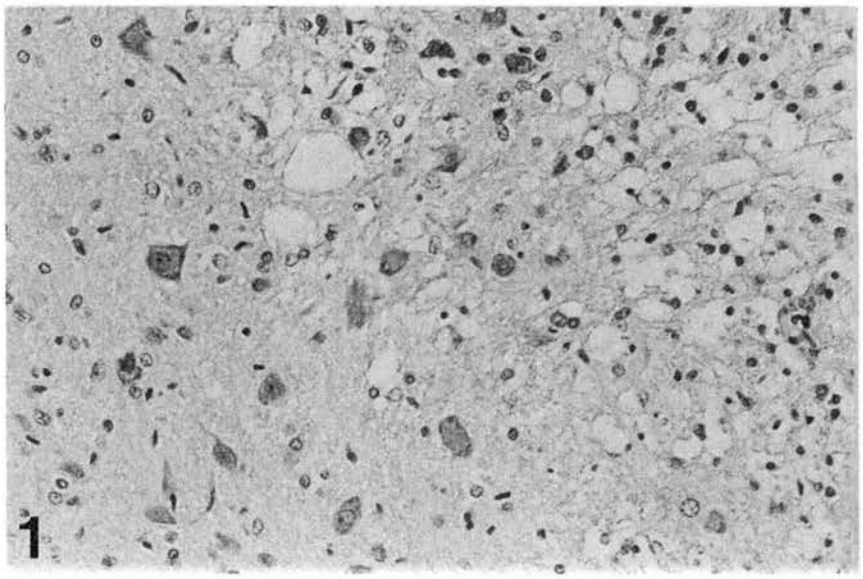
Watanabe I, Iwasaki Y, Aikawa H, Satoyoshi E, and Davis JW (1981a) Hemorrhage of thiamine deficient encephalopathy. J Neuropathol Exper Neurol 40:566-580

Watanabe I, Tomita T, Hung KS and Iwasaki Y (1981b) Edematous necrosis in thiamine deficient encephalopathy of the mouse. J Neuropathol Exper Neurol 40:454-471

Watanabe T, Ishihara N, and Ikeda M (1976) Toxicity of and biological monitoring for 1,3 diamino-2,4,6 TNB and other nitro-amino derivatives of benzene and chlorobenzene. Intl Arch Occup Environ Health 37:157-168

Figure 1. Photomicrograph of the nucleus interpositus. Note the focus of malacia comprised of numerous vacuoles containing infiltrates of glial cells. HE. X175

Figure 2. Photomicrograph of the medial vestibular nuclei. Hemorrhagic blood vessel with expanded Virchow-Robin space and extravasated erythrocytes forming cuffs. The erythrocytes also infiltrate the vacoulated neuropil. HE. X180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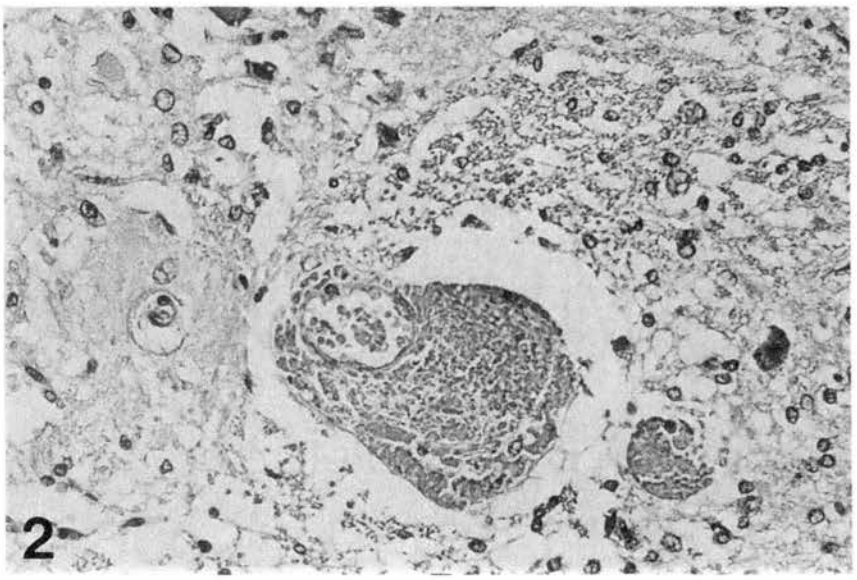
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CHAPTER VI

NEUROTOXICITY OF 1,3,5-TRINITROBENZENE (TNB): IMMUNOHISTOCHEMICAL STUDY OF CEREBROVASCULAR PERMEABILITY, NEURONAL DAMAGE AND GLIAL REACTION

INTRODUCTION

The nitroaromatic compound 1,3,5-trinitrobenzene (TNB) is used primarily in explosive compositions and munitions, and is also a by-product of both 2,4,6-trinitrotoluene (TNT) synthesis and photolysis. Previously we have reported encephalopathy with TNB which is manifested morphologically as bilaterally symmetrical malacia, gliosis and circumscribed hemorrhage, with a unique topographic distribution in the brain of rats (Chandra et al., 1995).. The lesion was dorsal and lateral to the fourth ventricle involving the cerebellar nuclei, medial and lateral vestibular nuclei, and inferior colliculi. Furthermore, the distribution of the lesions within the CNS are reminiscent of those of pyrithiamine-induced acute thiamine-deficient encephalopathy (Watanabe, 1978), 1,3-dinitrobenzene (DNB) encephalopathy (Philbert et al., 1987) and nitrobenzene encephalopathy (Morgan et al., 1985).

In conjunction with the scant general toxicity data on TNB, the pathogenesis of the CNS lesion is poorly understood. It has been suggested that the vascular bed may play an important role in the pathogenesis of the DNB-induced encephalopathy (Romero et al., 1991). Prior studies with the structurally analogous nitroaromatics DNB or

nitrobenzene have not addressed the breakdown of the blood brain barrier (BBB) in the evolution of these tissue changes.

During the past few years, a number of experimental and clinical studies have suggested that extravasated plasma constituents may exert a harmful effect on the brain tissue. The association between leakage of plasma constituents across the BBB (vasogenic edema) on the one hand and nerve cell death on the other hand has been demonstrated in several animal studies. Salahuddin et al (1988) showed that opening of the BBB by intracarotid infusions of hyperosmolar solutions caused nerve cell injury in areas where leakiness of the BBB was evident from extravasation of plasma proteins. Fredriksson et al (1988a) found cytolytic neurodegeneration in areas that showed leakage of plasma proteins in stroke prone hypertensive rats. Short-lasting (transient) blood brain barrier opening induced by adrenaline infusion, aortic clamping, epileptic seizures (Sokrab et al., 1988a, 1988b, 1990), or cerebral infarction (Nordborg et al., 1991), may cause neuronal damage with a spatial relationship to the extravasation of plasma proteins. Cerebellar Purkinje cells that are known to degenerate in epileptic patients are among those which are most heavily exposed to plasma constituents after epileptic seizures (Sokrab et al., 1990). Extravasation of plasma proteins has been demonstrated with the type D epsilon toxin of *Clostridium perfringens* (Finnie and Hajduk, 1992). Additionally it has been shown that rat albumin *per se* is neurotoxic in a concentration-dependent manner when injected into rat neostriatum (Hassel et al., 1994). Taken together these studies suggest that plasma components may be neurotoxic. Immunohistochemistry is an established method for demonstrating extravasated serum-proteins in histological sections (Salahuddin et al., 1988; Sokrab et al., 1988a; Loberg and Torvik 1991, 1992; Loberg et al., 1992, 1993).

Astrocytes play a role in the maintenance of the BBB (Janzer and Raff, 1987), homeostasis of water and ion balances (Kimelberg, 1983; Hertz and Schousboe, 1975) and repair after tissue damage (Montogomery, 1994). Astrocytic response can be evaluated by immunohistochemical staining for the intermediate filament, glial fibrillary acidic protein (GFAP) (Norenberg, 1994).

This study focused upon, 1) relationship between the selective topographic distribution of the TNB-induced lesions and vascular permeability to albumin in the tissue changes, and 2) the long term sequelae.

MATERIALS AND METHODS

Chemicals

1,3,5-trinitrobenzene (99.83 % purity) was obtained from Naval Surface Warfare Center (Silver Springs, MD) and the purity of the compound was confirmed by HPLC. TNB was ground to a fine powder and then mixed with corn oil to form a solution in a Potter-Elvehjem grinder. The TNB corn oil mixture was prepared daily just prior to dosing. The oral LD_{50} value for TNB in rats has been determined to be 284 mg/kg for combined sexes (Fitzgerald et al., 1992). The one-fourth dose (71 mg/kg) was selected for this study since brain damage was not evident at the one-eighth dose (35.5 mg/kg) in our earlier experiment (Chandra et al., 1995).

Animals

After a 2 week acclimatization to laboratory conditions (12 h light/12h dark cycle, $72^0 \pm 2^0$ F, 50±10% relative humidity), F-344 rats (initial body weight 220 g) were ranked by body weight and randomly distributed among the experimental groups. The animals were

housed one per cage and were provided with Purina Laboratory Chow and tap water *ad libitum*.

Experimental

A histopathologic evaluation was done after multiple oral doses of TNB at 71 mg/kg. Thirty five male Fischer-344 rats were randomly assigned by body weight to seven experimental groups (5/group). Within each experimental group four rats were gavaged with 71 mg/kg of TNB and the control rat was gavaged with corn oil (vehicle). Rats in groups I, II, III, IV, and V, were killed 24 hours after 4, 5, 6, 8, and 10 doses of TNB respectively. The remaining 10 rats were used to study the long term sequelae. Rats in group VI and VII were killed after 10 consecutive daily oral doses of TNB (at 71 mg/kg) with a recovery period of 10 and 30 days respectively. At the end of each exposure period, rats were deeply anesthetized with sodium pentobarbital (20-30 mg/kg) and perfused transcardially with 10% neutral buffered formalin. The skull was opened, and the brain was left in-situ until the next day when it was removed from the skull and immersed in the same fixative.

Histology

After removal, each brain was carefully sectioned into 2.0 mm thick coronal slices (according to Paxinos and Watson 1986) with a sharp razor blade for macroscopic examination. The brains slices were then dehydrated, routinely processed and embedded in paraffin. Paraffin sections were cut at 4-6 μm and stained with hematoxylin and eosin (H&E). Parallel serial sections of the brain were prepared for immunohistochemical studies.

Immunohistochemistry ABC Staining Procedure

For immunohistochemisry 4-6 μm thick paraffin-embedded (serial) sections were immunohistochemically stained to detect albumin and GFAP. Tissue sections were placed onto poly-L-lysine coated slides and dried overnight. The following day the sections were dewaxed in xylene and hydrated via a graded ethanol series. Endogenous peroxidase activity was blocked with a 30 minute incubation in methanol containing 3% hydrogen peroxide. Between the remaining steps, sections were washed twice for 10 minutes each in isotonic phosphate buffered saline (pH = 7.4). Sections were pre-incubated for 30 minutes in normal goat serum to decrease nonspecific binding. Excess serum was blotted before primary antibody application. For the determination of extravasated serum albumin, sections were incubated with rabbit anti-rat albumin (1:16000) (Cappel, Organon Teknika Corporation, Durham NC, Catalog No. 55711). The glial reaction was demonstrated with antiserum to bovine glial fibrillary acidic protein (1:1000) which cross reacts with rat GFAP (product information, Dako Corp., Carpinteria, CA). The incubation with the primary antibodies (albumin and GFAP) was carried out at room temperature for 60 minutes. The bound antibodies were visualized with a commercially available avidin-biotin-peroxidase complex (Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA). Immunolabelled peroxidase was visualized by using the commercially available chromogen Vector VIP (Vector Laboratories, Burlingame, CA) which yields an intense purple color. Harris' hematoxylin was used as a light nuclear counterstain. After a brief rinse with tap water, sections were dehydrated and coverslipped.

The specificity of the immunohistochemical reaction was evaluated by the omission of individual steps. Normal rabbit serum was substituted instead of the primary antibody (i.e. rabbit anti-albumin or anti-GFAP). Other control procedures included omission of biotinylated secondary antibody and avidin-biotin peroxidase complex. Additionally, sections from all the rats were immunohistochemically stained (on the same day) using the same diluted antibody solution throughout to avoid variation in staining between sections.

RESULTS

Clinical Signs

The earliest clinical signs were observed approximately 30 minutes after dosing every day. The TNB-treated rats showed depression, rapid breathing, and cyanotic ears, eyes and feet. These clinical sign were due to the high methemoglobin levels observed after TNB exposure, which have been reported earlier (Chandra et al., 1995). The rapid breathing and depression usually subsided 2-3 hours after dosing. The cyanotic ears, eyes, and feet were visible up to 5 hours after dosing.

A rat belonging to group VI started showing clinical signs of neurotoxicity after receiving four doses of TNB. This rat showed cardinal signs of TNB encephalopathy, which included walking on toes and hunched back. With progression of time, the rat became recumbent and died. Rats killed after 4, 5, 6, and 8 doses (i.e. groups I-IV) did not exhibit any clinical signs related to the central nervous system (CNS), except for the methemoglobinemia described earlier. On day 9 a rat belonging to group VI (10 day recovery) and group VII (30 day recovery) exhibited the cardinal signs including a generalized depression and flaccidity in the rear legs. On day 10 (i.e. after 10 doses) all treated rats in groups V, VI, and VII exhibited signs ranging from mild head shaking, walking on toes, and a pivotal movement (keeping the rear legs stationary, while moving in semi-circles with the fore legs), which was probably related to the flaccid paralysis of the rear legs. The fore limb grip reflex, righting reflex, and pinch reflex (in limbs and tail) did not appear to be impaired at any point in time. Signs persisted 24 hours after the last dose in these rats. During the recovery phase, rats in groups VI and VII had minimal clinical signs only on days 11-13, including dullness, lethargic movements and subtle tremors. No clinical signs were apparent during the subsequent recovery period in either group.

Gross Findings

There were no gross lesions in the brain of rats belonging to groups I-IV. All four TNB-treated rats in group V had grossly visible, symmetrical petechial hemorrhages around the cerebellar roof nuclei (cerebellar peduncle) and the inferior colliculi. The cerebral cortex was spared of hemorrhages. Hemorrhages (or other abnormalities) were not visible macroscopically in rats killed after a 10 day (group VI) or 30 day recovery (group VII) period.

General Histology

The brain of all control rats were histologically normal. Histopathologic examination of the brains from rats killed after 4, 5, 6, and 8 doses (i.e. groups I-IV) of TNB had no abnormalities. Rats killed after 10 daily doses (group V) had vacuolated neuropil and widened Virchow-Robin spaces containing erythrocytes. The vacuolated lesions were confined to the cerebellar roof nuclei, medial and lateral vestibular nuclei, superior olivary nuclei and inferior colliculi. These lesions observed at 10 days, have been documented previously (Chandra et al., 1995) and will not be restated here. In the group VI rats, killed after a 10 day recovery period, resolution of the brain damage was evident. The affected foci in the cerebellar roof nuclei, vestibular nuclei, olivary nuclei and inferior colliculi (the predominantly affected regions), of the brain had an intense dense infiltrate of foamy macrophages and glial cells (Fig 1 & 2). Occasional shrunken intensely eosinophilic (necrotic) neurons were observed in the resolving necrotic foci. Yellow pigment (hemosiderin) was present within vessel walls. Also a few scattered Purkinje cells were intensely eosinophilic and shrunken with a clear halo around the cell boundary. In the group VII rats, killed after a 30 day recovery period, lesion resolution was complete, and vacuolation was minimal. The infiltrate of macrophages and glial cells was attenuated in two of the four rats. Few neurons in the susceptible nuclei were shrunken and eosinophilic

(Fig 3). The most dramatic change was observed in the cerebellum. There was scattered loss of Purkinje cells in the cerebellar folia leaving gaping holes and the cerebellum had a spongiotic appearance (Fig 4). This necrosis and loss of Purkinje cells was not uniform or diffuse, since normal healthy Purkinje cells were occasionally present next to dead or lost cells. Rarely, the cerebellar granular cells were also necrotic. The cerebral cortex, including the hippocampus, did not have any histopathologic changes in any treated rats.

Immunohistochemistry

Albumin immunoreactivity

The brain of all control rats sacrificed at varying times were immunohistochemically normal. Immunoreactive albumin was present in the meninges, choroid plexus and some vessel walls in the neuropil of the control rats. The ependymal cells rarely stained positive. Neurons and glia were never stained in the controls. Rats killed after 4, 5, and 6 doses of TNB had a staining pattern identical to the control rats (Table 1). In 2 of the 4 rats killed after 8 doses of TNB, immunoreactive albumin was faintly detected in the neuropil around the inferior colliculi and the olivary nuclei. Neurons and glial cells did not take up the stain.

In group V (10 doses) in addition to the vacuolated lesion observed on routine H&E stain, there was a marked increase in the intensity and distribution of immunoreactive albumin in all four treated rats. Widespread areas of purple reaction product were seen in the cerebellar peduncle and the brain stem. The extravasation of albumin was not confined solely to those regions having a vacuolated appearance. Instead, the reaction product had a centrifugal spread to a limited distance into the white matter of the adjacent cerebellar folia and diffusely throughout the brain stem. Rat albumin immunoreactivity was present in the meninges and in the choroid plexus (positive

control). Numerous vessels, particularly arterioles, contained an extensive amount of immunoreactive albumin in the vessel wall (Fig 5). Immunoreactivity in the vessels was particularly pronounced in those regions of the brain susceptible to damage by TNB (cerebellar roof nuclei, inferior colliculi and olivary nuclei). An intense reaction product was present in arterioles as halos in the perivascular spaces. The neurons in the vacuolated lesion had intraneuronal albumin, which varied between individual cells. Some neurons contained purple fine cytoplasmic granules exclusively in the cell body sparing the nucleus and nucleolus (granular pattern) (Fig 6). These neurons had normal shape and size. The other pattern was observed in the shrunken neurons, wherein immunoreactvity completely obliterated nuclear and cytoplasmic detail (diffuse pattern) (Fig 6). Glial cells (astrocytes, oligodendrocytes and microglia) in the spongy lesion contained albumin; however this was inconsistent among rats. In addition to the neurons in the vacuolated foci, the cerebellar Purkinje cells also had granular and diffuse patterns of immunoreactivity (Fig 7). The staining of the Purkinje cells was confined only to those cells in the immediate vicinity of the cerebellar roof nuclei indicating a centrifugal spread. Albumin positive Purkinje cells were brightly eosinophilic and somewhat distorted in morphology on the H&E stained sections. The cerebellar granule cells and the molecular layer did not stain positively for albumin, even though the Purkinje cells and the white matter were positive. The entire cerebral cortex was notably spared of immunoreactivity in all four rats.

In contrast to the group V rats, group VI rats (i.e. those killed after a 10 day recovery period), the immunoreactivity to albumin was no longer present as a diffuse pattern in neuropil. Immunoreactivity to albumin in these rats appeared as scattered granular purple debris between and within the numerous macrophages, glial cells and new capillaries. It is noteworthy that the granular immunoreactivity to albumin was confined only to those regions of the brain susceptible to TNB encephalopathy. The staining

intensity of neurons and Purkinje cells varied among the rats, with some showing intense immunoreactivity to albumin and others faint. Animals given a 30 day recovery period (group VII) did not have any albumin immunoreactivity in the susceptible regions of the brain or in the neurons. These rats exhibited identical immunoreactivity to that observed in the control rats.

GFAP Immunoreactivity

In rats belonging to groups I-IV, the GFAP immunoreactivity appeared as faintly staining fibers scattered throughout the entire brain (Table 1). An identical staining pattern was also observed in the control rats of all the groups. Rats killed after 10 doses, had increased GFAP immunoreactivity characterized by the presence of markedly hypertrophic astrocytes with numerous prominent cell processes (Fig 8). These reactive astrocytes were present in the white matter of the cerebellar folia, and the margins of the vacuolated lesions. There was a complete loss of GFAP staining within vacuolated foci. In group VI rats (10 day recovery), a partial glia limitans characterized by intense immunoreactivity and thick bundles of glial filaments delineated the vacuolated foci from the adjacent neural tissue (Fig 9). In addition, reactive astrocytes were densely distributed in the surrounding neural tissue. Rats belonging to group VII (30 day recovery), had variable immunoreactivity to GFAP. Although not intense, astrocytic processes were more prominent in the cerebellar roof nuclei, ventral cochlear nuclei, and olivary nuclei.

DISCUSSION

The observations in this study confirm our previous clinical and histological descriptions of the encephalopathy with TNB. The TNB induced encephalopathy is accompanied by widespread vacuolation and necrosis of the neuropil in white and gray matter of the brain confined to the cerebellar roof nuclei, vestibular nuclei, inferior colliculi, and olivary nuclei. In the present investigation we identified the regional distribution of the TNB induced lesions with the immunohistochemical stain for albumin.

In the present study, we used an immunohistochemical reaction for extravasated serum albumin as a method of evaluating BBB. An exogenous tracer like horseradish peroxidase (HRP) was used to study vascular permeability in encephalopathy reported with DNB (Romero et al., 1991), nitrobenzene (Morgan et al., 1985), and pyrithiamine (Watanabe et al., 1981). The immunohistochemical method is considered to be the most sensitive one available for the detection of extravasated albumin and is valuable for morphological investigations. This method detects the extravasation of endogenous serum protein, which is the most direct marker *in vivo*, and is thought to reflect the accumulation of extravasated serum albumin up to the time when animals are killed (Chui et al., 1981; Kitagawa et al., 1991).

BBB and extravasation of plasma proteins

The results of this study indicated that a transient opening of the BBB (increased cerebrovascular permeability) occured in rats treated with TNB for 10 days. The extravasation occurred both in the gray and white matter of the susceptible regions/nuclei, with spread of plasma proteins in the surrounding tissue to variable distances. Similarly, transient opening of the BBB, leading to brain damage has been reported with infusion of hyperosmolar solutions (Salahuddin et al., 1988), hypertension (Sokrab et al., 1988b;

Fredricksson et al., 1988a), adrenaline induced hypertension (Sokrab et al., 1988a), epileptic seizures (Johansson and Nilsson, 1977; Mihaly and Bozoky, 1984), cerebral ischemia (Kitagawa et al., 1991; Loberg et al., 1993), and bicuculline-induced experimental seizures (Sokrab et al., 1990).

After 10 days of recovery, the immunoreactivity was present as granular debris, and at 30 days post treatment, immunoreactivity was confined to the choroid plexus and meninges (similar to the controls). These results indicating a complete restoration of the BBB. A practical question at this point is whether the BBB in the 30 day recovered animals had been opened at all? In the absence of extravasated albumin, other observations from this group and 10 day recovered animals confirm that BBB had been opened. In these rats the speckled immunoreactivity indicating active clearance of protein debris at 10 day posttreatment indicate a transient opening of the BBB, and more importantly the loss (necrosis) of neurons and Purkinje cells confirms previous irreversible neuronal damage. One can postulate that initial damage was followed by a complete closure of the BBB, disposal of extravasated proteins, and normalization of the eventual cellular changes. Identical results were obtained by Sokrab et al (1988b), where extravasated serum albumin was not detected 7 days after an episode of acute hypertension, and also in rats subjected to experimental seizures with bicuculline (Sokrab et al., 1990). Kitagawa et al (1991) did not detect albumin in the neuropil or neurons 3 or 7 days after cerebral ischemia in gerbils even though other changes were present. Studies with isotope-labeled albumin indicated that extravasated proteins are cleared from the brain within a short time after a transient opening of the BBB (Lorenzo et al., 1972).

Endothelial cell damage or a frank vascular rupture was not observed in this study or in our previous study. Even though the mechanism of action of TNB is not known, we believe that vascular distention leading to increased permeability of the cerebral

vasculature is the most likely cause. The increased blood flow in the brain of rats treated with the analogous compound DNB (Romero et al., 1991); and the use of organic nitrates, nitrites, and nitrosocompounds like nitro-glycerin, sodium nitroprusside as therapeutic vasodilators (Murad, 1990), suggest that the vasodilatation due to TNB may be linked to its exaggerated pharmacological action. An alternative hypothesis is the possibility that the guanylyl cyclase:nitric oxide vasodilator system (Collier and Vance, 1989) may have been stimulated by the altered redox potential within endothelial cells caused by TNB intoxication. Vascular distension has been implicated in the opening of the BBB by hyperosmolar solutions (Salahuddin et al., 1988). The vascular leakage probably occurred between separated endothelial cells as reported for HRP (Brightman et al., 1970; Nagy et al., 1979; Lehtosalo et al., 1982) or possibly by vesicular transfer as well (Lehtosalo et al., 1982). Very close similarities exist between TNB induced encephalopathy and pyrithiamine induced encephalopathy (Wernicke's encephalopathy) in mice (Watanabe et al., 1978). Hemorrhagic lesions and increased vascular permeability (to HRP) were observed by Watanabe et al (1981) in these mice. However transmission electron microscopy revealed intact endothelial cells and tight junctions. To explain this phenomena, the authors suggest a transendothelial transport as the likely route (Watanabe et al., 1981).

Fate of extravasated proteins

Neurons, including Purkinje cells, were prime targets for the extravasated proteins. The neuronal and glial uptake of albumin was usually seen within and close to points of extravasation, but strongly positive neurons (and Purkinje cells) were also present outside the actual leakage sites. Accumulation of protein tracers and albumin in neurons have been observed repeatedly in various experimental models of BBB damage (Brightman et al., 1970; Klatzo et al., 1962; Nag, 1984; Olsson and Kristensson, 1979; Tengvar, 1986; Chui et al., 1981; Sokrab et al., 1988a, 1988b; Salahuddin et al., 1988). In these studies,

the possibility was emphasized that transient opening of the BBB induced neuronal damage.

The distribution of albumin within the neurons in our study was similar to other reports (Salahuddin et al., 1988; Sokrab et al., 1988a, 1988b; Loberg and Torvik 1991, 1992; Loberg et al., 1992, 1993). The albumin immunostaining in the neurons was both granular (cytoplasmic only) and diffuse (entire cell). It is suggested that neurons with cytoplasmic albumin represent living cells which have accumulated proteins in lysosomes after vesicular transfer across the plasma membrane (Loberg and Torvik, 1991; Kristensson, 1984; Kristensson and Olsson, 1971; LaVail and LaVail, 1972; Mesulam, 1982; Tengvar, 1986), whereas the diffuse form is likely the result of a protein influx in a cell with a severe cell membrane injury (Tengvar and Olsson, 1982; Clark, 1984; Klatzo et al., 1962; Olsson and Hossman 1970). In agreement with the interpretation that albumin is actively taken up by living cells, a large number of neurons which had a granular (only cytoplasmic) staining with the anti-albumin serum looked normal. On the other hand, many neurons with diffuse immunoreactivity for albumin appeared shrunken and stained intensely with eosin suggesting a more severe injury. The mechanism proposed by Kitagawa et al (1991) to explain neuronal staining include a) passive diffusion through the damaged membrane as observed in the dead neurons, b) damaged neuronal processes and c) active uptake to reduce the vasogenic edema. The authors further state the granular immunostaining with intact nuclei are viable and responding actively to the extravasation. If the BBB opening persists for a longer period, these neurons could become irreversibly damaged. Hence the pathological mechanism of the irreversible nerve cell injury is related to the extravasation of plasma constituents.

Glial reaction

Astrocytic response as assessed by immunohistochemical staining for GFAP has not been evaluated earlier with the analogous nitro-heterocyclic compounds DNB or nitrobenzene. GFAP immunoreactivity is present in normal rat brain at a moderate level (Bignami and Dahl, 1974), but increases progressively after brain damage. The most dramatic features of the acute lesion caused by TNB at 10 days is the astroglial hypertrophy and the strict limitation of this change to the defined brain areas. Serum entry into the neural tissue was one trigger for the glial response (Norenberg, 1994) as demonstrated by the fact that GFAP-staining coincided with the regions showing albumin extravasation. The second phase at 10 day post recovery had a glia limitans with thick filaments entrapping the resolving foci. This distribution is similar to that reported with alpha-chlorohydrin toxicity (Cavanagh et al., 1993), traumatic stab injury (Bignami and Dahl, 1976, Bignami et al., 1980) and around cysts in hypertensive rats (Fredricksson et al., 1988b). In this situation several tissue factors could be liberated which are known to stimulate glial cells, including hormones, second messengers, and several macromolecules (Nieto-Sampedro et al., 1985; Duffy, 1983; Cancilla et al., 1992). At 30 day post recovery the GFAP immunoreactivity, though moderate, was seen within more remote areas of the cerebellar peduncle and brain stem. This is probably related to factors stimulating glial proliferation (Giulian et al., 1986; Giulian and Young, 1986) or neuronal and dendritic injury (Yamamoto et al., 1986). These observations suggest a complex relationship between edema formation, neuronal injury, and glial response.

The results of this study confirms the causal relationship between the extravasation (BBB breakdown) and the development of the changes. The presence of widespread vacuolation and associated extravasated serum proteins within the cerebellum and brain stem of TNB treated rats is an indication of vasogenic brain edema. The immunohistochemical demonstration of extravasated serum proteins confirms vasogenic

edema as opposed to cytotoxic edema in which no protein extravasation is present (Klatzo, 1967). Further disruption of the blood brain barrier appears to be a critical event in TNB toxicity with neuronal damage following later.

In conclusion, we successfully established a reproducible encephalopathy in rats administered TNB and investigated albumin extravasation and glial reaction by immunohistochemical technique. In this study, we clarified the following points. 1) rats receiving daily oral dose of TNB had a 'transient' opening of the BBB characterized by leakage of protein. 2) distribution of albumin occurred selectively in regions susceptible to damage by TNB. 3) areas adjacent to extravasation contained neurons immunopositive for albumin, leading to permanent neuronal injury and loss. 4) gliotic response coincided with the vasogenic edema and vacuolated (spongy) lesion.

REFERENCES

Auer RN, Kalimo H, Olsson Y, and Siesjo BK (1985) The temporal evolution of hypoglycaemic brain damage. Light and electron microscopic findings in the rat cerebral cortex. Acta Neuropathol 67:13-24

Bignami A and Dahl D (1974) Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. J Comp Neurol 153:27-36

Bignami A and Dahl D (1976) The astroglial response to stabbing. Immunofluorescence studies with antibodies to astrocyte-specific protein (GFA) in mammalian and submammalian vertebrates. Neuropathol Appl Neurobiol 2:99-110

Bignami A, Dahl D and Rueger DC (1980) Glial fibrillary acidic protein (GFA) in normal neural cells and in pathological conditions. Adv Cell Neurobiol 1:285-319

Brightman MW, Klatzo I, Olsson Y, Reese TS (1970) The blood-brain barrier to proteins under normal and pathological conditions. J Neurol Sci 10:215-239

Cancilla PA, Bready J, and Berliner J (1992) Expression of mRNA for glial fibrillary acidic protein after experimental cerebral injury. J Neuropathol Exp Neurol 51:560-565

Cavanagh JB, Nolan CC and Seville MP (1993) The neurotoxicity of alpha-chlorohydrin in rats and mice: 1. Evolution of the cellular changes. Neuropathol and Appl Neurobiol 19:240-252

Chandra AMS, Qualls CW Jr. and Reddy G (1995) 1,3,5-Trinitrobenzene induced encephalopathy in male Fischer-344 rats . Toxicol Pathol (In Press)

Chui E, Wilmes F, Sotelo JE, Horie R, Fujiwara K, Suzuki K, and Klatzo I (1981) Immunocytochemical studies on extravasatoin of serum proteins in cerebrovascular disorders. In: J. Cervos-Navarro and E. Fritschka (eds.). Cerebral Microcirculation and Metabolism, Raven Press, New York, pp. 121-127

Clark PG (1984) Identical population of phagocytes and dying neurons revealed by intravascularly injected horseradish peroxidase,and by endogenous glutaraldehyde-resistant acid phosphatase, in the brains of chick embryos. Histochem J 16:955-969

Collier J and Vance P (1989) Second messenger role for nitric oxide widens to neurons and immune systems. Trends Pharm Sci 10:428-431

Duffy PE (1983) Astrocytes. Normal Reactive and Neoplastic. Raven Press, New York

Finnie JW and Hajduk P (1992) An immunohistochemical study of plasma albumin extravasation in the brain of mice after the administration of Clostridium perfringens type D epsilon toxin. Aust Vet J 69:261-262

Fitzgerald G B, DiGuilio N, Desai LS, Reddy G (1992) Acute toxicity evaluation of 1,3,5-trinitrobenzene. J Amer Coll Toxicol Part B. Acute Toxicity Data. 1:169-170

Fredriksson K, Kalimo H, Nordborg c, Johansson BB and Olsson Y (1988a) Nerve cell injury in the brain of stroke-prone hypertensive rats. Acta Neuropathol 76:227-237

Fredriksson K, Kalimo H, Nordborg c, Johansson BB and Olsson Y (1988b) Cyst formation and glial response in the brain lesions of stroke-prone hypertensive rats. Acta Neuropathol 76:441-450

Giulian D, Allen R, Baker A, and Tomozawa Y (1986) Brain peptides and glial growth I. Glia-promoting factors as regulators of gliogenesis. J Cell Biol 102:803-811

Giulian D, and Young D (1986) Brain peptides and glial growth II. Identification of cells that secrete glia-promoting factors. J Cell Biol 102:812-820

Hassel B, Iversen EG and Fonnum F (1994) Neurotoxicity of albumin in vivo. Neurosci Lett 167:29-32

Hertz L and Schousboe A (1975) Ion and energy metabolism of the brain at the cellular level. Int Rev Neurobiol 18:141-211

Janzer RC and Raff MC (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. Nature 325:253-257

Johansson BB and Nilsson B (1977) The pathophysiology of the blood-brain barrier dysfunction induced by severe hypertension and by epileptic brain activity. Acta Neuropathol 38:153-158

Kimbelberg HK (1983) Primary astrocyte cultures-a key to astrocyte function. Cell Mol Cardiol 3:1-16

Kitagawa K, Matsumoto M, Tagaya M, Ueda H, Oku N, Kuwabara K, Ohtsuki T, Handa N, Kimura K, and Kamada T (1991) Temporal profile of serum albumin extravasation following cerebral ischemia in a newly established reproducible gerbiil model for vasogenic edema: a combined immunohistochemical and dye tracer analysis. Acta Neuropathol 82:164-171

Klatzo I, Miquel J, Otenasek R (1962) The application of fluorescein labelled serum protein (FLSP) to the study of vascular permeability in the brain. Acta Neuropathol 2:144-160

Klatzo I (1967) Neuropathological aspects of brain edema. J Neuropathol Exp Neurol 26:1-14

Klatzo I, Chui E, Fujiwara K and Spatz M (1980) Resolution of vasogenic brain edema Adv in Neurol 28:359-373

Kristensson K and Olsson Y (1971) Retrograde axonal transport of protein. Brain Res 29:363-365

Kristensson K (1984) Retrograde signalling after nerve injury. In: Elam JS, Cancalon P (eds) Axonal transport in neuronal growth and regeneration. Plenum, New York, pp. 31-43

LaVail JH and LaVail MM (1972) Retrograde axonal transport in the central nervous system. Science 176:1416-1417

Laursen H, HansenAK, and Sheardown M (1993) Cerebrovascular permeability and brain edema after cortical photochemical infarcts in the rat. Acta Neuropathol 86:378-385

Lehtosalo J, Panula P and Laitinen LA (1982) The permeability alteration of brain and spinal cord vasculature to horseradish peroxidase during experimental decompression sickness as compared to alteration in permeability induced by hyperosmolar solution. Acta Neuropathol 57:179-187

Loberg EM and Torvik A (1993) Neuronal uptake of plasma proteins in brain contusions An immunohistochemical study. Acta Neuropathol 84:234-237

Loberg EM, Brorson SH, Skjorten F, and Torvik (1992) Neuronal uptake of plasma proteins in cryogenic brain lesions. An immunelectron microscopic study. APMIS 100:1033-1040

Loberg EM and Torvik A (1991) Uptake of plasma proteins into damaged neurons. An experimental study on cryogenic lesions in rats. Acta Neuropathol 81:479-485

Loberg EM, Karlsson BR, and Torvik A (1993) Neuronal uptake of plasma proteins after transient cerebral ischemia/hypoxia. Immunohistochemical studies on experimental animals and human brains. APMIS 101:777-783

Lorenzo AV, Shirahige I, Liang M, Barlow CF (1972) Temporary alterations of cerebrovascular permeability to plasma protein during drug-induced seizures. Am J Physiol 231:483-488

Mesulam MM (1982) Tracing neuronal connections with horseradish peroxidase. In: Smith AD (ed) Methods in the neurosciences. Wiley, Chichester

Mihaly A and Bozoky B (1984) Immunohistochemical localization of extravasated serum albumin in the hippocampus of human subjects with partial and generalized epilepsies and epileptiform convulsions. Acta Neuropathol 65:25-34

Montgomery DL (1994) Astrocytes: Form, functions, and roles in disease. Vet Pathol 31:145-167

Morgan KT, Gross EA, Lyght O and Bond JA (1985) Morphologic and biochemical studies of a nitrobenzene-induced encephalopathy in rats. Neurotoxicology 6:105-116

Murad F (1990) Drugs used for the treatment of angina: Organic nitrates, calcium channel blockers and β adrenergic antagonists. In: Goodman and Gilman's The Pharmacological Basis of Thereapeutics, Gilman AG, Rall TW, Nies AS, and Taylor P (Eds). Pergamon Press, Elmsford, New York

Nag S (1984) Cerebral changes in chronic hypertension: combined permeability and immunohistochemical studies. Acta Neuropathol 62:178-184

Nagy Z, Pappius HM, Mathieson G, Huttner I (1979) Opening of tight junctions in cerebral endothelium. 1. Effect of hyperosmolar mannitol infused through the internal carotid artery. J Comp Neurol 158:569-578

Nevander G, Ingvar M, Auer R, Siesjo BK (1985) Status epilepticus in well-oxygenated rats causes neuronal necrosis. Ann Neurol 18:281-290

Nieto-Sampedro M, Saneto RP, deVellis J, and Cotman CW (1985). The control of glial populations in brain: Changes in astrocytic mitogenic and morphogenic factors in response to injury. Brain Res 343:320-328

Nordborg C, Sokrab TEO, and Johansson BB (1991) The relationship between plasma protein extravasation and remote tissue changes after experimental brain infarction. Acta Neuropathol 82:118-126

Norenberg MD (1994) Astrocyte responses to CNS injury. J Neuropathol Expl Neurol 53:213-220

Olsson Y, and Hossman KA (1970) Fine structural localization of exudated protein tracers in the brain. Acta Neuropathol 16:103-116

Olsson Y, and Kristensson K (1979) Recent applications of tracer techniques to neuropathology with paricular referance to vascular permeability and axonal flow. Recent Adv Neuropathol 1:1-26

Paxinos G and Watson C (1986) The rat brain in stereotaxic coordinates. Academic Press, San Diego, CA

Persson L, Hansson HA, Sourander P (1976) Extravasation, spread, and cellular uptake of Evan's blue-labelled albumin around a reproducible small stab-wound in the rat brain. Acta Neuropathol 3:125-136

Philbert MA, Nolan CC, Cremer JE, Tucker D, and Brown AW (1987) 1,3-Dinitrobenzene-induced encephalopathy in rats. Neuropathol Appl Neurobiol 13:371-389

Romero I, Brown AW, Cavanagh JB, Nolan CC, Ray DE and Seville MP (1991) Vascular factors in the neurotoxic damage caused by 1,3-dinitrobenzene in the rat. Neuropathol Appl Neurobiol 17:495-508

Salahuddin TS, Kalimo H, Johansson BB and Olsson Y (1988) Observations on exudation of fibronectin, fibrinogen and albumin in the brain after carotid infusion of hyperosmolar solutions. An immunohistochemical study in the rat indicating longlasting changes in the brain microenvironment and multifocal nerve cell injuries. Acta Neuropathol 76:1-10

Schmidt-Kastner R, Szymas J, and Hossmann KA (1990) Immunohistochemical study of glial reaction and serum-protein extravasation in relation to neuronal damage in rat hippocampus after ischemia. Neurosci 38:527-540

Sokrab TEO, Johansson BB, Tengvar C, Kalimo H, and Olsson Y (1988a) Adrenaline-induced hypertension: morphological consequences of the blood-brain disturbance. Acta Neurol Scand 77:387-396

Sokrab TEO, Johansson BB, Kalimo H, and Olsson Y (1988b) A transient hypertensive opening of the blood brain barrier can lead to brain damage. Extravasation of serum proteins and cellular changes in rats subjected to aortic compression. Acta Neuropathol 75:557-565

Sokrab TEO, Kalimo H, and Johansson BB (1990) Parenchymal changes related to plasma protein extravasation in experimental seizures. Epilepsia 31:1-8

Tengvar C (1986) Extensive intraneuronal spread of horseradish peroxidase from a focus of vasogenic edema into remote areas of central nervous system. Acta Neuropathol 71:177-196

Tengvar CH, and Olsson Y (1982) Uptake of macromolecules in neurons from a focal vasogenic brain edema and subsequent axonal spread to other brain regions. A preliminary study in the mouse with horseradish peroxidase as a tracer. Acta Neuropathol 57:233-235

Vorbrodt AW, Lossingsky AS, Wsniewski HM, Suzuki R, Yamaguchi T, Maseoka H, and Klatzo I (1985) Ultrastructural observations on the transvascular route of protein removal in vasogenic edema. Acta Neuropathol 66:265-273

Watanabe I (1978) Pyrithiamine-induced acute thiamine-deficient encephalopathy in the mouse. Exp Mol Pathol 28, 381-394

Watanabe I, Iwasaki Y, Aikawa H, Satoyoshi E, and Davis JW (1981) Hemorrhage of thiamine deficient encephalopathy. J Neuropathol Exp Neurol 40:566-580

Yamamoto K, Yoshimine T, Homburger HA, and Yanagihara T (1986) Immunohistchemical investigation of regional cerebral ischemia in the gerbil: Occlusion of the posterior communicating artery. Brain Res 371:244-252

Table 1. Histopathological pattern of lesions in F-344 rats orally administered TNB at 71 mg/kg

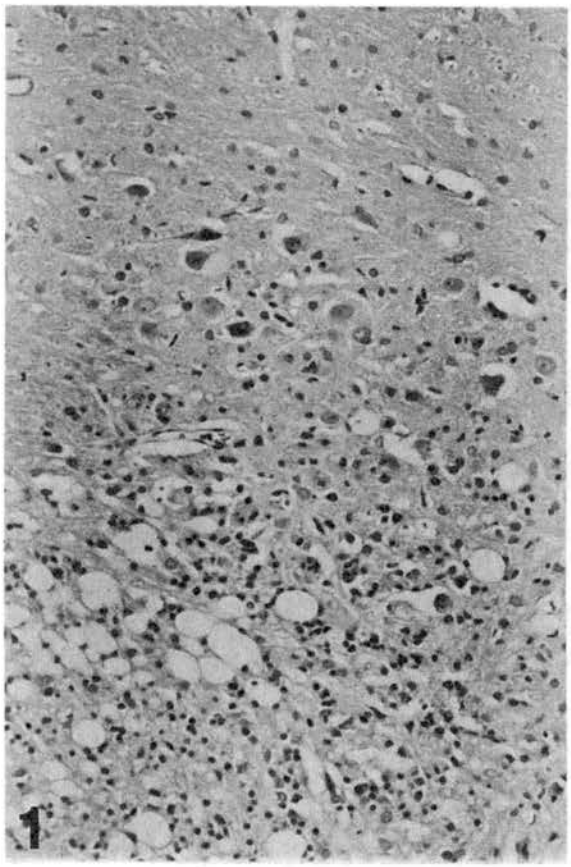
Exptl. gps.	I	II	III	IV	V	VI	VII
Doses of TNB	4	5	6	8	10	10 + 10d recovery	10 + 30d recovery
Neurologic signs	no	no	no	no	yes*	yes*	yes*
H&E lesion	none	none	none	none	Vacuolated neuropil, necrotic neurons	Vacuoles, intense aggregates of gitter cells and glia, necrotic neurons	Necrotic Purkinje cells and neurons. Spongiosis of cerebellum
Anti-albumin (neuropil)	-	-	-	+ (2/4), minimal focal	+ (4/4), severe, diffuse	+ (3/3), moderate scattered debris	-
Anti-alumin (neuronal)	-	-	-	-	+ (4/4), intense, multi-focal	+ (3/3), attenuated, random	-
Anti-GFAP	-	-	-	-	+ (4/4), reactive, hypertrophied	+ (3/3), glia limitans, thick filaments	+ (4/4), intense scattered glia

* see text for explanation.

Figure 1. Dense aggregates of macrophages and glial cells in the resolving necrotic focus from a rat given a 10 day recovery. Note the adjacent less affected neuropil. H&E. X160

Figure 2. A higher magnification of the same lesion as in Fig 1. H&E. X260

Figure 3. Dark shrunken neurons (arrowhead) of cerebellar roof nuclei from a rat given a 30 day recovery period. The vacuolation is negligible. Gliosis is mild. Compare with fig 1. H&E. X160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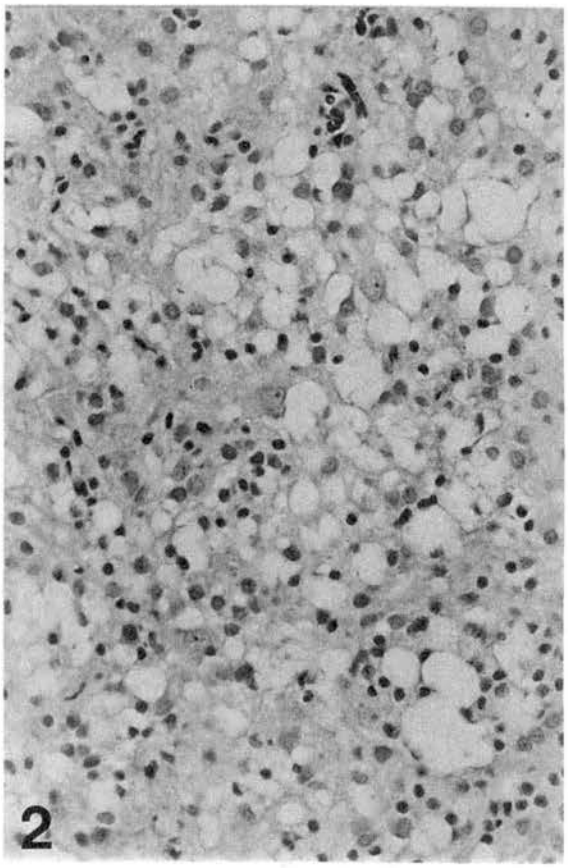
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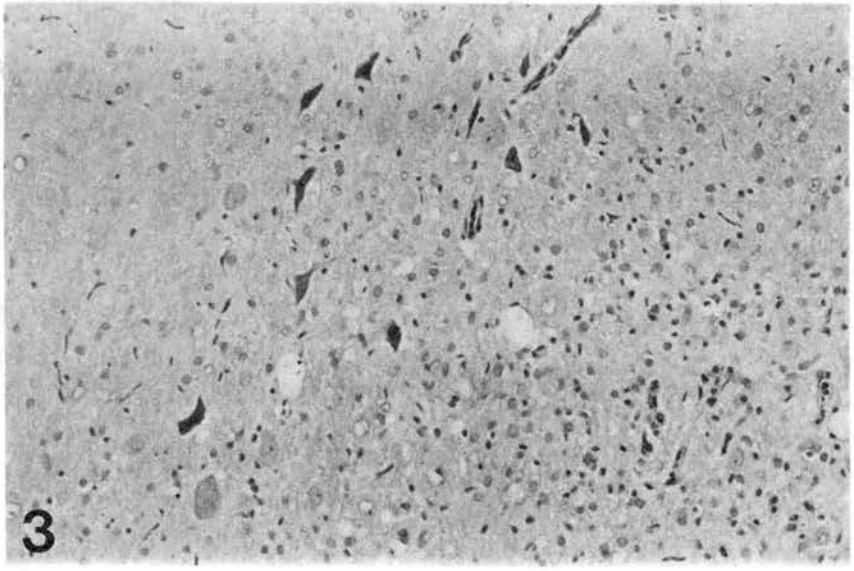
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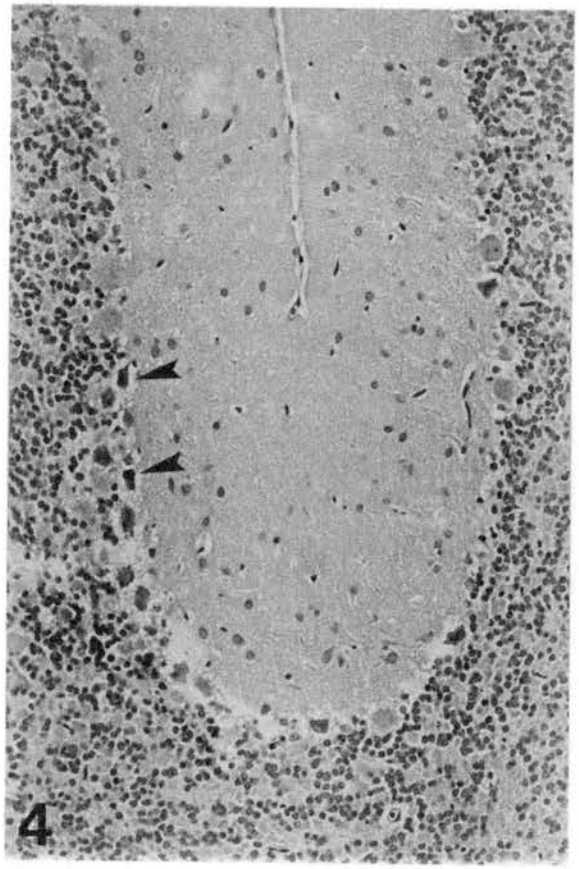
Figure 4. A typical lesion in the cerebellum from a rat given a 30 day recovery period. There is loss of Purkinje cells. Pyknotic Purkinje cells (arrowheads), which stained negatively with albumin. H&E. X160

Figure 5. Pronounced albumin immunoreactivity in vessel from a rat killed after 10 doses of TNB. Arrowheads indicate albumin positive necrotic neurons. Anti-rat albumin. Hematoxylin counterstain. X300

Figure 6. Immunostained neurons from the olivary nucleus in a rat killed after 10 doses of TNB. Note the granular (arrow) and diffuse staining (arrowhead) of the cells. Anti-rat albumin. Hematoxylin counterstain. X160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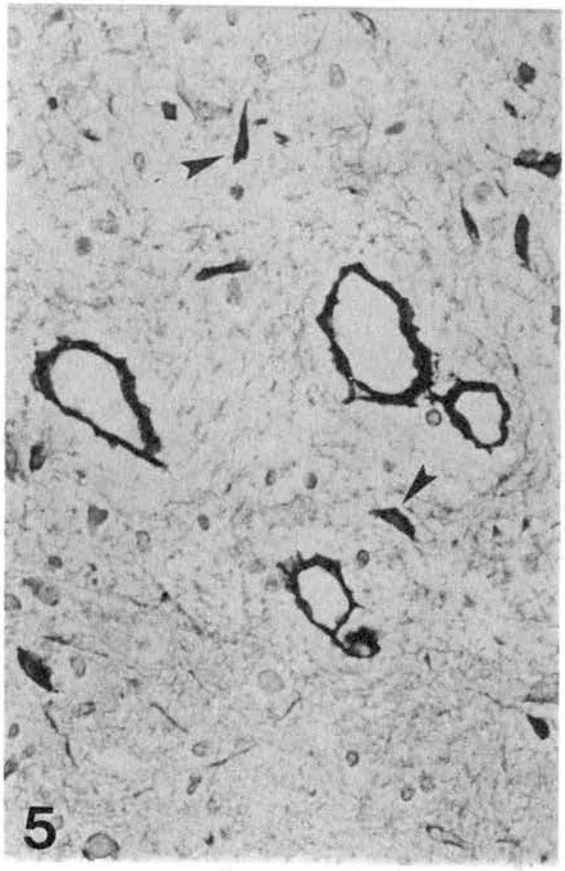
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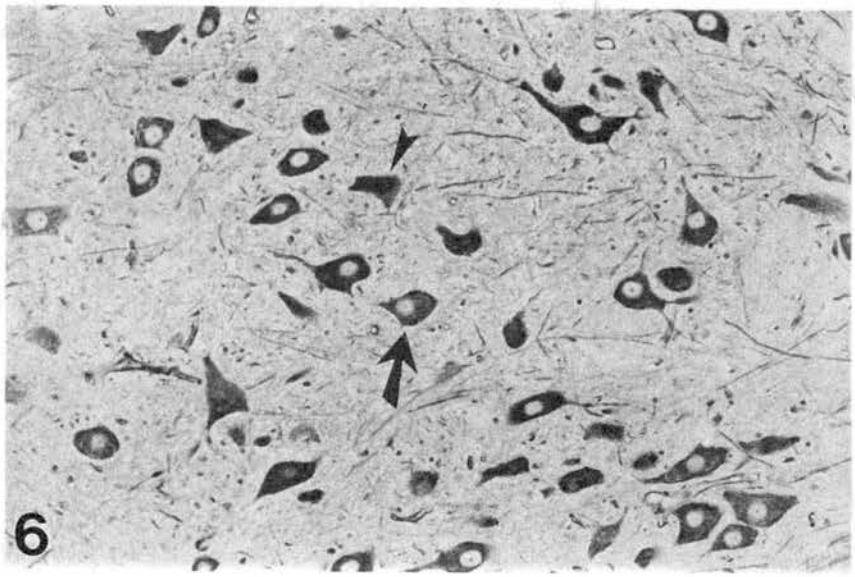
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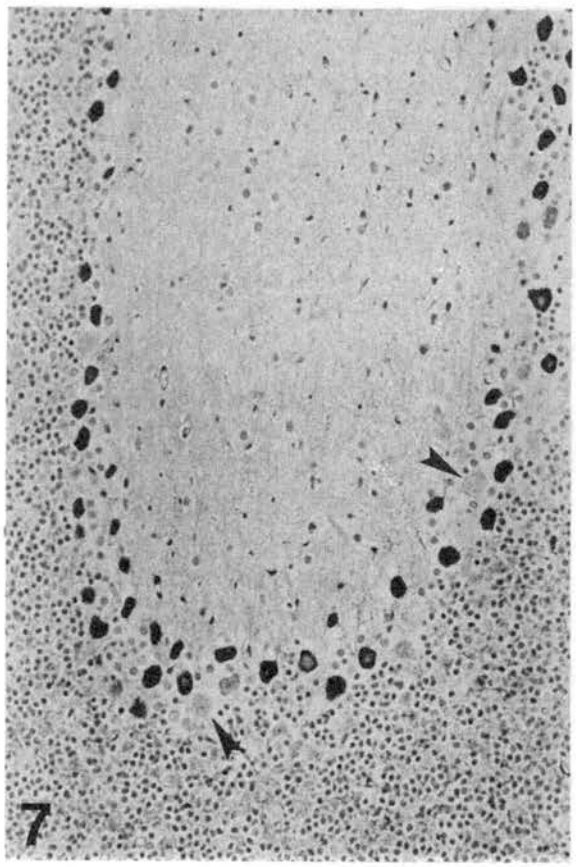
Figure 7. Immunostained Purkinje cells in a rat killed after 10 doses of TNB. Negatively staining Purkinje cells (arrowheads) are amidst the positive Purkinje cells. Anti-rat albumin. Hematoxylin counterstain. X160

Figure 8. Astrocytic response after 10 doses of TNB. Note the negative staining in the lesion (asterisk). Anti-GFAP. Hematoxylin counterstain. X130

Figure 9. Astrocytic response in a rat given a 10 day recovery. Note the sharp demarcation between the thick glial filaments and the resolving focus. Anti-GFAP. Hematoxylin counterstain. X200



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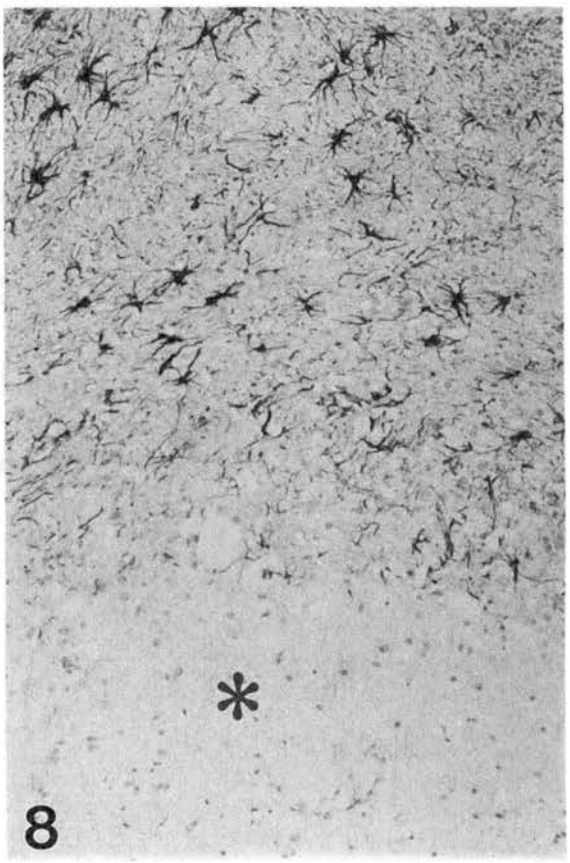

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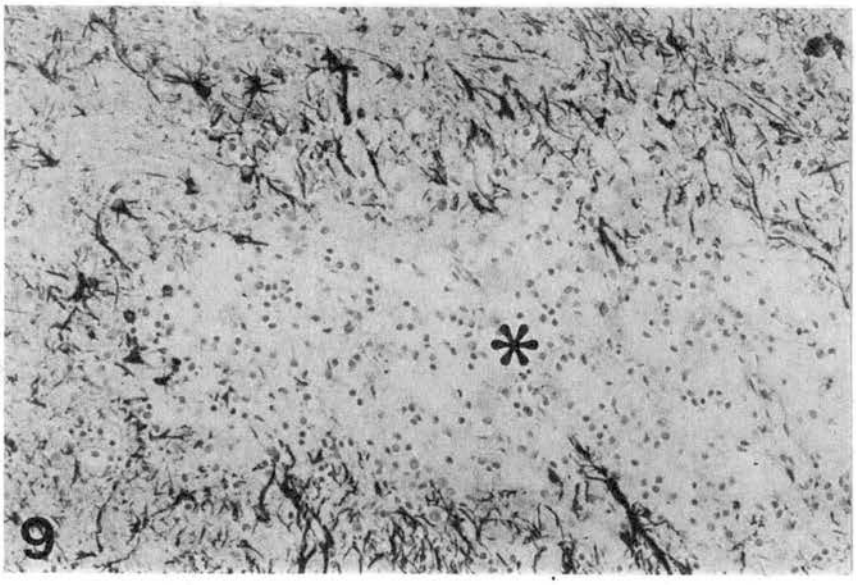

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CHAPTER VII

SUMMARY AND CONCLUSIONS

1,3,5-Trinitrobenzene (TNB) is a nitroaromatic compound that is usually associated with the production of munitions and armaments. TNB has been classified as a high explosive and it is used in military and commercial explosive compositions. TNB can enter the environment in wastewater effluents from facilities that synthesize, produce or demilitarize munitions, or from the disposal of solid 2,4,6-trinitrotoluene (TNT) wastes. TNB is classified as an EPA hazardous waste. TNB has been identified in 14 of the 1300 hazardous waste sites on the National Priorities List (NPL). An anthropogenic environmental contaminant, exposure to TNB can occur through contact with wastewater effluents released from facilities that synthesize, produce or demilitarize munitions, or from the disposal of solid TNT wastes..

Toxicity data on oral, dermal, or inhalation exposure of experimental animals or humans to TNB are limited to a few Russian and English reports. Most of the toxicity data derived for TNB is by analogy to two structurally similar compounds 1,3-dinitrobenzene and 2,4,6-trinitrotoluene. Therefore the broad objectives of this study were to characterize the acute toxic effects in rats orally exposed to TNB.

The specific objectives of this study were to characterize the hematological effects, testicular effects and neurological effects and elucidate the mechanism of toxicity for the observed effects. Structurally analogous nitroaromatic compounds exert their principle affected with TNB also.

The first objective was to characterize the hematological effects both in vivo and in vitro. Male F-344 rats were gavaged with TNB at 35.5 and 71 mg/kg in corn oil. Blood was collected 5 h and 24 h after a single oral dose or 24 h after daily oral doses for 4 or 10 d in four different set of experiments. A dose-dependent methemoglobinemia was present only in blood collected 5 h after a single dose. A highly significant dose-dependent anemia with reduced red cells, hemoglobin, and hematocrit was present in rats receiving TNB for 4 or 10 d. A dose dependent decrease in serum triglyceride was present in rats receiving TNB for 10 d. To determine whether TNB is an inducer of methemoglobinemia in vitro, rat erythrocytes were incubated with TNB for 9 h. There was a progressive increase in methemoglobin formation in vitro. These results suggest, that unlike nitrobenzene, TNB can form methemoglobin in vitro and cecal microbial metabolism is not a prerequisite. There was no hemolysis when red cells were incubated with TNB. These results suggest that TNB is not directly hemolytic even with high methemoglobin levels and a different mechanism was probably responsible for the hemolytic anemia.

Further studies were conducted to elucidate a possible mechanism for the hemolytic anemia. Since methemoglobinemia and hemolytic anemia were salient features of TNB toxicity, it was hypothesized that TNB causes denaturation of hemoglobin forming hemichromes leading to premature destruction of red cells. Hemichromes can cross-link the major erythrocyte membrane-spanning protein, band 3, into clusters which provide the recognition site for antibodies directed against the erythrocyte. Experiments were conducted in vitro to determine whether TNB can cause hemichrome formation. Changes induced in hemoglobin by addition of TNB were spectroscopically recorded to identify hemichromes. Repetitive scanning of the TNB spectra revealed the formation of methemoglobin and hemichromes. There was only methemoglobin formation with sodium nitrite used as a negative control. Based on the in vivo and in vitro results, a hypothesis for the hemolytic anemia was proposed for the first time with nitroaromatics.

Testicular toxicity of TNB are confined to a few abstracts and detailed histopathologic evaluations were lacking. In this study testicular effects of TNB were characterized after single and multiple doses of TNB. Testicular effects were not evident at the light microscope level in rats killed after a single dose of TNB or after 4 daily doses at 35.5 mg/kg of TNB. Rats receiving 4 daily doses of TNB at 71 mg/kg had the earliest evidence of testicular damage with necrosis and degeneration of pachytene spermatocytes including a significant decrease in testicular weight. When rats were dosed at 35.5 mg/kg for 10 days, severe testicular lesions were present, in addition to the decrease in testicular weight. There was degeneration of round and elongate spermatids, and formation of multinucleate syncytial cells. The epididymis was devoid of sperm, instead contained exfoliated syncytial spermatids. Rats dosed at 71 mg/kg of TNB for 10 days had testicular atrophy and cessation of spermatogenesis. Histopathologic examination of the ventral prostate revealed apoptic cells. To assess the extent of reversibility in these atrophied testis, rats were allowed to recover for 10 or 30 days after 10 doses of TNB. A significant regenerative attempt with proliferating spermatocytes were present at 10 days and elongate spermatids were evident at 30 days. These reversibility studies indicate testicular effects of TNB are partially reversible.

The closely related compound 1,3-dinitrobenzene exerts its principle effect on the Sertoli cell and it was hypothesized that Sertoli cell is the primary target with TNB also. To understand more about the target cell, testicles were immunohistochemically stained using proliferating cell nuclear antigen (PCNA) which detects cells in the S-phase. Testicles from treated and control rats had PCNA positive spermatogonia and spermatocytes. PCNA positive cells were also observed in atrophied testis. These results suggest that spermatogonia is probably not a target cell and spermatogonial mitosis occurs

even in the absence of spermatogenesis. This gave credence to the hypothesis that germ cell loss was an indirect effect due to Sertoli cell dysfunction.

Neurotoxicity of nitroaromatic compounds is of particular importance. Encephalopathy has been reported with analogous compounds nitrobenzene and 1,3-dinitrobenzene, but not with TNB. Male F-344 rats treated with TNB at 71 mg/kg for 10 days had clinical signs of neurotoxicity with head tilt, ataxia, hind-limb splay, and walking on toes. Histologic examination of the brain from these rats revealed a structural damage associated with the functional change. Light microscopic examination revealed petechial hemorrhages in the brain stem and cerebellum, bilaterally symmetric degeneration and necrosis (malacia) with reactive gliosis in the cerebellar peduncles. The malacia was lateral and dorsal to the fourth ventricle involving the cerebellar nuclei, medial and lateral vestibular nuclei, and inferior colliculi. Blood vessels associated with the lesion had widened Virchow spaces, occasionally with extravasated erythrocytes.

Further experiments were conducted to elucidate the pathogenesis of the encephalopathy. The cerebral vasculature has been suggested to play an important role in the pathogenesis of the 1,3-dinitrobenzene-induced encephalopathy. This study was conducted to understand the contribution of the blood brain barrier (BBB) and to assess the long term sequelae. In this experiment F-344 rats were killed after 4, 5, 6, 8, or 10 daily doses of TNB (71 mg/kg). Also 5 rats were allowed to recover for 10 or 30 days after receiving 10 doses. Integrity of the BBB was assessed immunohistochemically for extravasated plasma albumin on paraffin sections. Serial sections were stained for glial fibrillary acidic protein (GFAP) and with H&E. Rats killed after 4-8 doses had no lesions. In the 10 dose group, lesions were multifocal often confluent foci of extravasated albumin

in susceptible nuclei. Albumin was present in vascular walls, extracellular space, neurons and Purkinje cells. Immunoreactivity in neurons was granular representing pinocytic uptake or diffuse due to nerve cell injury with uncontrolled albumin leak into the cytoplasm. Shrunken hypereosinophilic neurons were seen in areas of albumin extravasation with sponginess of the neuropil. There was no GFAP staining in the vacuolated foci, with hypertrophied astrocytes around the foci. After 10 d of recovery, vacuolated foci were infiltrated by glial and gitter cells. Albumin immunoreactivity was present as granular debris and neuronal staining was decreased. A glia limitans around the resolving foci had thick bundles of GFAP positive astrocytic processes. In rats allowed a 30 d recovery, immunoreactivity to albumin was not seen and GFAP was mild. Instead, these rats had necrotic neurons in the susceptible nuclei and loss of Purkinje cells. These observations demonstrated a causal relationship between increased vascular permeability (vasogenic edema) and neuronal damage. Further disruption of the BBB appears to be a critical event with neuronal damage following later.

VITA

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