

Naval Health Research Center Detachment (Toxicology)

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**ABSORPTION, DISTRIBUTION, AND CLEARANCE OF
2,6-Di-tert-Butyl-4-nitrophenol (DBNP)**

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PREFACE

This technical report summarizes the findings of the research project entitled "Toxicokinetic Evaluation of 2,6-DI-*tert*-butyl-4-nitrophenol (DBNP) through oral gavage in male rats." The research described in this technical report began in February 2001 and was completed in April of the same year. This research was supported by the Office of Naval Research (ONR) under the direction of the Officer-in-Charge (OIC) of the Toxicology Detachment, Naval Health Research Center, CAPT Kenneth R. Still, MSC, USN. The objective of this project was to gain information on the distribution and excretion of DBNP post an oral gavage. The study was carried out at NHRC/TD Building 433 Area B Wright-Patterson Air Force Base Ohio 45422-7903 by Navy and contract scientists under the scientific supervision of CAPT Kenneth R. Still, MSC, USN (principle investigator) and G. Bruce Briggs, DVM (co-investigator). The animals used in this study were treated in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, DHHS, Publication No. (NIH) 86-23 (1996), and the Animal Welfare Act of 1966 as amended. The opinions expressed herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Navy or the Naval Service at large.

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PREFACE

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ABBREVIATIONS

¹⁴ C-DBNP	Ring-labeled 2,6-Di- <i>tert</i> -Butyl-4-Nitrophenol-UL- ¹⁴ C
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DBNP	2,6-DI- <i>tert</i> -butyl-4-nitrophenol
DBP	2,6-DI- <i>tert</i> -butylphenol
DMSO	Dimethyl sulfoxide
DPM	Disintegration per minute
EBD	General Dynamics Electric Boat Division
H ₂ O ₂	Hydrogen Peroxide
HPLC	High pressure liquid chromatography
ip	Intraperitoneal
iv	Intravenous
Na-EDTA	ethylenediaminetetraacetic acid sodium salt
NHRC/TD	Naval Health Research Center Toxicology Detachment
ppb	parts per billion
ppm	parts per million

INTRODUCTION

In 1992, the Navy Environmental Health Center in Norfolk, VA was informed of about the discoloration or yellowing of the interiors in the US submarine fleet and possible exposure of Navy personnel to this unknown substance. The discoloration was predominant on the bulkheads of underway submarines but was apparent throughout the ship. It would preferentially accumulate on all internal painted surfaces but could also be found on dishes, glasses, flatware, bedding, etc. In extreme cases, the outlines of sleeping sailors could be visible on the bed sheets and a progressive yellowing of the skin of some submariners was reported. The submarine surface yellowing was reported to General Dynamics Electric Boat Division (EBD), who determined that the phenomenon was related to presence of the nitrophenol, 2,6-Di-*tert*-butyl-4-Nitrophenol (DBNP, CAS #728-40) (General Dynamics, 1992). DBNP is an intensely yellow crystalline material (melting point = 157°C) resulting from the nitration of 2,6-di-*tert*-butylphenol (DBP). Nitration occurs when oil mist vapor/aerosol from hydraulic lines (containing DBP) passes through the electrostatic vent fog precipitators used to remove particulate oil and other contaminants from the submarine atmosphere. DBP is used as an antioxidant additive to 2190 TEP synthetic steam turbine lubricating oils (MILSPEC-L-17331H), as well as some synthetic hydraulic fluids. While provisions have been made to remove DBP from the manufacturing specifications of 2190 oils, the stockpile of DBP-containing 2190 lube oil is large enough that the potential for Navy personnel to be exposed to DBNP will exist for several more years.

EBD data indicated that although the yellowing was most pronounced in submarine engineering compartments, DBNP was capable of movement throughout the submarine *via* the ventilation system. The air concentrations of DBNP in several locations in the submarine ranged from less than 3 ppb to 13 ppb. Laboratory simulations of the submarine environments found DBNP concentrations as high as 122 ppb. A recent replication of the EBD study (Alexander *et al.*, 2001) indicated that airborne DBNP concentrations of less than 2 ppb to 122 ppb were present.

Both DBP and DBNP may function physiologically as uncouplers of mitochondrial oxidative phosphorylation, resulting in inhibition of mitochondrial respiration and ATP production (Jonsson *et al.*, 1985; de las Alas, 1990; Castilho, 1997). Oxidative phosphorylation of ADP to ATP in the mitochondria is highly dependent on the maintenance of a proton electrochemical gradient generated by the inner

mitochondrial membrane for respiration. If the mitochondrial membrane is rendered permeable to protons by a protonophoric reagent such as DBNP, the organelle is no longer capable of sustaining ATP synthesis. As a result, mitochondrial respiration and ATP synthesis is uncoupled, leading to increased respiratory rates and excess energy that is released as heat (Hemker, 1962 de las Alas, 1990). Further, uncoupling of oxidative phosphorylation may alter the production of oxygen reactive species (Okuda *et al.*, 1992). Clinical effects (i.e. prostration, extreme hyperthermia, muscle rigidity, and death) seen in rats exposed to DBNP are consistent with this hypothesis (Rivera-Nevarés *et al.*, 1995; MacMahon *et al.*, 1998, 1999; Alexander *et al.*, 2001). Rivera-Nevarés *et al.* (1995) estimated that DBNP was second only to 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile (SF 6847, Yoshikawa *et al.*, 1980) in its action as an ATP uncoupler, based on quantum-mechanical calculations.

Because DBNP is known to be highly lipophilic and thus nearly insoluble in water, it is assumed that the human risk from dermal or respiratory exposure is minimal. Indeed, previous research suggests a very low toxicity for acute dermal exposure (up to 1000 mg/kg) in rats (Vesselinovitch *et al.*, 1961; MacMahon *et al.*, 1999) and that the insolubility of DBNP essentially precludes its absorption by the lung (Alexander *et al.*, 2001). The solubility of nitrophenols in lipid carriers provides a mechanism by which DBNP deposited on foods, food-processing equipment, eating utensils or in drinking water might ultimately be delivered to humans, particularly during ingestion of foods (*i.e.*, salad oils, *etc.*) high in lipid content.

However, the data for oral gavage acute LD₅₀'s in rats, mice or guinea pigs suggests species, strain and gender differences. Further, the vehicle (*i.e.*, corn oil, canola oil/DMSO, ethanol or carboxymethylcellulose) used to dissolve or suspend the DBNP is critically important to the dose response. Table 1 lists the LD₅₀ levels found by researchers in several different species.

Table 1 Survey of studies of DBNP toxicity, as a function of number of administrations, vehicle, route of administration, gender, species and strain.

SINGLE ADMINISTRATION OF DBNP				
Vehicle	Study	Gender, Species & Strain	Route	LD ₅₀ (Male/Female)
None (Crystalline Powder)	Alexander <i>et al.</i> , 2001	Male Rabbits	Dermal (Taped)	No Deaths at 2 g/kg
None (Crystalline Powder)	Vesselinovich <i>et al.</i> , 1961	Male Sprague-Dawley	Dermal (Taped)	No Deaths at 1 g/kg

0.2% CMC:99.8% water	Vesselinovich <i>et al.</i> , 1961	Male Guinea Pigs	og	800 mg/kg
0.2% CMC:99.8% water	Vesselinovich <i>et al.</i> , 1961	Male/Female Mice	ip	700/850 mg/kg
0.2% CMC:99.8% water	Vesselinovich <i>et al.</i> , 1961	Male Guinea Pigs	ip	580 mg/kg
0.2% CMC:99.8% water	Vesselinovich <i>et al.</i> , 1961	Male/Female Sprague-Dawley	og	500/450 mg/kg
0.2% CMC:99.8% Water	Vesselinovich <i>et al.</i> , 1961	Male/Female Sprague-Dawley	ip	270/260 mg/kg
Unknown	Deichman and Gerarde, 1969	Unknown	og	250 mg/kg
100% Corn Oil (12-Hr Fasting)	Alexander <i>et al.</i> , 2001	Male/Female Fischer-344	og	<100 mg/kg (100% Deaths at 275 mg/kg)
100% Corn Oil (12-Hr Fasting)	Alexander <i>et al.</i> , 2001	Male Sprague-Dawley	og	98 mg/kg (100% Deaths at 250 mg/kg)
100% Corn Oil (5-Hr Fasting)	MacMahon <i>et al.</i> , 1999	Male Sprague-Dawley	og	93 mg/kg
100% Corn Oil (5-Hr Fasting)	MacMahon <i>et al.</i> , 1998	Male Fischer-344	og	82 mg/kg
2% CMC:98% Water (12-Hr Fasting)	Alexander <i>et al.</i> , 2001	Male Sprague-Dawley	og	(20% Deaths at 98 mg/kg)
Methylcellulose	Holder <i>et al.</i> , 1970	Male Wistar	og	1 or 20 mg/Rat (Not Reported)
Aqueous Ethanol	Holder <i>et al.</i> 1970	Male Wistar	iv	Not Reported
Aqueous Ethanol	Holder <i>et al.</i> 1970	Male Wistar	ip	0.192-1 mg/Rat ("Some" Deaths at 1 mg/Rat)

CMC, Carboxymethylcellulose; og, oral gavage

REPEATED ADMINISTRATION OF DBNP

Vehicle	Study	Gender Species/Strain	Route	LD ₅₀
Crystalline in Food for 112 Days	Vesselinovich <i>et al.</i> , 1961	Male/Female Sprague-Dawley Weanlings	Feeding	0.2% DBNP (100% Deaths at 0.4%)
0.2% CMC:99.8% Water for 60 Days	Vesselinovich <i>et al.</i> , 1961	Female Sprague-Dawley	ip	25-50 mg/kg/d (100% Deaths at 50 mg/kg/d)
80% DMSO:20% Water for 10 Days	Carpenter <i>et al.</i> , 1997	Male/Female Fischer-344	ip, iv or oral	(No Deaths at 10 mg/kg/d)
80% DMSO:20% Water for 30 Days	Carpenter <i>et al.</i> , 1997	Male/Female Fischer-344	ip	(No deaths at 25 mg/kg)

More recent research by MacMahon *et al.*, 1999 reports a much lower LD₅₀ of 93 mg/kg and NOAEL of below 62.5 mg/kg in male Sprague-Dawley rats. Death occurred within 2.5 hours post exposure for all rats gavaged with 250 mg/kg. The rats that died exhibited core temperatures as high as 108.1°F, while those surviving did not exceed 106.8°F. However, only 40-80% of rats dosed with 79 or 98 mg/kg died, and all of the rats exposed to 62.5 mg/kg experienced prostration, labored breathing and

hyperthermia, without death. Exposed rats exhibited degenerative lesions in skeletal, smooth and cardiac muscle, and epithelial histopathology in renal and hepatocellular ducts. Similarly, MacMahon *et al.* (1999) reported an LD₅₀ of 82 mg/kg, NOAEL of 50 mg/kg, and clinical histopathology in male Fischer-344 rats dosed orally with DBNP in 100% corn oil.

Route of exposure appears to be a critical factor in evaluation of DBNP clearance as well. ¹⁴C-DBNP in 80% DMSO was 85% cleared from the blood within 60 min and 87% was cleared by 120 min after a single ip dose (Carpenter *et al.*, 1997). However, the remaining 13% of the administered DBNP was still in the blood 16-hr later. Fifty-eight percent of a single iv dose was cleared from the blood in 15 min, but 42% remained in blood after 7 hours. The clearance of DBNP from the body occurs rapidly for the first 30 minutes until it reaches a steady state where 4%-6% remains in the blood for up to 1 week. The tissue distribution of a single ip dose of DBNP is shown in Table 2.

Table 2. Tissue Distribution 24 Hrs post single ip 0.4 mg/kg ¹⁴C-DBNP dose

TISSUE	RANGE OF % OF DOSE
LIVER	14-16
FAT	11-13
KIDNEY	8-10
BLOOD	6-8
SPLEEN	3.5
HEART	2-5
BRAIN	0.8-1.2
MUSCLE	0.5-1

DBNP is eliminated from the body primarily through the urine and feces as a glucuronide conjugate, with a small amount found in the bile (3-5%) (Holder *et al.*, 1970; Macys, 1994). With ip or iv administrations, no unconjugated DBNP was found in the urine, feces or bile, indicating that excretion cannot occur until the parent compound is conjugated by phase-II metabolism (Carpenter *et al.*, 1997). With oral administrations, approximately 70% of the DBNP in an 80% DMSO vehicle is absorbed from the digestive system into the blood. Thirty percent is excreted unchanged through the feces. Within the first 10 days following a single oral gavage at 0.4 mg/kg, 82-90% is excreted in urine and feces. Urinary excretion of orally administered DBNP is, however, reduced by approximately 30% when animals are given 20 mg of Neomycin first. This would indicate that intestinal microflora might contribute to the absorption of DBNP after metabolism by the microflora (Vesselinovitch *et al.*, 1961). The concentrations

of DBNP found in the blood after 24 hrs may reflect release of the DBNP or its metabolite from fat stores. Accumulation of DBNP in the fat would further explain the toxic consequences of repeated exposure to relatively low concentrations of DBNP as reported by both Vesselinovitch *et al.* (1961) and Carpenter *et al.* (1997).

MATERIALS AND METHODS

Chemicals. Ring-labeled 2,6-Di-*tert*-Butyl-4-Nitrophenol-UL-¹⁴C (¹⁴C-DBNP), ethylenediaminetetraacetic acid sodium salt (Na-EDTA), and dimethyl sulfoxide (DMSO) were purchased from the Sigma Chemical Company (St. Louis, MO). The specific activity of the ¹⁴C-DBNP was 10.2 mCi/mmol. Dr. T.K. Narayanan (methods, Rivera-Nevares, 1995; Barnes and Higginbottom, 1961) synthesized unlabeled DBNP at the Naval Health Research Center Toxicology Detachment. Solvable and Ultima Gold Scintillation Cocktail were purchased from Perkin Elmer (Boston, MA). Hydrogen peroxide was purchased from the Fisher Scientific Chemical Company. Mazola Canola Oil, manufactured by Unilever Best foods, was purchased through local retail.

Animals. Male Sprague-Dawley rats (220-250 g) were purchased from the Charles River Laboratories (Raleigh, NC) and were quarantined for 14 days after delivery. Twelve rats (12) rats were randomly assigned to each treatment group, and eight (8) additional rats were selected as controls. Animals were housed in individual Nalgene metabolic cages with free access to food and water except for the 12 hours prior to dosing. Animal rooms were maintained at 21-25°C with 12 hour light/dark cycles.

Dosing. Rats were weighed and dosed by oral gavage with DBNP in corn oil/DMSO or vehicle at a volume of 1mL/100 g rat weight. The first group of twelve rats was given a single oral dose of 40 mg/kg DBNP in 99.2% canola oil:0.8% DMSO, which contained 4 μ Ci/rat ¹⁴C-DBNP. One rat from the high dose group died after being given the oral gavage. The second group (12 rats) received a 15 mg/kg DBNP dose. Eight additional rats were dosed with the vehicle only.

Collection of Samples. Two rats from each dosing group were used for blood collection. Blood was collected from the tail vein (~20 μ L) at 10-min intervals post dosing for the first hour. In the second hour, samples were collected every fifteen min. During the third and fourth hours, blood was drawn every 30 min. From the fifth hour to the tenth, samples were taken once an hour. After ten hours, a blood

sample was collected every 24 hours for 10 days. The urine and feces from each rat was collected at 24 hr intervals.

For 10 days, at 24-hour intervals, two rats were euthanized by CO₂. In the high dose group, eight of the experimental rats died the day after dosing. Due to the high loss rate experienced in this group, the euthanasia schedule was changed. The remaining animals were euthanized one per day for the following nine days. Two of the control animals were euthanized on the first day of the study and the other two on the last day. The tissues collected from the animals included the brain, heart, lungs, liver, kidney, testis, spleen, and a 1 g section each of striated muscle and fat. All samples were stored at 4°C until they could be processed for scintillation counting.

Solubilization of Tissue and Feces Samples. All tissues and feces were minced with a scalpel blade in a glass Petri dish and thoroughly mixed before solubilization. A sample of 100 mg of each tissue and feces was placed in individual 20 mL borosilicate glass scintillation vials. All samples were processed in triplicate. One mL of Solvable was added to each vial (Thompson and Burns, 1996). The vials were incubated for 1-1.5 hours in a gently shaking 60°C water bath. Once the sample was completely digested, it was removed from the water and allowed to cool to room temperature. Aliquots of 0.1 mL of H₂O₂ were added to a volume of 0.2 mL. The vials were swirled between additions and the next aliquot was not added until the reaction had subsided. The vials were then placed back in the gently shaking 60°C water bath to complete the decolorization. The samples were cooled to room temperature and 10 mL of Ultima Gold Scintillation Cocktail was added.

Preparation of Blood Samples. A 100-μL sample of blood was placed in a 20 mL scintillation vial along with 1 mL of Solvable. The entire 20-μL of blood collected immediately after dosing was used for counting. The vials were incubated in a gently shaking 60°C water bath for one hour. Addition of 0.1 mL of 0.1M Na-EDTA to the vials reduced the foaming action that occurred upon the addition of the hydrogen peroxide. Aliquots of 0.1 mL of hydrogen peroxide were added to the vials until the sample was decolorized (0.3-0.5 mL final volume). Once the hydrogen peroxide reaction had subsided, the samples were allowed to stand at room temperature for 15-30 minutes. They were then placed back in the gently shaking 60°C water bath for 1 hour. After cooling to room temperature, 10 mL of Ultima Gold Scintillation Cocktail was added to the vials. The vials were shaken until clear to ensure complete mixing and were

then placed into the Packard 1600TR Liquid Scintillation Analyzer. Samples were allowed to adapt to the light inside the closed analyzer for 1 hour before being counted. The analyzer protocol collected counts from each vial for 15 minutes.

Preparation of Urine Samples. Urine samples were analyzed in triplicate. An aliquot of 1mL was placed in a borosilicate glass scintillation vial and 10 mL of Ultima Gold Scintillation cocktail was added to it. The vials were shaken to until clear to ensure complete mixing and were then placed into the Packard 1600TR Liquid Scintillation Analyzer. Samples were allowed to adapt to the light inside the closed analyzer for 1 hour before being counted. The analyzer protocol collected counts from each vial for 15 minutes.

Statistics. Results are given in DPM/g (disintegrations per min/g of tissue) unless otherwise noted. Data is presented as the mean of assays conducted in triplicate, and includes the standard error of the mean (S.E.M.).

RESULTS

While none of the 16 rats orally gavaged with 15 mg/kg DBNP died from the treatment during the 1-11 day post-exposure period, 6/15 rats successfully gavaged with 40 mg/kg DBNP died within 24 hr post-exposure. Because of this, it must be assumed the LD₅₀ for this treatment in male Sprague-Dawley rats is approximately 40 mg/kg. It should be noted that because 1 rat was euthanized each day post-exposure, a true LD₅₀ could not be calculated. In all cases of death, necropsy indicated edema and/or hemorrhage of the lungs or congestion of the chest cavity. Only one of the six rats was directly observed during the debilitation immediately preceding death. Prostration, reduced auditory startle response, reduced locomotor activity, extreme hyperthermia, and muscular rigidity were observed before death. Rats dosed with 15 or 40 mg/kg DBNP that did not die exhibited lethargy, reduced startle response, and hyperthermia.

One or two rats from each treatment group were sacrificed at 1-11 days post-exposure, and nine tissues (brain, spleen, fat, striated muscle, testis, lungs, liver, kidneys, heart) and the blood sampled for presence of the ¹⁴C radiolabel (DPM/g tissue). Four control rats were sacrificed 24 hr following administration of the vehicle to allow calculation of background levels of ¹⁴C. Figure 1 shows the distribution of DBNP in the tissues following the 40 mg/kg dose over the 10-day study. The brain, muscle, spleen, and heart tissues were not collected from the eight rats that died 24 hours after dosing. Most of the DBNP concentrates in the fat, kidney, lung, and liver within 24 hours after dosing. By the third day, the majority of the dose remains in the fat, kidney, or liver. The lung tissue has dropped back down to near baseline values. The higher concentration of DBNP in these three tissues is clearly visible for the first 96 hours of the study. After 120 hours, the tissue concentrations are only slightly elevated. Results are given in DPM/g (Disintegrations per minute) unless otherwise noted. The counts from each triplicate of samples were averaged and the standard errors calculated.

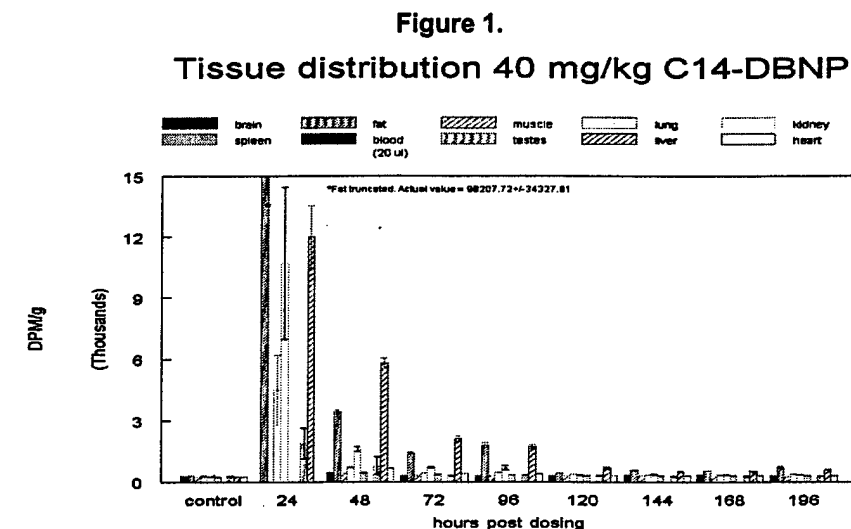
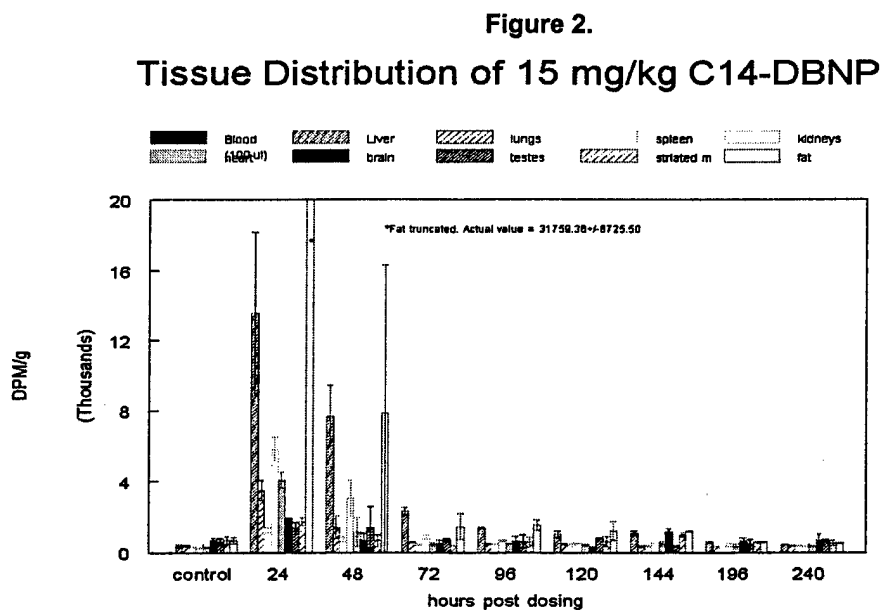


Figure 2 shows the tissue distribution in the low dose group, 15 mg/kg ^{14}C -DBNP. The same pattern appears in the tissue distribution of this dose group as was seen in the high dose group. The liver, kidneys, and fat show the highest concentration of DBNP which slowly tapers off over time until it approaches baseline values.



Figures three (40 mg/kg dose group) and four (15 mg/kg dose group) isolate the distribution of DBNP in the liver, kidneys, and fat. Here the spike in DBNP concentration 24 hours post dosing can be clearly seen. The elevated levels taper down to baseline levels in the first 96 hours of the study.

Figure 3.

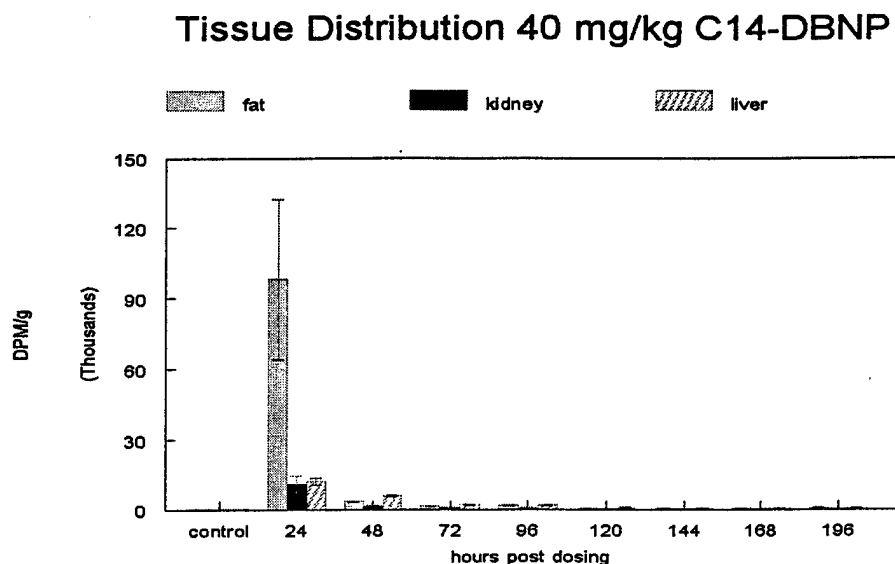
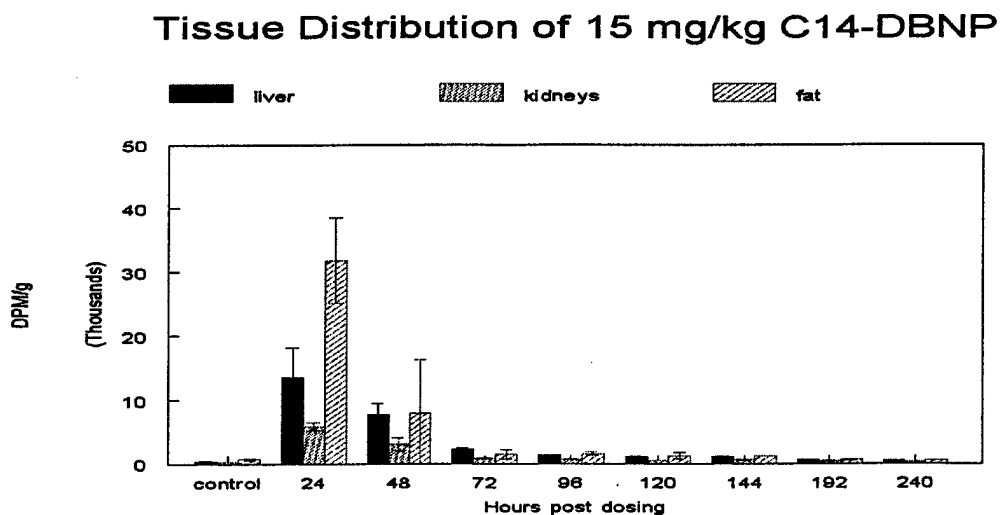


Figure 4.

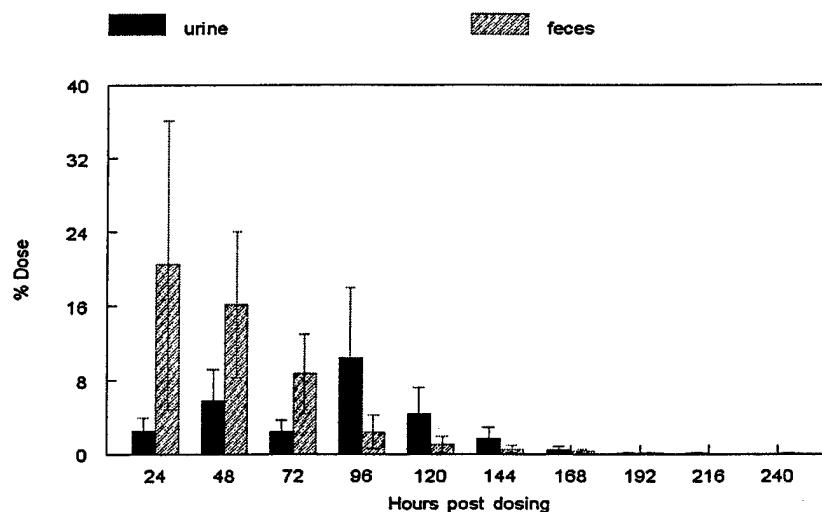


For both the 40 and 15 mg/kg doses, it was shown that the ^{14}C radiolabel is differentially present in all tissue compartments sampled at levels higher than control at 24-96 hr post-exposure, but that accumulation beyond 24 hr does not occur from a single dose. At 24 hr post-exposure, a high concentration of the radiolabel was identified in the fat, liver, and kidneys. By 48 hr post-exposure, the concentration of the radiolabel in the fat and kidneys was reduced by more than 75%, while the concentration in the liver was reduced by less than 50%. Generally, fat contains the greatest amount of

radiolabel (as summarized in Table 3), although the concentrations measured in the fat and liver were significantly greater than in other organs. It can be seen that DBNP or its metabolites were generally cleared from all tissue compartments, including the fat, by 96 hr post-exposure.

As is indicated in Fig. 5, the majority of orally gavaged ^{14}C -DBNP is excreted in the feces. Approximately 24% of the ingested dose was eliminated in the feces within 24 hr; 43% with 48 hr; 59 within 96 hr; and 62% within 168 hr. Clearance of DBNP in the urine accounted for up to 37% of the orally administered dose. As is indicated, urinary excretion peaks (12% of ingested dose) approximately 96 hr post-exposure, possibly indicating reabsorption from the fat and other tissue compartments. For both urinary and fecal excretion, baseline levels are observed after approximately 192 hr following a single oral gavage exposure. Due to the deaths of 6 rats gavaged with 40 mg/kg with 24 hr of exposure, and the morbidity of the remaining living subjects, urine and feces samples were not collected in sufficient quantities to allow a meaningful assay.

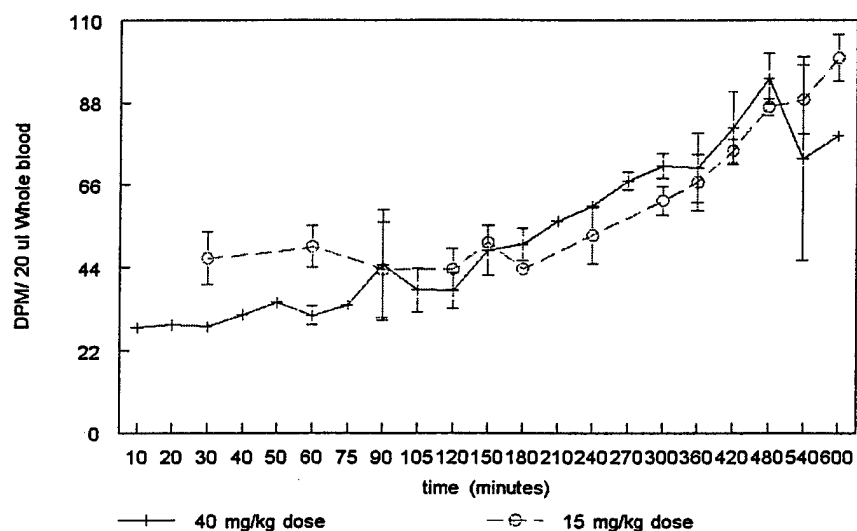
Figure 5.
Excretion of ^{14}C -DBNP



Blood samples (20 μL) were collected from the tail vein of two rats per treatment group, beginning 10-30 min post-exposure, and continuing for 10 hr. These data established quantities of ^{14}C - radiolabel in the blood as a function of time post-exposure, and are presented in Fig. 6. ^{14}C -DBNP delivered by oral gavage was detectable in the blood at 10 min post-exposure (data was not collected for the 15 mg/kg

group until 30 min post-exposure). Absorption of DBNP continued in an approximately linear manner throughout at least the first 600 min post-exposure. Both the 15 mg/kg and 40 mg/kg groups exhibited approximately equal concentrations of the ^{14}C radiolabel at all time points sampled throughout the initial 600 min. DBNP spikes in the blood and then tapers off as it is absorbed into the fat or excreted. Oral gavage administration of DBNP resulted in identifiable levels of the parent compound in the blood as soon as 10 min post-exposure (high dose). Blood concentration increased approximately linearly for at least the first 10 hr, with little difference observed between the low dose and high dose administrations at any post-administration time point.

Figure 6.
DBNP Appearance in Blood Stream Post Dosing



DISCUSSION

In the present study, Sprague-Dawley rats were orally gavaged with a single dose of ^{14}C -DBNP in a Canola Oil/DSMO vehicle. Canola oil was selected to represent a lipid carrier typical of those commonly ingested by both military and civilian personnel during normal dining. A small percentage of DMSO was added to the vehicle to maximize the presence of the radiolabeled compound in the tissues assayed. The results of the current study appear generally consistent with previously published animal studies for oral ingestion of DBNP, and complete data gaps on absorption, tissue disposition, and clearance. There can be no question that orally ingested DBNP is rapidly absorbed from the GI tract of rats, is differentially distributed to tissue compartments as a function of lipid content and other factors, and is generally eliminated in the feces and urine within 72-96 hr. While the gender, species, and strain of animals exposed are minor factors in the toxicity observed, the far more important consideration is the vehicle utilized. Further, it would appear that the persisting presence of DBNP in various tissues for as long as 5-6 days provides a mechanism by which accumulation with repeated exposure could occur. These considerations are important for at least military risk assessment issues. For example, submarine personnel can orally ingest crystalline DBNP (or the precursor DBP) mixed with food high in lipid content on a daily basis during continuous assignments lasting up to 6 months.

The considerable increase in core temperature associated with DBNP exposure, and the severe adverse health effects associated with both lethal and non-lethal doses provides evidence for the hypothesis that DBNP, like some other nitrophenols, is a potent uncoupler of oxidative phosphorylation (Klingenberg, 1990). What remains to be determined is whether humans, like mice, rats and guinea pigs, are susceptible to consequential health effects from DBNP ingestion, and whether such effects can occur in response to "real world" exposures scenarios. It has been shown that DBNP ingested for as little as one week by rodents in crystalline form (in pelleted rat chow) is lethal at high concentrations (*i.e.*, $\leq 0.4\%$ of food volume) [Vesselinovich *et al.*, 1961]. Based on this observation, it might be assumed that if an adult rat normally consumes 20-25 g of pelleted rat chow/day, then the ingestion of 80-100 mg undissolved DBNP/day (0.4% DBNP) will be sufficient to induce death in <16 weeks, while ingestion of 40-50 mg/day (0.2% DBNP) will induce "significant toxicity". Further, because the rats exposed to DBNP (Vesselinovich *et al.*, 1961) reduced mean food intake by 25% during the 16-wk exposure, it might be

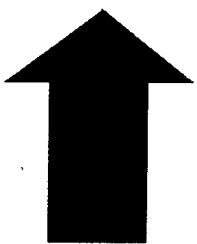
assumed that repeated intake of 30-37.5 mg/kg/day crystalline DBNP was sufficient to induce "severe" health consequences. If the rat is assumed to be a linear model for human DBNP toxicity, then repeated oral ingestion of 2.6 g/day crystalline DBNP ($37.5 \text{ mg/kg} \times 70 \text{ kg body weight} = 2,594 \text{ mg}$, with no lipid carrier) would be sufficient to produce "significant" health consequences (WHO report, 1962). It must, of course, be considered that repeated human ingestion of 2.6 g DBNP aboard a submarine would be highly unlikely, and further that data from limited exposure of rat and human hepatocyte cultures to DBNP led Carpenter *et al.* (1997) to conclude that DBNP may be more toxic to rodents than to humans. However, based on the animal research discussed, it must be considered that oral ingestion of much smaller quantities of DBNP suspended in food oils or dissolved in ethanol might provide a real world human risk.

It has been shown (Table 1) that the vehicle used to deliver DBNP to the GI tract is critically important to the toxicity observed. In the present study, DBNP dissolved in 0.8% DMSO, then orally gavaged (40 mg/kg) in canola oil (99.2%) resulted in the deaths of 40% of the subjects within 24 hr. It would appear that this vehicle increases the toxicity of DBNP as compared to either 0.2-2% aqueous CMC or even 100% corn oil. Human risk from the possible ingestion of DBNP or the precursor DBP accidentally mixed with food would appear to vary as a function of the specific chemical characteristics of the food. For example, foods containing a high fat content (*i.e.*, salad or cooking oils, *etc.*) might be expected to be far more effective in delivering DBNP to the blood than foods with low fat content. Further, based on limited data from Holder *et al.* (1970), it must be considered that mixture of crystalline DBNP with small amounts of ethanol might substantially yet increase DBNP absorption and hence human risk.

It was clear that orally ingested DBNP in Canola Oil/DMSO is detectable in the blood at 10 min post-exposure (40 mg/kg dose), and increases in concentration in blood for at least the first 10 hr post-exposure. There was very little difference between the 15 mg/kg and 40 mg/kg doses in the time it takes for it to appear in the blood. When measured at 24-hr post-exposure, the radiolabeled marker was identified in each of the nine tissue compartments measured, but varied substantially in concentration among tissues. Table 3 shows the increasing concentration of DBNP in the tissues. However, the concentrations measured in the fat and liver were from 200-1000% greater than in other organs.

Table 3. Percentage (DPM/g) of the initial dose of ^{14}C -DBNP (15 mg/kg) measured in each of nine tissues 24 or 48 hr post-dosing.

Percentage (Per Gram Tissue) of Initial Dose		
Tissue	Hours Post Dosing	
	24 Hours	48 Hours
1. Fat	0.352%	0.082%
2. Liver	0.149	0.084
3. Kidneys	0.062	0.031
4. Heart	0.043	0.011
5. Lungs	0.036	0.012
6. Brain	0.014	0.003
7. Spleen	0.012	0.006
8. Striated Muscle	0.012	0.003
9. Testes	0.009	0.010

Increasing Concentration of ¹⁴ C Radiolabel in Tissues Measured 24 hrs post dosing	
	Fat
	Liver
	Kidneys
	Heart
	Lungs
	Brain
	Spleen
	Striated Muscle
	Testes

The radiolabeled marker was identified at concentrations numerically higher than controls in most organs for approximately 8 days post-exposure. For the first 48 hr post-exposure, there remained high concentrations in both fat and the liver. The lung tissue of the 40 mg/kg dose group indicates a high concentration of DBNP 24 hrs post exposure. In the first 24 hours after dosing this group, about half of the test animals died. They were found in advanced rigor and, when the lung tissue was removed, it contained a great deal of blood. Therefore, a portion of the high concentration shown in the lung tissue was actually due to the blood that collected in the lungs. When measured at 24-hr post-exposure, the radiolabeled marker was identified in each of the nine tissue compartments evaluated, but varied substantially in concentration among tissues (Table 3). Due to known lipid solubility of DBNP, its hypothesized metabolism in the liver, and partial elimination through the kidneys, the proportionately higher presence in the fat, liver and kidneys was predictable. The relatively small presence in the brain, a highly lipophilic tissue compartment, was unexpected, and perhaps suggests failure of DBNP to cross the blood-brain barrier (BBB) in significant concentrations. It must be considered that various stressors (*i.e.*, heat, psychological stress, oxidative stressors) that can alter the permeability of the BBB can occur

commonly in a submarine environment (Esposito *et al.*, 2001) and may alter delivery of DBNP to the brain.

The identification of DBNP in the liver, kidneys, heart, lungs, striated muscle and spleen is consistent with previous reports of DBNP-induced histopathology in at least those organs (Vesselinovitch *et al.*, 1961; Carpenter *et al.*, 1997; MacMahon *et al.*, 1998, 1999) including:

- Congestion in the liver, with fatty accumulation in hepatocytes; increased liver weight and liver:body weight ratio
- Abortive mitoses, lymphorrhesis, and reduced tissue volume in the spleen
- Epithelial degeneration and necrosis accompanied by the formation of obstructive casts in the tubule cells of the outer region of the kidney medulla
- Patchy, waxy degeneration of the myocardium, with palpable firmness of the heart
- Edema and congestion of the lungs

Minimal degeneration of skeletal and gastrointestinal tract muscle

The radiolabeled marker was identified at concentrations numerically higher than controls in most of the tissue compartments measured for at least five days post-exposure. With the single dose protocol used in the present study, clearance of the radiolabeled marker occurred rapidly over the first 48 hr post-exposure, minimizing the possibility that increasing accumulation in any organ system occurs following a single oral dose. However, the persisting presence of smaller quantities of the radiolabeled marker across as many as five days suggests that repeated exposure, as may occur in a closed submarine environment, may result (additively or synergistically) in accumulation that may exceed the highest concentration expected with a single dose. Further, the substantial clearance of DBNP from the fat observed during the periods 24-48 hr post-exposure following a single dose might be expected to combine with subsequent doses to ultimately produce a higher concentration in other tissue compartments than would be predicted from single exposure data.

The clearance of DBNP from the body involves a complex process following oral ingestion. In the first 72 hours of the study, the majority of the DBNP was excreted through the feces. However, after 72 hours, the major portion of the excretion was via the urine. It has been suggested in previous research that approximately 30% of orally ingested DBNP is excreted as the parent compound in the feces, and

that the remaining 70% is absorbed from the GI tract into the blood. Of the portion absorbed into the blood, research has indicated that all of it is metabolized to the glucuronide form before it can be excreted (Holder *et al.*, 1970). Our data agrees with this. Initially, the DBNP passes through the gut and is excreted in the feces. The remaining portion that was absorbed from the GI tract is metabolized to the glucuronide conjugate and then excreted in the urine.

The daily exposure of Navy personnel to DBNP during 90-day submarine activities is unquestionable. Due to the insolubility of DBNP, it would appear that dermal exposure or respiratory exposure are minimally important routes of exposure. Because DBNP in crystalline form can be absorbed directly from the GI tract (Vesselinovitch *et al.*, 1961), and suspension of DBNP in lipid carriers (i.e., shipboard foods with high lipid content) significantly increases its uptake into the blood, exposure through oral ingestion may present a human toxicology risk. It is known that exposure of laboratory animals to high doses of DBNP induces tissue histopathology in multiple organ systems, and possibly death (MacMahon *et al.*, 1999). Further, it has been suggested that repeated exposure to DBNP may result in significant accumulation in lipid tissue compartments. For these reasons, it becomes important to understand the tissue compartment distribution and clearance of the compound following oral administration, as this route appears to provide the greatest human risk. While there have been no reports of acute medical symptoms or long-term illness among personnel exposed to DBNP, it is unknown whether repeated exposure over months or years to low doses of DBNP is capable of inducing negative health effects. It, of course, cannot be assumed that the rodent is an adequate model for human DBNP toxicity or, in fact, that DBNP presents a toxic risk to humans. To date, no military person exposed repeatedly to DBP or DBNP aboard a submarine has reported illness that has been associated with such an exposure. However, it also must be considered that submariners represent the extreme of a "healthy worker" population, and that some subtle health effects possibly induced by repeated DBNP exposure may not be readily detected for many months or years of exposure.

References

- Alexander WK, Briggs GB, Still KR, Jederberg WW, MacMahon K, Baker WH and Mackerer C (2001). Toxicity of 2,6-Di-*tert*-butyl-4-Nitrophenol (DBNP). *Appl. Occup. Environ. Hyg.* **16**:487-495.
- Barnes TJ, and Higginbottom WJ (1961). 4-Nitro,2,6-di-*t*-butylphenol and its thermal decomposition. *J. Chem. Soc.* **83**: 953-956.
- Carpenter RL, Narayanan TK, Jung AE, Prues S and Still KR (1997). Characterization of the metabolism, distribution and toxicity of 2,6-di-*tert*-butyl-4-nitrophenol. *Naval Medical Research Institute Technical Report No. NMRI-97-39*, Wright-Patterson Air Force Base, OH.
- Castilho RF, Vicente JAF, Kowaltowski AJ and Vercesi AE (1997). 4,6-dinitro-*o*-cresol uncouples oxidative phosphorylation and induces membrane permeability transition in rat liver mitochondria. *Int. J. Biochem. Cell. Biol.* **29**:1005-1011.
- de las Alas V, Voorhees WD, Geddes LA, Bourland JD, and Schoenlein WE (1990). End-tidal carbon dioxide concentration, carbon dioxide production, heart rate, and blood pressure as indicators of induced hyperthermia. *J. Clin. Monit.* **6**:183-185.
- Deichman, WB, and Gerarde, HW (1969). *Toxicology of Drugs and Chemicals*. Academic Press, Inc. New York, NY.
- Esposito P, Gheorghe D, Kandere K, Pang X, Connolly R, Jacobson S, and Theoharides TC (2001). Acute stress increases permeability of the blood-brain barrier through activation of brain mast cells. *Brain Res.* **888**:117-127.
- General Dynamics/Electric Boat Division (1992). *Submarine Yellowing Problem Report*. 9510/61-225-X-2, 6110/429.

Hemker HC (1962). Lipid solubility as a factor influencing the activity of uncoupling phenols. *Biochim. Biophys. Acta* 62:46-54.

Holder GM, Ryan AJ, Watson TR and Wiebe LI (1970). A note on the excretion of 2,6-Di-*tert*-butyl-4-nitrophenol. *Food Cosmet. Toxicol.* 9:531-535.

Jonsson GG, Erikson G, and Pero RW (1985). Influence of hyperthermia and gamma radiation on ADP-ribosyl transferase NAD⁺, and ATP pools in human mononuclear leukocytes. *Radiat. Res.* 102:241-253.

MacMahon KL, Baker WH, Alexander WK, Jederberg WW, Still KR, Briggs GB, and Godfrey RJ (1998). *Acute Oral and Dermal Toxicity Evaluation of 2,6-Di-Butyl-4-Nitrophenol. Air Force Research Laboratory Technical Report AFRL/HEST-TR-1998.*

MacMahon KL, Baker WH, Alexander WK, Still KR, Briggs GB, and Godfrey RJ (1999). *Acute Oral Toxicity Evaluation of 2,6-Dibutyl-4-Nitrophenol in Male Sprague-Dawley Rats. Air Force Research Laboratory Technical Report AFRL-HE-WP-TR-1999-0173*

Macys DA and Wyman J (1994). Study of in vitro and in vivo effects of DBNP on rat liver fatty acid binding protein and rat liver sulfotransferases. *Naval Medical Research Institute Report F3360194MT601*, Wright-Patterson Air Force Base, OH.

Okuda M, Lee HC, Kumar C and Chance B (1992). Comparison of the effect of a mitochondrial uncoupler, 2, 4-dinitrophenol and adrenaline on oxygen radical production in the isolated perfused rat liver. *Acta Physiol. Scand.* 145:159-168.

Rivera-Nevarés JA, Wyman JF, von Minden DL, Lacy N, Chabinye MC, Fratini AV and Macys DA (1995). Facile synthesis and physical and spectral characterization of 2,6-Di-*tert*-butyl-4-Nitrophenol, a potentially powerful uncoupler of oxidative phosphorylation. *Env. Tox. Chem.* 14:251-256.

Thompson J and Burns DA (1996). *LSC Sample Preparation by Solubilization*.
<http://www.packardbioscience.com/pdf/appnotes/cs003.pdf>.

Vesselinovitch D, Dubois KP, Fitch FW and Doull J (1961). Mammalian toxicity and histopathologic effects of 2,6-dibutyl-4-Nitrophenol. *Toxicol. Appl. Pharmacol.* 3:713-725.

World Health Organization (WHO) (1962). *Principles Governing Consumer Safety in Relation to Pesticide Residues. WHO Tech Rep. Ser. 240*.

Yoshikawa KN, Kumazawa N, and Terada H (1980). Physico-chemical properties of SF 6847, a potent uncoupler of oxidative phosphorylation in mitochondria in relation to its activity. *Int. J. Quantum Chem.* 18:539-544.

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