AFRL-HE-WP-TR-1999-0173

UNITED STATES AIR FORCE RESEARCH LABORATORY

ACUTE ORAL TOXICITY EVALUATION OF 2,6-DIBUTYL-4-NITROPHENOL IN MALE SPRAGUE-DAWLEY RATS

K.L. MacMahon
W.H. Baker
OPERATIONAL TOXICOLOGY BRANCH
HUMAN EFFECTIVENESS DIRECTORATE
AIR FORCE RESEARCH LABORATORY
WRIGHT-PATTERSON AFB, OH 45433-7400

W.K. Alexander
K.R. Still
NAVAL MEDICAL RESEARCH INSTITUTE DETACHMENT
NMRI/TD BLDG 433
2612 FIFTH STREET
WRIGHT-PATTERSON AFB, OH 45433-7903

G.B. Briggs
GEO-CENTERS, INC.
7 WELLS AVENUE
NEW CENTRE, MA 02159

R.J. Godfrey
MANTECH ENVIRONMENTAL TECHNOLOGY, INC.
PO BOX 31009
DAYTON, OH 45437-0009

June 1999
Final Report - February 1999 - June 1999

Human Effectiveness Directorate
Deployment and Sustainment Division
Operational Toxicology Branch
2856 G Street
Wright-Patterson AFB OH 45433-7400

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TECHNICAL REVIEW AND APPROVAL

AFRL-HE-WP-TR-1999-0173

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

STEPHEN R. CHANNEL, Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Research Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1999	Final Report - February 1999 - June 1999

4. TITLE AND SUBTITLE
Acute Oral Toxicity Evaluation of 2,6-Dibutyl-4-nitrophenol in Male Sprague-Dawley Rats

5. FUNDING NUMBERS
Contract F41624-96-C-9010
PE 62202F
PR 7757
TA 7757A1
WU 7757A108

6. AUTHOR(S)
K.L. MacMahon, W.H. Baker, W.K. Alexander, G.B. Briggs, K.R. Stills, and R.J. Godfrey

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ManTech Geo-Centers Joint Venture
PO Box 31009
Dayton, OH 45437-0009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Human Effectiveness Directorate
Air Force Research Laboratory
Wright-Patterson AFB, OH 45433-7400

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AFRL-HE-WP-TR-1999-0173

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This study investigated the acute oral toxicity of 2,6-di-t-butyl-4-nitrophenol (DBNP) in male Sprague-Dawley rats. DBNP is formed in U.S. submarines when lube oil containing the antioxidant additive 2,6-di-t-butylphenol (DBP) is nitrated after passing through an electrostatic precipitator. DBNP has triggered human health concerns due to its adherence to painted surfaces causing a noticeable yellowing. Male Sprague-Dawley rats were dosed with DBNP in corn oil. Survivors at each dose level were 5/5 at 62.5 mg/kg, 4/5 at 78 mg/kg, 2/5 at 98 mg/kg, and 0/5 at 250 mg/kg. The LD50 of DBNP in corn oil is 93 mg/kg. The No Observable Effect Level for DBNP is less than 62.5 mg/kg. Hyperthermia and severe rigor were found in treated animals.

14. SUBJECT TERMS
Acute toxicity; Limit test; LD50; LC50; Oral gavage; Sprague-Dawley rats; 2,6-dibutyl-4-nitrophenol

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

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PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Operational Toxicology Branch, Human Effectiveness Directorate, Air Force Research Laboratory (AFRL/HEST), Wright-Patterson Air Force Base, Ohio by the ManTech/Geo-Centers Joint Venture contract. This document serves as a final report on the acute oral toxicity evaluation of 2,6-dibutyl-4-nitrophenol in corn oil in male Sprague-Dawley rats. The research described in this report began in February 1999 and was completed in May 1999. This study was funded by the Naval Health Research Center, Toxicology Detachment, Wright-Patterson AFB, OH. Lt Col (Sel) Stephen Channel served as the Contracting Officer's Representative for the U.S. Air Force, AFRL/HEST. Darol E. Dodd, Ph.D., served as Program Manager for ManTech/Geo-Centers Joint Venture. Capt Kathleen MacMahon served as Task Director for the Operational Toxicology Branch. Expert statistical consultation was provided by Mr. Chuck Goodyear. Invaluable technical assistance was provided by Ms. Margaret Parish.

The animal use described in this study was conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

ABBREVIATIONS

AFRL	Air Force Research Laboratory
°C	Degrees centigrade
DBNP	2,6-Dibutyl-4-nitrophenol
DBP	2,6-Di-t-butylphenol
EBD	Electric Boat Division
EPA	Environmental Protection Agency
°F	Degrees Fahrenheit
F-344	Fischer 344 (rat)
g	Gram
HEPA	High efficiency particulate air
hr	Hour
kg	Kilogram
LD_{50}	Median lethal dose
min	Minute
mg	Milligram
n	Sample size
NRL	Naval Research Laboratory
NOAEL	No Observable Adverse Effect Level
SD	Standard deviation

SECTION I

INTRODUCTION

This study investigated the acute oral toxicity of 2,6-di-tert-butyl-4-nitrophenol (DBNP) in male Sprague-Dawley rats. This study is part of the evaluation of the toxicity of DBNP requested by the Navy Environmental Health Center due to the expressed concerns of U.S. Navy submarine personnel. Within the submarine fleet, especially in newer boats, a yellowing of painted surfaces was noted both in the builders yard and during trails at sea. In a number of survey reports from the Naval Research Laboratory (NRL) and the Electric Boat Division (EBD) of General Dynamics Inc. during 1992-1993 (unpublished data) researchers determined that the cause of the yellowing was 2,6-di-t-butyl-4-nitrophenol. They found that 2,6-di-t-butylphenol (DBP), an antioxidant in 2190 lubricating oils, was easily nitrated to DBNP by passage through electrostatic precipitators installed in the engineering spaces on submarines. Aerosolized DBP could be nitrated through the electrostatic precipitators or as a reaction on the surface of the metal. Nitration might also be possible on the surface of an aerosol droplet. DBP and DBNP showed a greater affinity for painted surfaces over bare metal, however, it was also found on pillowcases and other bedding materials on submarines.

Based on these findings the specification for 2190 lube oil was changed to remove DBP. Removal of DBP from the oil should resolve the yellowing in the future. However, the stockpile of 2190 lube oil remaining in the system will supply the fleet for a number of years with DBP-containing 2190 oils. Questions arose from the fleet submarine community regarding the toxicity and human health risk of DBNP. The removal of DBP from the oil, the low toxicity of DBNP reported by Vesselinovitch *et al.* (1961), and the minimal exposure findings reported by the NRL and EBD studies caused the Navy to conclude that the yellowing phenomenon was a cosmetic rather than a health concern. Submarine force personnel remained concerned about yellowing of their skin when they came in contact with surfaces coated with DBNP. They requested assistance and evaluation of the toxicity of DBNP from the Navy Environmental Health Center. This study is a portion of that evaluation.

This study investigated the acute oral toxicity of DBNP in male Sprague-Dawley rats using the carrier corn oil. Previous work had demonstrated that a solution of DBNP in corn oil resulted in consistent dosing for oral gavage (MacMahon *et al.*, 1998). Based on this previous study and other data available in the literature (Vesselinovitch *et al.*, 1961) the dose selected for initial acute oral testing was 250 mg/kg DBNP in corn oil. Additional doses selected included 62.5 mg/kg, 78 mg/kg, and 98 mg/kg

DBNP in corn oil. The purpose of this study was to evaluate whether the acute toxicity data obtained in a previous study in Fischer 344 rats was reproducible in Sprague-Dawley rats. This acute toxicity data in combination with additional research on the proposed mechanism of action of DBNP will be valuable in evaluating the human health risks of DBNP. The information obtained in these experiments when combined with inhalation and subchronic toxicity testing will provide a database for a human health risk assessment.

SECTION II

MATERIALS AND METHODS

Test Material

2,6-Di-tert-butyl-4-nitrophenol was synthesized from 2,6-di-tert-butylphenol according to the techniques of Rivera-Nevares *et al.* (1995). The chemical was synthesized by personnel at the Naval Health Research Center, Toxicology Detachment, Wright-Patterson Air Force Base, Ohio. The test material was determined by gas chromatography-mass spectrophotometry analytical methods to be 92.4% pure DBNP. The test material also contained 7.6% DBP. DBNP (CAS 728-40-5) is a yellow powder with a melting point of 157°C and a pKa value of 6.8. The DBNP was brought into solution in corn oil (Mazola brand) by pulverization and intense mixing.

Test Animals

Male Sprague-Dawley rats weighing between 100 and 150 g were purchased from Charles River Laboratories, Wilmington, MA. All animals were subjected to a two-week quarantine period. Rats were individually housed in clear plastic cages with Cell-Sorb Plus® laboratory bedding. Water and feed (Purina Formula #5008) were available *ad libitum*, except for 12 hours prior to oral dosing. Animal room temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12 hour intervals.

Experimental Approach

Five male Sprague-Dawley rats were fasted 12 hours prior to the administration of each oral dose. Each rat was weighed prior to oral gavage dosing. Groups of five male rats were orally gavaged sequentially at doses of 250, 98, 78, or 62.5 mg/kg DBNP in corn oil. Five male control animals were gavaged with corn oil only. All animals were dosed at a volume of 1 ml per 100g body weight. Body temperatures were monitored pre- and postdosing with a Thermoscan® (Thermoscan Inc., San Diego, CA) instant thermometer placed in the ear canal. Clinical observations were also noted at these times. Gross necropsies were performed on all animals dying during the initial dosing phase. Select tissues were collected from two animals from each dosage level that died from exposure to the test agent. Tissues collected included the lung, heart, liver, spleen, kidney, adrenal gland, skeletal muscle, stomach, duodenum, ileum, colon and pancreas. Tissues were preserved in four parts formalin: one part glutaraldehyde and examined histologically. Surviving animals were weighed at 1, 7, and 14 days

postexposure and signs of toxicity recorded at least twice daily. On the 14th day postexposure, rats were sacrificed by overexposure of halothane inhalation and gross pathologic examination was performed. Tissues were collected from two control animals and fixed as previously stated.

Statistical Analysis

The percent change in body weight from day 0 to days 1,7, and 14 was determined for each animal that survived. These percent changes were used as the dependent variable in a 2-factor (day x dose) mixed design analysis of variance. Paired comparisons for dose used the Bonferroni procedure with a 0.05 overall error level (per comparison error level = 0.05/6 = 0.0083). The LD50 was calculated using probit analysis (Finney, 1980).

SECTION III

RESULTS

All male rats orally gavaged with 250 mg/kg DBNP in corn oil died about 2.5 hours after dosing (Table 1). Clinical signs prior to death included prostration, rapid respiration, lethargy, and hyperthermia. High body temperatures after dosing ranged from 102.8°F to 107.3°F (mean= 104.4°F). At the time of death the animals' bodies were in a state of rigor. Gross examination at necropsy revealed congestion of livers and lungs to be a common finding. The hearts were palpably firm. Histologic analysis of skeletal muscle demonstrated minimal degeneration of muscle fibers with occasional contraction bands. Similar changes were noted in the cardiac musculature and in the smooth mucle layers of sections of the gastrointestinal tract. Additional degenerative and necrotic changes were noted in individual hepatocytes and in kidney ductal epithelial cells.

Two of five male rats gavaged with 98 mg/kg DBNP in corn oil died approximately 4 hours after dosing; a third died overnight (Table 1). Maximum recorded body temperatures between the times of dosing and death ranged from 103.9°F to 108.0°F (mean= 105.6°F). The two animals that survived attained body temperatures of over 104.0°F by 5.5 hours after dosing. Their body temperatures returned to normal levels by 30 hours postdosing. Clinical signs were similar to those noted above in the high dose group during the period immediately following dosing and had returned to normal by 24 hours postdosing.

One of five male rats orally gavaged with 78 mg/kg DBNP died within 7.25 hours of dosing (Table 1); its body temperature was 108.1°F at death (Table 4). The high temperatures of all treated animals ranged from 102.6°F to 108.1°F (mean= 105.3°F). Rapid respiration and prostration were observed as in higher doses. Normal body temperatures and activity levels were observed by 24 hours postdosing.

All animals treated by oral gavage with 62.5 mg/kg DBNP survived dosing and the 14-day post-dose observation period (Table 1). All treated animals were prostrate in their cages for several hours postdosing and had labored breathing. The maximal body temperatures after dosing, seen 4.5 to 7.5 hours postdosing, ranged from 103.4°F to 105.2°F (mean=104.1°F). All animals had returned to a normal body temperature and activity level by 24 hours postdosing.

All control rats (n=5) showed no clinical signs during the 14-day post-dose observation period, continued to gain body weight (Table 2) and had no gross lesions at necropsy. The two control animals assessed for microscopic lesions were relatively free of comparable lesions. Maximum recorded body temperatures in the control rats after dosing ranged from 99.7°F to 101.7°F (mean= 100.7°F, Table 5).

The body weight of most animals continued to increase throughout the 14 day observation period (Table 3). The 98 mg/kg study group lost weight the first day after dosing but thereafter increased body weight, although at a slower rate than the others. As there were no survivors at the 250 mg/kg dose range their body weights could not be monitored. A 2-factor (day x dose) mixed design analysis of variance analyzing body weight revealed a significant main effect of dose. Paired comparisons for dose showed the only significant difference to be between the control group and 98 mg/kg.

TABLE 1. ACUTE ORAL TOXICITY OF DBNP

Dose Level (mg/kg)	Carrier	Mortality Ratio	Time to Death (Maximum)
250	Corn oil	5/5	3 hr, 26 min
98	Corn oil	3/5	22 hr, 25 min[a]
78	Corn oil	1/5	7 hr, 13 min
62.5	Corn oil	0/5	--------

a= one animal died overnight; exact time of death unknown;
2 animals died at 3 hours, 56 min and 4 hours, 7 min

TABLE 2. BODY WEIGHTS (GRAMS) OF CONTROL MALE SPRAGUE-DAWLEY RATS AFTER ORAL GAVAGE WITH CORN OIL

	Study Day 0	1	7	14
CORN OIL CONTROL				
Animal #				
19	244.7	267.7	330.6	415.6
2	227.6	248.0	315.3	396.5
9	215.7	238.1	262.6	274.5
11	209.6	237.6	290.5	358.5
14	220.5	243.9	307.3	373.7
Mean	223.6	247.1	301.3	363.8
SD	13.5	12.3	26.0	54.4

TABLE 3. BODY WEIGHTS (GRAMS) OF MALE SPRAGUE-DAWLEY RATS AFTER ORAL GAVAGE WITH DBNP IN CORN OIL

	Study Day			
	0	**1**	**7**	**14**
250 mg/kg				
Animal #				
3	212.9	-----	-----	-----
4	210.0	-----	-----	-----
17	222.8	-----	-----	-----
8	210.9	-----	-----	-----
12	229.3	-----	-----	-----
Mean	217.2			
SD	8.5			
98 mg/kg				
Animal #				
7	284.4	-----	-----	-----
15	302.2	287.8	348.9	422.5
5	303.8	288.4	352.6	419.6
10	236.6	-----	-----	-----
13	288.2	-----	-----	-----
Mean	283.0	288.1	350.8	421.0
SD	27.3	0.4	2.6	2.1
78 mg/kg				
Animal #				
8	243.3	246.8	302.0	353.3
4	216.7	217.5	272.9	322.0
11	226.5	232.8	279.1	325.0
12	219.4	225.8	280.5	366.0
14	228.7	-----	-----	-----
Mean	226.9	230.7	283.6	341.6
SD	10.4	12.4	12.7	21.5
62.5 mg/kg				
Animal #				
18	237.4	252.0	301.1	347.0
6	221.8	228.8	292.3	360.4
16	231.2	234.4	300.2	366.3
1	234.4	248.1	310.2	396.2
20	240.2	239.4	308.0	382.6
Mean	233.0	240.5	302.4	370.5
SD	7.1	9.6	7.1	19.2

TABLE 4. BODY TEMPERATURES (°F) OF MALE SPRAGUE-DAWLEY RATS PRE- AND POSTDOSING WITH DBNP

ANIMAL #	PRE-DOSE	POST DOSE 0.5 HR	1.5 HR	2.5 HR	3.5 HR	4.5 HR	5.5 HR	6.5 HR	24 HR	48 HR
250 mg/kg DBNP in corn oil										
3	99.0	100.7	101.9	103.2	97.7[a]					
4	101.1	101.9	103.7	107.3[a]						
17	100.7	101.9	103.9	97.4[a]						
8	99.0	100.7	101.0	102.8[a]						
12	100.0	102.1	100.6	104.8	97.3[a]					
98 mg/kg DBNP in corn oil										
7	100.6	101.0	100.7	101.4	103.7	103.9	102.7	102.5	-----	-----
15	101.1	101.8	102.4	103.3	104.4	103.4	104.0	104.3	100.0	99.0
5	100.4	101.4	101.0	102.6	102.2	104.0	104.3	104.1	101.3	99.7
10	100.3	102.5	103.2	105.9	107.4	106.9[a]				
13	100.8	100.6	101.4	105.3	105.7	108.0[a]				
78 mg/kg DBNP in corn oil										
8	98.6	101.8	99.8	99.8	100.5	100.7	101.6	102.1	98.4	98.2
4	98.0	100.0	100.6	100.8	100.0	101.6	103.9	106.8	99.4	99.2
11	98.7	100.9	101.9	101.8	103.2	104.7	103.8	105.7	98.9	101.0
12	98.8	100.3	99.9	100.1	99.9	102.1	101.6	102.6	98.8	98.9
14	98.8	101.8	102.0	100.8	103.1	104.0	104.4	105.4[b]	-----	-----
62.5 mg/kg DBNP in corn oil										
18	99.2	99.2	101.7	102.1	102.4	101.7	103.4	101.2	99.4	99.7
6	101.8	101.5	100.5	101.3	101.4	103.7	103.3	102.2	101.4	99.1
16	101.2	100.6	102.7	100.9	102.5	103.2	104.4	102.6	99.9	99.4
1	100.8	102.7	102.3	101.2	101.7	102.1	103.7	99.8	100.0	99.0
20	101.2	101.6	101.2	101.1	103.4	103.4	103.4	104.1[c]	100.7	99.1

a= Postmortem temperature.
b= Temperature at death (7.25 hours) =108.1°F.
c= Temperature recorded at 7.5 hrs= 105.2°F.

TABLE 5. BODY TEMPERATURES (°F) OF CONTROL MALE SPRAGUE-DAWLEY RATS PRE- AND POST ORAL GAVAGE WITH CORN OIL

ANIMAL #	PRE-DOSE	POST DOSE 0.5 HR	1.5 HR	2.5 HR	4.5 HR	5.5 HR	6.5 HR	7.5 HR	24 HR	48 HR
CORN OIL										
1	97.4	97.8	96.9	95.1	101.2	100.4	101.2	97.2	98.2	95.6
3	96.1	97.6	96.1	96.0	99.8	99.4	100.9	98.2	98.3	96.8
4	96.1	99.4	100.5	97.7	99.8	101.7	100.1	98.0	98.6	98.5
8	98.9	99.6	97.2	98.7	97.4	98.6	99.8	98.8	97.6	97.2
10	98.8	96.2	98.9	96.9	99.7	98.3	99.4	97.8	97.8	95.8

SECTION IV

DISCUSSION

Oral gavage of DBNP in corn oil at 250 mg/kg in male Sprague-Dawley rats resulted in complete mortality of rats. Oral gavage with 78 mg/kg and 98 mg/kg DBNP in corn oil resulted in partial mortality of animals. The LD_{50} of DBNP in corn oil in Sprague-Dawley rats as calculated by probit analysis is 93 mg/kg. Even at the lowest dose tested, 62.5 mg/kg, the treated animals showed clinical signs including prostration, labored breathing, and hyperthermia. The No Observable Adverse Effect Level (NOAEL) of DBNP is less than 62.5 mg/kg body weight. These results are comparable to the LD_{50} of 82 mg/kg and NOAEL of 50 mg/kg reported in Fischer-344 rats (MacMahon *et al.,* 1998). Vesselinovitch *et al.* (1961) reported a substantially less toxic LD_{50} value of DBNP in carboxymethylcellulose of 500 mg/kg in male Sprague-Dawley rats. The difference in toxicity reported here may be due to the carrier corn oil permitting increased absorption or bioavailability of DBNP as compared to carboxymethylcellulose. The more potent toxicity of DBNP demonstrated in corn oil in both strains of rats indicates the need for more detailed toxicity studies on DBNP.

MacMahon *et al.* (1998) reported hyperthermia in Fischer-344 rats exposed to DBNP in corn oil. Hyperthermia was also evident in this study, with animals dying with temperatures as high as 108.1°F and surviving animals attaining temperatures as high as 106.8°F. Two earlier studies reported results of toxicity testing of DBNP in rats (Vesselinovitch *et al.,* 1961; Deichman and Gerarde, 1969); neither case noted hyperthermia as an observation. Studies of dinitrophenol (DNP) demonstrated that induction of hyperthermia is associated with an increased metabolism (De las Alas *et al.*, 1990) and uncoupling of oxidative phosphorylation with a resultant decrease in ATP synthesis (Jonsson *et al.,* 1985). The exact mechanism of action of DBNP remains unknown, although it is speculated that it may have a mechanism of action similar to dinitrophenol (DNP). Additional work in this area would increase our understanding of the effects of DBNP.

Microscopic changes observed in Sprague-Dawley rats were similar to those reported in Fischer-344 rats in an earlier study (MacMahon *et al.* 1998). Due in part to the nature of the putative insult and resultant rapid deaths, histopathologic changes were few and subtle but apparent. Noteworthy lesions included degenerative changes in skeletal, smooth, and cardiac muscles. In some animals hepatocellular and renal ductal epithelial changes were seen. The clinical observations linked with assessment of microscopic lesions suggest that metabolic derangement quickly translated into cardiac

failure and death. The subtle histopathologic changes seen indicate the need to use sensitive methods to assess the toxic effects of DBNP. Further research on the pathogenesis of this chemical will help to elucidate the mechanisms of action of DBNP. Further toxicity testing is recommended to ensure the safety of the human health of personnel exposed to DBNP.

SECTION V

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