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GENERATION OF *p*-DINITROBENZENE ATMOSPHERE AND METHEMOGLOBIN FORMATION IN RATS

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ABSTRACT: *A new exposure system was developed to generate p-dinitrobenzene (p-DNB) containing atmosphere. A glass column was filled with small glass beads coated with the chemical. The p-DNB containing medium was heated to a temperature beyond the boiling point of p-DNB. A stream of air flow was forced to pass through the column and let it mixed with fresh air before introducing into an inhalation chamber. The concentration of p-DNB in the chamber air was measured by direct assaying the air directly and by sampling the chemical using a microfilter installed in the chamber. Approximately 90% of the total p-DNB introduced in the chamber gained access to the chamber in first 60 min. Availability of this system was examined by exposing rats to the chamber atmosphere. Marked elevation in methemoglobin (MetHb) levels was observed in the rats when exposed. When the air was filtered through a microfilter prior to introduction into the chamber, however, no p-DNB was detected in the chamber air and little increase in the MetHb blood levels was noted. The results suggest that the chamber atmosphere contained p-DNB as respirable particulates rather than gas.*
Key words: *p-Dinitrobenzene, Inhalation chamber, Exposure, Methemoglobin (MetHb), Particulates.*

INTRODUCTION

p-Dinitrobenzene (*p*-DNB) is widely used in the synthesis of dyestuffs, other dyestuff intermediates, in explosive, and as a camphor substitute in celluloid production (Beard and Noe, 1981). Its physical state is colorless to yellow monoclinic needles, and boiling point 299°C. Its major toxicity lies on the formation of methemoglobin (MetHb). This chemical is the most potent MetHb former among various aromatic amino or nitro compounds of commercial importance (Kim, 1985). The major route of exposure of workers to *p*-DNB is through inhalation. This chemical is one of some 600 airborne toxicants for which the Threshold Limit Values (TLVs) have been established by the American Conference of Governmental Industrial Hygienists (ACGIH). The TLV for *p*-DNB is 0.15 ppm or 1 mg/m^3 (ACGIH, 1988).

In order to estimate the human risk to *p*-DNB exposure, availability of results from experiments using inhalation as the route of administration is strongly recommended. Most inhalation experiments in laboratory settings have been conducted for gaseous chemicals or chemicals which can be evaporated easily. In this study attempts were made to generate a chamber atmosphere containing *p*-DNB, and to examine the ability of *p*-DNB to induce a methemoglobinemia in rats when administered by inhalation. This necessiated development of a new exposure system because of the physical state of this chemical.

MATERIALS AND METHODS

Animals

Male adult Sprague-Dawley rats (200-250g) were used. Rats were housed in stainless steel cages (10 to 12 rats per cage) in environmentally controlled rooms with alternating light-dark cycle (L: 08:00-20:00, D: 20:00-08:00) and an ambient temperature of 22 ± 1°C. Lab chow and tap water were allowed *ad libitum*.

Chemicals

Chemicals used in this study included *p*-DNB (Eastman Kodak Co., Rochester, NY, U.S.A.), acetone (Eastman Kodak Co.), tris (hydroxymethyl) aminomethane (Sigma Chemical Co., St. Louis, MO, U.S.A.), sodium dithionite (Fisher Scientific Co., Fair Lawn, NJ, U.S.A), potassium cyanide (Fisher Scientific Co.), and potassium ferricyanide (J.T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.). All chemicals used were analytical grade or better except for reagent grade acetone.

Generation of Atmosphere

p-Dinitrobenzene, 2-4 g, was dissolved in 250 m*l* acetone. One pound of glass beads with 4 mm diameter (Fisher Scientific Co., Pittsburg, PA, U.S.A.) was added to the mixture in a metal pan ($10'' \times 15'' \times 2''$), and the acetone was allowed to evaporate in a hood overnight. The glass beads coated with *p*-DNB were packed densely in a cylindrical glass column 2.5 cm in diameter and 55 cm long. The column was wrapped with a heating tape (Thermolyne Corp., Dubuque, IW, U.S.A.) connected to a voltage regulator (Superior Electric, Co., Bristol, CT, U.S.A.). The column was preheated to 480°C for an hour before a stream of air flow, approximately 500 m*l* per min, was allowed to pass through the column. The *p*-DNB containing air was mixed with fresh air, and the mixture was introduced into a cylindrical glass chamber (diameter: 29 cm, length: 44 cm). The chamber was fitted with a plexiglass front containing inlets and outlets for the gas, and rubber septa for chamber gas sampling. Air lines were constructed of nalgene and tygon tubing. The total air flow introduced into the chamber was maintained at approximately 4 to 6 liters per min. Rapidly precipitating *p*-DNB carried by the air flow from the column was trapped in a 250 m*l* filtering flask. The gas mixture was filtered by a 0.45 μm pore size mixed cellulose ester filter (Millipore Corp., Bedford, MA, U.S.A.) to remove remaining *p*-DNB in par-

ticulate state (Filter 1). A second 0.45 μm filter, connected to a pump (Bendix, Baltimore, MD, U.S.A.), was utilized to sample the particulate *p*-DNB in the chamber air (Filter 2). A thermometer was inserted into the chamber to monitor the temperature. A schematic diagram of this system is depicted in Fig. 1. A minor modification was made in some experiments, and this is so indicated for each experiment.

Measurement of Chamber Concentrations

The chamber concentration of *p*-DNB was determined using a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. A 1 m*l* sample withdrawn from the chamber was injected into the GC. The glass column was packed with 5% OV-17 on 80/100 Chromosorb G-HP. Nitrogen (30 m*l*/min) was used as the carrier gas, air (300 m*l*/min) and hydrogen (30 m*l*/min) were utilized in the detector. The column temperature was set at 180°C, the detector 320°C, and the injection port at 310°C. The filter utilized to collect the particulate *p*-DNB in the chamber was replaced every hour and assayed for *p*-DNB. The filter was placed in a vial containing methanol and the vial was shaken in an Eberbach Shaker (Eberbach Corp., Ann Arbor, MI, U.S.A.) set on low for 30 min. A 10 μl of samples was injected into the GC. Standards were prepared by dissolving *p*-DNB in methanol.

Measurement of MetHb.

The MetHb concentration in blood was measured using a modification of the method of Rodkey *et al.* (1979) as described by Kim and Carlson (1986). A blood sample obtained from the orbital sinus was diluted with 1500-fold with 0.01M Tris solution containing potassium cyanide and saturated with carbon monoxide. The absorbance was determined at 420 nm in a spectrophotometer (Beckman Instruments Inc., Irvine, CA, U.S.A.). After addition of a few milligrams of sodium dithionite, the absorbance at 420 nm was again determined. The fraction of the total hemoglobin present as MetHb was calculated from these measurements using molar absorptivities of carboxyhemoglobin and cyanmethemoglobin determined in this laboratory.

RESULTS

The objective of this study was to generate a chamber atmosphere containing *p*-DNB and to examine the ability of *p*-DNB to induce a methemoglobinemia when administered by inhalation. Since *p*-DNB has a boiling point of 299°C, it was necessary to develop a new exposure system to evaporate the chemical by increasing the temperature and the surface area of the *p*-DNB-containing medium.

The first experiment was conducted to test the capability of this system to generate *p*-DNB containing atmosphere. Neither the Filter 1 on the gas line or the Filter 2 in the chamber was used in this experiment. Air samples were obtained from the tubing right next to the outlet of the trapping bottle and assayed for *p*-DNB. The results indicated that the *p*-DNB concentration declined rapidly in an exponential fashion (Fig. 2). In

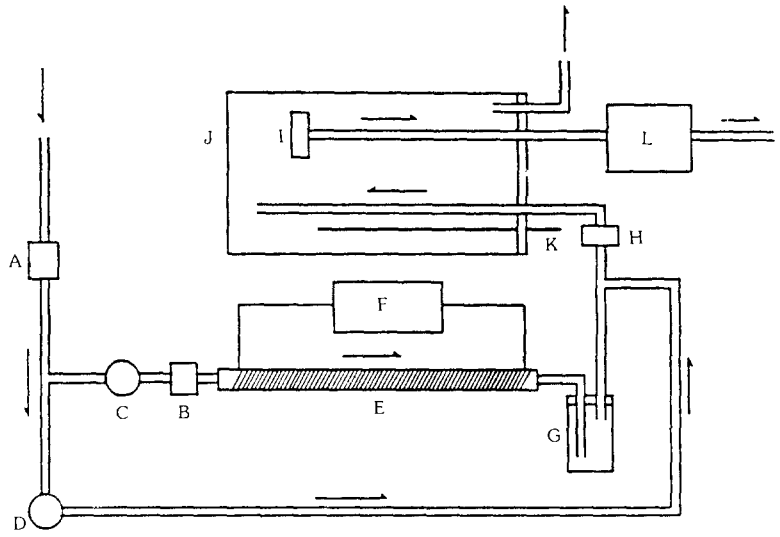

Fig. 1. Schematic diagram of *p*-DNB atmosphere generation system. Arrows indicate the direction of air flow. A and B rotameters, C and D valves, E a glass column wrapped with a heating tape, F a voltage regulator, G a trapping bottle, H and I microfilters, J an inhalation chamber, K a thermometer, L a pump. See the methods for details.

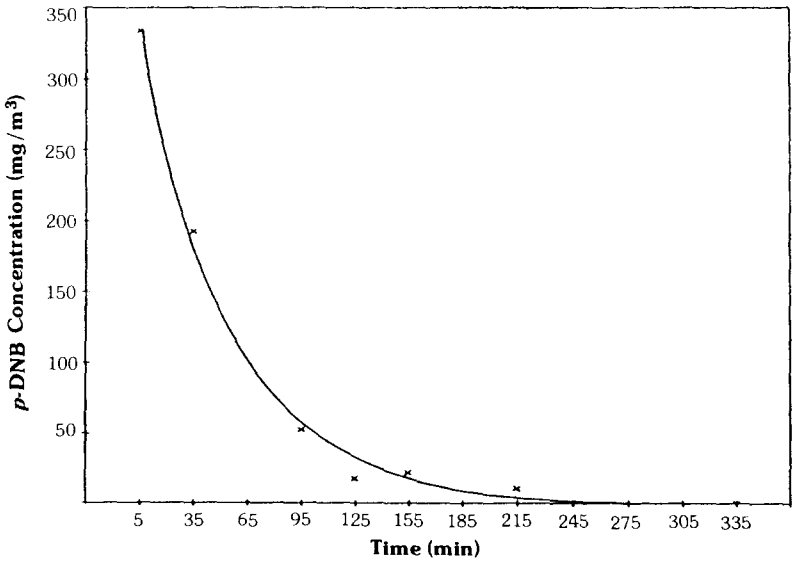

Fig. 2. Decline of *p*-DNB concentration in the air flow to the chamber. Air samples were taken from the tubing connecting the trapping bottle and the chamber, and assayed for *p*-DNB gas chromatographically. X axis represents the time after the initiation of passing air through the *p*-DNB containing column.

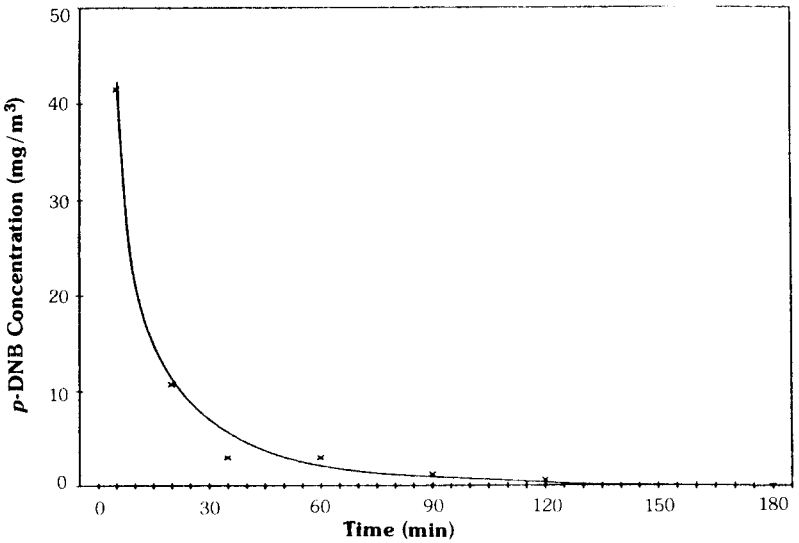

Fig. 3. Decline of *p*-DNB concentration in the chamber air.
Chamber atmospheres were sampled through septa on the chamber door and assayed for *p*-DNB gas chromatographically. X axis represents the time after the initiation of passing air through the chamber.

Table 1. Chamber concentration of *p*-DNB determined by amount of *p*-DNB recovered from the filter and MetHb formation in rats[a]

p-DNB (mg/m^3)[b]			MetHb (%)[c]
First Hour	Second Hour	Third Hour	
14.61	0.49	0.51	64.3 ± 1.8

[a]The exposure chamber atmosphere was generated for 3 hours according to the method described in the text. A microfilter installed in the chamber was replaced every hour and assayed for *p*-DNB. The flow rate of the pump connected to the microfilter was approximately 1.7 liters per min.
[b]Concentration of *p*-DNB was expressed as mg of *p*-DNB recovered from the filter per volume of air (m^3) filtered.
[c]MetHb level was measured in rats at the end of the 3 hour exposure. Value given is mean ± S.E. for five rats.

another experiment the Filter 2 connected to a pump was installed and replaced every hour to sample the *p*-DNB existing as dusts larger than the size of 0.45 μm. The results in Fig. 3 indicate that approximately 90% of the total *p*-DNB introduced into the chamber gained access to the chamber in the first 60 min. This is in good agreement with the results from the experiment measuring the amount of *p*-DNB accumulated every hour on the Filter 2 (Table 1). Exposure of rats to the test atmosphere for 3 hour resulted in a significant methemoglobinemia indicating that the *p*-DNB in the atmosphere was respirable. The chamber air temperature was slowly elevated from 26 to 29.3°C during the experiment.

In order to determine the physical state of *p*-DNB in the chamber atmosphere, mixed air flow from the trapping bottle and fresh air was filtered using the Filter 1 prior to

Table 2. Concentrations of *p*-DNB in the chamber and in the air line determined by amount of *p*-DNB recovered from the filters.[a]

	p-DNB (mg/m^3)[b]		
	First Hour	Second Hour	Third Hour
Filter 1[c]	9.90	0.35	0.45
Filter 2[d]	0.86	0	0

[a]The chamber atmosphere was generated for 3 hours according to the method described in the text. Both filters were replaced every hour and assayed for *p*-DNB. The total air flow was 6.8 liters/min and the flow rate of the pump connected to the filter 2 was 0.7 liter/min. The temperature of the chamber air slowly increased from 26 to 27.8°C during the experiment.
[b]Concentration of *p*-DNB was expressed as mg of *p*-DNB recovered from the filter per volume of air (m^3) filtered.
[c]Filter 1 was installed in the tubing between the trapping bottle and the chamber. Values in this group represent the particulate *p*-DNB concentrations in the air flow prior to the first filtration.
[d]Filter 2 was installed in the chamber and connected to the pump. Values in this group represent the particulate *p*-DNB concentrations in the chamber.

Table 3. Concentration of *p*-DNB in the air line determined by amount of *p*-DNB recovered from the filter and MetHb formation in rats[a]

Rats	MetHb (%)[b]		*p*-DNB (mg/m^3)[c]
	t = 0 min	t = 90 min	
Group I[d]	1.2 ± 0.7	0.3 ± 0.3	2.40
Group II[e]	2.9 ± 1.3	0.1 ± 0.3	

[a]The chamber atmosphere was generated for 3 hours according to the method described in the text. A microfilter was installed in the tubing between the trapping bottle and the chamber during the whole experiment and assayed for *p*-DNB accumulated for that period. The total air flow was 6.8 l/min.
[b]MetHb level was determined immediately following termination of the one hour exposure and 90 min after the first measurement.
[c]Concentration of *p*-DNB in the air flow prior to the filtration was expressed as mg of *p*-DNB recovered from the filter per volume of air (m^3) filtered.
[d]Group I was exposed to the chamber atmosphere for the first hour of the experiment. Value given is mean ± S.E. for three rats.
[e]Group II was exposed to the chamber atmosphere for the last hour of the experiment. Value is mean ± S.E. for three rats.

introduction into the chamber (Fig. 1). Both the Filter 1 and 2 were replaced every hour and assayed for *p*-DNB. The results indicate that the ratio of the time weighted concentration of particulate *p*-DNB in the air, prior to the first filtration, to that in the chamber for the first hour was 1:0.09 (Table 2). No *p*-DNB was collected on the Filter 2 from the second hour of exposure. Two groups of rats were exposed to the chamber atmosphere for either the first one hour or the last one hour of the three hour experiment. The MetHb levels were measured twice, immediately after the termination of exposure and 90 min following the first measurement. No significant elevation of MetHb level was observed in either group (Table 3). The chamber air was sampled during the

exposure and assayed for *p*-DNB, however, no measurable response of the GC to *p*-DNB was observed.

DISCUSSION

A new exposure system was developed to generate a chamber atmosphere containing a chemical with low volatility. *p*-DNB was chosen as the test chemical because it has a high boiling point and has a very potent MetHb producing activity, a characteristic which makes a rapid identification of exposure of animals to the chemical possible. In order to increase the volatility of the chemical, preheating of the chemical and maximizing the surface area of the *p*-DNB-containing medium by coating the chemical on glass beads were needed. Suitability of this system was determined by 1) direct measurement of the *p*-DNB concentration in the chamber air, 2) measurement of the *p*-DNB sampled on the microfilters installed in the exposure chamber, and 3) MetHb generation in the blood of rats exposed to the chamber air.

The *p*-DNB concentration in the chamber air declined rapidly. Approximately 90% of the total *p*-DNB dicharged into the chamber gained access to the chamber in the first one hour as determined by measurement of *p*-DNB in the chamber air. This result was supported by the amount of *p*-DNB accumulated hourly on the surface of the microfilter installed in the chamber. Significant increases in MetHb level were observed in rats exposed to the air indicating that the *p*-DNB in the air was respirable to rats regardless of the physical state of this chemical.

The *p*-DNB concentration in the chamber air was 0.6 mg/m^3 120 min following the initiation of passing the *p*-DNB air through the chamber (Fig. 3). The time weighted average *p*-DNB concentration in the chamber air filtered for the third hour of exposure was 0.51 mg/m^3 (Table 1), suggesting that most of the *p*-DNB in the chamber air was filtered out. In the experiment a filtered air is introduced to the chamber in order to determine the physical state of the *p*-DNB in the air, only minute amount of *p*-DNB was detected from the filter installed in the chamber indicating that most of the *p*-DNB, if not all, in the air has the form of particulates rather than gas (Table 2). Also negligible levels of MetHb were observed in the rats exposed to the filtered air suggesting that little *p*-DNB gained access to the inhalation chamber.

In an experiment a chemical is administered to animals by inhalation, it would be required to maintain a constant concentration of the airborne chemical in the chamber for certain periods. In the chamber atmosphere generated by the method employed in the present study, *p*-DNB levels declined rapidly. In order to obtain a constant concentration of *p*-DNB, a computer controlled exposure system with variable air flow rates through the *p*-DNB containing medium without affecting the total flow rate in an exposure chamber would be recommended.

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