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#### Authors:

#### Performing Organization:
US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDT-N
3100 Ricketts Point Road

#### Sponsor:
Defense Threat Reduction Agency
8725 John J. Kingman Road STOP 6201
Fort Belvoir, VA 22060-6201

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Determination of LC₅₀s in anesthetized rats exposed to aerosolized nerve agents

Jennifer L. Collins¹, Xinqi Peng², Robyn Lee³, Alicia Witriol⁴, Zdenka Pierre⁵, and Alfred M. Sciuto²

¹27 SOCES/CEAN Cannon Air Force Base, NM 88103, USA and ²United States Army Medical Research Institute of Chemical Defense Analytical Toxicology Division, Medical Toxicology Branch, Aberdeen Proving Ground, MD 21010, USA

Abstract
Nerve agents pose a threat to the respiratory tract with exposure that could result in acute compromised lung performance and death. The determination of toxicity by inhalation is important for the rational development of timely therapeutic countermeasures. This study was designed to deliver aerosolized dilute nerve agents in a dose-response manner to investigate the extent of lethality of nerve agents: soman, sarin, VX and VR. Male rats (240-270 g) were anesthetized intramuscularly with 10 mg/kg xylazine and 90 mg/kg ketamine. Following anesthesia, rats were intubated with a glass endotracheal tube (ET) and placed in a glove box. The ET was connected to a closed circuit nebulizer system (Aeroneb, Aerogen, Inc.) that delivered a particle size of < 2.0 μm and was in series between the ventilator and the ET. Nerve agents were delivered by a small animal ventilator set for a volume of 2.5 ml x 60-80 breaths/min. VX or VR were nebulized and delivered in concentrations ranging from 6.25-800 μg/kg over a 10-min exposure time period. Sarin (GB) or soman (GD), 6.5-1250 μg/kg, were delivered in a similar manner. Lethality by inhalation occurred either during the 10-min exposure period or less than 15 min after the cessation of exposure. Survivors were euthanized at 24 h postexposure. LC₅₀ estimates (± 95% confidence intervals [CIs]) were obtained from the sequential stage-wise experiments using the probit analysis. Probit analysis revealed that the LD₅₀ for VX was 110.7 μg/kg (CI: 73.5-166.7), VR 64.2 μg/kg (CI: 42.1-97.8); soman (GD), 167 μg/kg (CI: 90-310), and sarin (GB), 154 μg/kg (CI: 98-242), respectively. Although VR is a structural isomer of VX, the compounds appear to be markedly different in terms of toxicity when delivered by aerosol. These relationships were converted to actual 10 min LC₅₀ equivalents: VX = 632.2, VR = 367, GD = 954.3 and GB = 880 mg·min/m³. Validation of exposure was verified by the determination of blood levels of acetylcholinesterase (AChE) across doses for the agent VR.

Keywords: Chemical warfare nerve agents, aerosol, inhalation LC₅₀ determination, acetylcholinesterase, anesthetized rodents

Introduction
Organophosphorus compounds (OPs) are a family of highly toxic pesticides that have been used since the 1940s. They pose a significant risk to the general public due to the high frequency of poisonings and are responsible for approximately 220,000 deaths worldwide annually (World Health Organization 1993). In Germany prior to WWII, OPs were initially developed for pesticide use, but because of their lethality in humans some were developed for use as chemical warfare agents. Schrader synthesized sarin (GB) in 1937; Kuhn synthesized soman (GD) in 1944 (Maynard & Beswick 1992). While nerve agents were not used during WWII against soldiers or civilians, their development continued. Since that time more lethal and persistent agents were developed, especially during the cold war years and in particular by the US and the former USSR. The V-agents, of which VX is a member, are a family of nerve agents distinct from the G-agents. The first V-agent, VX, was developed in the late 1950s by Tammelin from the insecticide Amiton. These V-agents such as VX and VR are sulfonated organophosphorus compounds, while the G-agents GB...
and GD are fluorinated organophosphorus compounds (Figure 1a–d). One of the primary physical characteristic differences between the two families is their volatility (Table 1). G-agents are known to be more volatile than the V-agents, but V-agents are more persistent than the G-agents in the environment extending their temporal toxicity.

Physiological and toxicological biomechanisms of the effects of traditional nerve agents following inhalation have not been comprehensively investigated using an established and consistent inhalation exposure model. The significance of this work is that a reliable aerosol inhalation model was developed to address the exposure responses.

Target organs of nerve agent toxicity are the eyes and skin; however, the primary route of exposure especially for the more volatile agents is by inhalation. Nerve agents function as anticholinesterases that inhibit the enzyme acetyl-cholinesterase that is necessary for degrading acetylcholine, a critical neurotransmitter active at neuromuscular junctions. As such, these can result in repetitive nerve misfiring and fasciculations. Nerve agents also affect many sites and receptors such as smooth muscle muscarinic and skeletal muscle nicotinic receptors. Symptoms of toxicity, known as “cholinergic crisis” include bronchoconstriction, miosis, rhinorhea, salivation, and urination to epileptic seizures, and ultimately death by asphyxiation. For a review of the physiological effects of nerve agent exposure see Tuorinsky (2008). These mechanistic data which are generated from consistent exposure models are vital for the development of appropriate therapeutic countermeasures.

The nerve agents represent a current threat as they have been used in terrorist attacks and in warfare in the Middle East (United Nations Security Council 1984; United Nations Security Council 1986; United Nations Security Council 1987). One of the best known cases is for GB which was used in a terrorist attack in Japan on 20 March 1995 in which over 5000 people were exposed to GB gas, and over 1000 people were listed as moderately to critically ill. Twelve people died in the attack. Most people are unaware that this same terrorist group previously killed 12 people with GB and also experimented with VX (Asai & Arnold 2003). Nerve agents were suspected to have been used in the conflict between Iran and Iraq (United Nations 1987). However, this has never been officially medically determined (Willems 1989).

Whether the victims are civilians or military, the fact remains that there are no antidotes with complete medical protection from or against the toxic effects of these agents if inhaled. This basic problem has lacked serious investigation and, as such, the paucity of fundamental research has not provided sufficient data to field products that counteract inhaled nerve agent toxicity. Investigation of the toxic effects of these low (VX, VR) and high (GB, GD) volatility agents outlined herein will expand the database in the development of rational medical countermeasures against the inhalation toxicity of these highly poisonous nerve agents. We have developed a reliable and consistent model for inhaled nerve agent. This model bypasses the nasal passages, where agent can be neutralized and instead delivers it directly to the lung and more closely simulates exposure in humans because the agents were delivered directly to the upper tracheal airway and lung. This method of exposure bypasses the detoxifying properties of the nasal passages. Using this more relevant and consistent model of aerosol inhalation exposure see Tuorinsky (2008).

Table 1. Nerve Agent physical/chemical properties.

<table>
<thead>
<tr>
<th>Property</th>
<th>Sarin (GB)</th>
<th>Soman (GD)</th>
<th>VX/VR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>140.1</td>
<td>182.18</td>
<td>267.36</td>
</tr>
<tr>
<td>Specific gravity at 25°C</td>
<td>1.0887</td>
<td>1.022</td>
<td>1.0083</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>−56</td>
<td>−80</td>
<td>−20</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>147</td>
<td>167</td>
<td>300</td>
</tr>
<tr>
<td>Vapor pressure (mmHg)</td>
<td>0°C</td>
<td>0.52</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>10°C</td>
<td>1.07</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>2.1</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>2.9</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>30°C</td>
<td>3.93</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>12.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Guinea pig LD₅₀ µg/kg (h.w.)</td>
<td>42</td>
<td>30</td>
<td>8/11.3</td>
</tr>
</tbody>
</table>

s.c.: subcutaneous administration.
Table adapted from Maynard and Chilcott (2009).
we have designed a study to ascertain the LCt_{50} for the nerve agents VX, VR, GD, and GB.

**Methods and materials**

All agents were delivered, using an aerosolization technique, directly into the intubated airway of anesthetized rats using a small animal ventilator (Harvard Model 683, Harvard Apparatus, Holliston, MA) set for a tidal volume of 2.5 mL and a respiratory rate of approximately 70 breaths/min (Figure 2). This allowed for a standardized agent delivery using narrow ventilation range. Agents were delivered within a closed circuit standard nebulizer system (Aeroneb, Aerogen, Inc., Marietta, GA) that allowed the nebulization of up to 10 mL of buffer or solvent. The nebulizer was in series between the ventilator and the endotracheal tube. Agent off-gassing from the lung into the expiration air outflow was trapped in 10% NaOH (or charcoal) prior to the ventilator and subsequently vented through the HEPA filter in the glovebox. Male rats (240–270 g) were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) intramuscular injections. Rats were intubated with a 10–12 gauge fire-polished glass Pasteur pipette that was secured with a gauze strip around the rostrum to prevent slippage (Figure 3). Anesthetized rats were exposed singly to an agent using a closed-to-atmosphere inhalation system via mechanical ventilation for 10 min. The entire exposure system fits well within the confines of the inhalation exposure chamber (glovebox). The aerosolization rate in this system was determined to be 0.2–0.3 mL/min with the delivery of a consistent particle size of 2.1 μm. This is in the middle range for distal lung deposition. All rats were removed from the ventilator and allowed to recover in the glovebox for 10–15 min after exposure for off-gassing purposes.

**Determination of a 50% lethal concentration at 24 h (LD_{50})**

The LD_{50} was determined by generating lethality dose response curves using a modification of the adaptive dose design described by Feder et al. (1991a, b, c). One to three rats were allocated randomly to each of 3–5 agent challenge levels per stage. In the first stage, a range of agent doses were selected to span the predicted range of lethality from 0 to 100%. The results of the first stage were used to select agent doses for the next stage. This approach allowed animals in this and later stages to be placed at doses that allow better estimation of the LD_{50} over time. The dosing stages continued until the half-width of the 95% CI, defined as (upper bound – lower bound)/(2 × LD_{50}) for the LD_{50}, was less than 0.40. After the last stage, probit dose-response models using maximum likelihood were fitted to the combined data for all stages (Feder et al. 1991a, b). These data were then used to calculate the LCt_{50}. Fieller’s method was used to compute a 95% CI for the LD_{50} (Finney 1971). The stage-wise adaptive dose
design is an efficient design, because it reduced overall animal use by eliminating the need of a separate range finding study (range finding is conducted in stage 1) and eliminated the chance of completely missing the lethality range of the agent thereby having to repeat the study.

**Determination of blood AChE**

To determine blood acetylcholinesterase (AChE) inhibition, blood was drawn either from the saphenous vein or by cardiac puncture from each euthanized animal into a heparinized syringe at 5–10 min preexposure and at 24 h post-exposure. Blood was then diluted 10-fold with deionized water and 20 μL of diluted sample was placed in the well of a 96-well microtiter plate for a modified microtiter assay for AChE activity (Ellman et al. 1961; Doctor et al. 1987). To this, 260 μL of phosphate buffer (pH 8.0) containing 4 μM of iso-OMPA (tetra monoiso-propyl pyrophosphor-tetramide (BChE inhibitor) was added, along with 10 μL of DTNB (dithionitrobenzoic acid) followed by 10 μL of acetylcholine iodide (ATCh). The plate was read in a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) at 412 nm. The AChE activity, measured in triplicate, was normalized to total blood protein measured by Bradford dye binding assay at 595 nm (Bradford 1976).

**Data analysis**

LD₅₀ estimates with 95% CI were determined using the stage-wise adaptive dose and the probit analysis methods of Feder et al (Feder 1991a,b,c). A one-way ANOVA statistical analysis was run on AChE data using the Bonferroni post-hoc test for significance. Significance was accepted at p ≤ 0.05.

LC₅₀s were calculated from the real-time LD₅₀ determinations using the following equation from Weston and Karel (1946):

\[
LD_{50} = V_m \times LC_{50} \\
\]

or

\[
LC_{50} = LD_{50} / V_m
\]

where LD₅₀s (μg) were obtained from ratio of the actual probit plot analyses of lethality for each agent (Figure 1a–1d) and Vₘ (minute ventilation) which is the product of respiratory rate (breaths/min) × tidal volume (mL). Vₘ is expressed as m³/min. During this study, Vₘ was held constant for each animal so that Vₘ = (70 breaths/min) × (2.5 mL/breath) = 0.175 L/min. This was then converted to 0.000175 m³/min.

**Results**

LD₅₀ assessments of VX, VR, GD, and GB were determined using an intratracheal aerosol delivery system. There were approximately 80–90 rats tested per agent. LC₅₀ assessments were critical to establish exposure-lethality standards for all future exposures. Each rat was singly exposed to agents for 10 min through the following range of concentrations 6.25–700 μg/kg for VX, and 6.25–800 μg/kg for VR, 6.25–1250 μg/kg for GD, and 12.5–1250 μg/kg for GB. All mortalities occurred within 15–30 min after the start of exposure. Probit analysis revealed that the LD₅₀ was estimated to be 110.7 μg/kg for VX (CI: 73.5–166.7), 64.2 μg/kg for VR (CI: 42.1–97.8), 154.3 μg/kg for GB (CI: 98–242) and 167.3 μg/kg for GD (CI: 90–310), Figures 4–7. These relationships were converted to actual 10 min LC₅₀ equivalents: VX = 632.2, VR = 367, GD = 954.3 and GB = 880 mg·min/m³.

Validation of the exposure model was assessed through the determination of blood AChE levels across inhaled agent dose. We selected VR for this estimation because it was the most toxic of the agents tested. It can be seen in Figure 8, that at the stated toxicity for VR (64.2 μg/kg) there was an approximately 55% decrease in AChE compared to preexposure levels.

**Conclusions**

To our knowledge, this study represents the first systematic comparative lethality study of the traditional nerve agents using direct aerosolization of agent into the lungs. Historically, studies have focused on the delivery of an agent via whole-body inhalation or a subcutaneously...
delivered exposure (Gates & Renshaw 1946; von Lohs 1960; Fleisher et al. 1963; Brimblecombe et al. 1970; Pazdernik et al. 1983; Boskovic et al. 1984). Whole-body inhalation in rodents is generally equivalent to a nose-only model. With regard to the present study we should emphasize that in mouth-breathing humans, the nasal passages are generally bypassed. A study by Morris and Hubbs (2009) looking at the inhalation dosimetry of diacetyl in rats showed that in the nose-breathing rat approximately 62% of the inhaled diacetyl reached the bronchi compared to 97% estimated for mouth-breathing humans (Morris 2009). This indicates that nose-breathing rodent studies may underestimate equivalent dosimetry exposure/response effects when extrapolated to mouth-breathing humans. As such, both the whole-body or subcutaneous techniques are not suitable comparisons with human exposure. Comparison of inter-laboratory methods with regard to lethality restricts how to interpret biological responses into meaningful treatment strategies is problematic at best. Animal exposure models vary considerably from laboratory to laboratory. Basic respiratory responses to what are termed traditional nerve agents have not been systematically investigated. The method presented herein does provide an alternate method to assess inhalational toxicity. Moreover, we have shown that nerve agents regardless of their inherent chemical characteristics can be studied with reasonable accuracy. The advantages of this method are several: 1) lethality was determined in singly-exposed rats thereby avoiding the competition for breathing space in confined whole-body studies, 2) the nasal passages were bypassed and direct inhalation of agent into the airways more closely resembles that of mouth-breathing humans, 3) all ventilator parameters such as tidal volume and respiration rates were tightly controlled and standardized based on the experimental animals’ own baseline values.

Our results indicate that while VR is a structural isomer of VX, the compounds are markedly different in terms of lethality when delivered by aerosolization with VR being more toxic than VX. We could speculate that the reason for this may be that the stereospecificity in the AChE molecule is affected in some manner by the rearrangement of agent-side groups. Molecular dissymmetry shows that VR has 4 propyl side groups compared to two for VX (Figure 1a and 1b), which could prolong the effects of VR. It has been suggested that exposure to VR may require higher doses of atropine compared to other organophosphate nerve agents (Chang et al. 2002). The Chang et al. study suggests that the toxicity of VR involves significant cardiorespiratory collapse, which may require aggressive rescue efforts. As such, our data generally agrees with their results. In addition, GB was shown to be as lethal as GD from probit analyses. The literature, however, suggests that GD is generally more lethal than GB, but these comparisons were done using unrealistic exposure models, as stated earlier. When our exposure...
concentrations were converted to mass/volume (mg/mL), GB was more toxic than GD. From these data, a feasible explanation for the difference between GB and GD may lie in the well-known capacity of GD to "age" on the enzyme AChE. This could be an indication that the lethal effects take more time as compared to more volatile GB.

One criticism of this work could be that the V-agents, generally considered percutaneous exposure hazards, are not valid inhalational threats due to their low vapor pressure. Sentence deleted. Low volatility agents can be inhaled as a vapor or aerosol if the environmental conditions are right. In addition, they could adhere to particles of dust or mix with mists or fog. As such, it makes them all the more dangerous in addition to their inherent toxicity. Taking into consideration the above, it becomes essential to have valid, standard, and relevant inhalation exposure models to assess temporal and dose-response exposure outcomes for all agents.

The current study has attempted to address inhalation research gaps by developing a consistent, realistic, and reliable state-of-the-art exposure system and establishing a dose-response rat inhalation model for traditional nerve agents. From these experiments the rapidity of lethality is quite remarkable and reinforces the continued development of timely and effective medical intervention as high priority both nationally and militarily. The validity of the exposure system is supported by the temporal decreases in AChE activity across the doses shown in Figure 8. While it is well known that decreases in AChE following nerve agent exposure are not necessarily aligned with mortality, they can be linked to toxicity (Munro 1994).

The next phases of this project will establish trigger points of injury and a therapeutic window in conjunction with evaluation of FDA-approved treatments. This is critically important when comparing the exposure-response effects for traditional agents against each other, which this study will allow us to do. In addition, testing therapies against inhaled aerosolized traditional agents has not been systematically investigated. These data will provide invaluable information for the medical therapy database through the comparison of the effects of non-volatile agents, such as VX and VR, vs. the effects of the volatile agents such as GB and GD. In conclusion, we have established a precise and accurate inhalation model to assess lethality in the rat. We intentionally bypassed the nares to avoid detoxification of the agent in the rat - an obligate nose-breather. We believe these data more accurately reflect a realistic human exposure in which the direct effects of exposure maybe reflected in the deeper airway.

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The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, Publication No. 85-23, 1996), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

Declaration of interest

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Inhalation lethality curves of nerve agents


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