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Inhalation Toxicity in Mice Exposed to Sarin (GB) for 20–720 min

R. W. Bide* and D. J. Risk
Defence R&D Canada — Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6

Key words: nerve agents; GB; longer exposures; mice; toxicity; inhalation.

Most of the historical data for the toxicity of sarin (GB) was collected for exposure times of <10 min in attempts to establish the utility of and defence against this agent in offensive military use. However, information concerning the toxicity of GB (and other nerve agents) from longer exposures of 1–12 h is critical for all personnel who must work in or close to low-level concentrations of chemical for extended periods and for all personnel, dressed in Individual Protective Equipment, who need to know when, and if, it is safe to take off these cumbersome garments.

The data presented for the toxicity of GB to mice for whole-body exposures of 20 min to 12 h are intended to form part of an ongoing, multi-species effort aimed at establishing toxicity estimates for humans for these longer exposure times: LCt_{50} values of 430, 540, 900, 1210 and 2210 mg·min m^{-3} or LC_{50} values of 21.5, 9.0, 5.0, 3.4 and 3.1 mg m^{-3} were obtained for mice for 20-, 60-, 180-, 360- and 720-min exposures to GB, respectively. The data for longer exposures do not follow Haber's rule ($LCt_{50} = CT$). The 20- and 60-min data fit the 'toxic load model' involving C^nT that was established previously from historical data for 0.17–30 min GB exposures to mice. The LCt_{50} and LC_{50} values for 3, 6 and 12 h are progressively higher (toxicity lower) than predicted by either Haber's rule or the toxic load model.

INTRODUCTION

When the nerve agents were first studied shortly after World War II, the experiments done to define toxicity were designed to describe the offensive utility of and the necessary defensive measures against attacks with these new agents. In these early experiments, many exposure times were short (1–30 s) to demonstrate the lethality resulting from a massive attack and incapacitation from a single breath. The result was that very few data were generated for exposures of >10 min (Bide *et al.*, 1998, 2004a). When the literature concerning nerve agents was re-examined in preparation for the 1991 Gulf War and following the terrorist use of sarin (GB) in Tokyo (Okumura *et al.*, 1996, 1998a,b,c), one of the main data gaps identified was the absence of data concerning the toxicity of the nerve agents at exposure times of >15 min (Bide *et al.*, 1998, 2004a; Mioduszewski *et al.*, 2000, 2001; Grotte and Yang, 2001). Information concerning the toxicity of chemical warfare agents at these longer exposure times is critical for:

(i) Setting limits for first responders functioning near an incident where contamination levels can be low and variable, and exposures of many hours are encountered.
(ii) Determining the boundaries of safety surrounding a spill or other incident.
(iii) Designing detection equipment, monitors, alarm systems and protective equipment of all kinds.
(iv) Hospital staff and their support workers:
    (a) who must work with contaminated victims who may or may not be sufficiently decontaminated;
    (b) who must establish the safe level of decontamination for the immediate activity.
(v) All personnel, dressed in Individual Protective Equipment, who need to know when, and if, it is safe to take off these cumbersome garments.

At exposure times up to 30 min the toxicity of GB in several species (Bide *et al.*, 1998, 2004a) follows an empirical relationship (tenBerg, 1986; Yee, 1996; Yee *et al.*, 1998), referred to as the 'toxic load model' and characterized by the equivalent planar surface equations

$$Y = b_0 + b_2 \log_{10} C^n T \quad \text{or} \quad Y = b_0 + b_1 \log_{10} C + b_2 \log_{10} T$$

involving the exposure time T explicitly. Here, Y is the PROBIT value, C is the exposure concentration, T is the exposure time, $n(= b_1/b_2)$ is the 'toxic load exponent' and b_0, b_1 and b_2 are experimentally derived regression coefficients (empirical constants). Note that constant b_0 is dependent upon the units of C and T whereas the other constants and n are not. With no toxicity data available for exposures of >30 min, it was not clear whether this relationship for GB would be valid at longer exposures. To test the hypothesis that the relationship would continue to longer exposures, a series of LCt_{50} studies were carried out with mice at exposures between 20 and 720 min. The resulting data presented here are compared with the historical information collected at shorter exposure times (Bide

* Correspondence to: R. W. Bide, Defence R&D Canada — Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6.
E-mail: Richard.Bide@drdc-rddc.gc.ca

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et al., 1998, 2004a). As part of an ongoing international cooperative effort, a similar study has been carried out in rats (Mioduszewski *et al.*, 2000, 2001).

MATERIALS AND METHODS

Chemicals

Sarin (isopropyl methylphosphonofluoridate, GB; CAS no. 107-44-8 or 50642-23-4; MW = 140) was synthesized *de novo* at DRDC Suffield and was freshly distilled shortly before use. The purity (>98%) was tested on a regular basis during the experiments using NMR.

Animals

The mice used were male CD-1® strain purchased from Charles River Canada, St. Constant, PQ. The mice were acclimatized for at least 7 days in the vivarium at DRDC Suffield before use.

Air supply

Particle- and chemical-free air was supplied by a breathing air system consisting of a water-sealed compressor (SIHI model KHPE 35504, SIHI-Halberg International Co., Ludwigshafen/Rhein, West Germany) fitted with HEPA filters on the air intake, a water separator and air dryer immediately following the compressor and then oil and particle filters in the compressed air line. The dry air was delivered at 60 psi (413 KN/m^{-2}) to the inhalation exposure system.

Exposure system

Whole-body animal exposures were carried out in a dynamic exposure system (Bide and Risk, 2002) consisting of (in progressive order) a Tylan flow controller (Model RO-28 with FC-262-V; Tylan General, Torrance CA) that regulated the total air flow in the system at 20 l min^{-1}, an injection port created from a stainless-steel Swagelok® T-union with a septum fitted to one arm of the 'T', a Miran analyzer (Model CVF general purpose gas analyser; Foxboro Analytical, South Norwalk, CT) and a custom-made stainless-steel and glass whole-body exposure chamber of 31.75 l interior volume and 30 × 30 cm interior cross-section (designed by BioResearch Inc., Senneville, PQ, under contact to DRDC Suffield). All piping connections were made with 3/8″ ID (9.53 mm) polypropylene tubing. The gas entered at the top and the exhaust from the bottom was passed through a carbon filter (US M18 military tank filter (NATO) 4240-00-828-3952). In the chamber, the mice were housed in a 26 × 26 × 8.5 cm six-compartment wire-mesh animal holder. The animal holder was supported on a close-fitting platform of 20-gauge stainless-steel, 0.25″ perforated at 0.375″ centres (40% open), suspended 7 cm above the bottom of the chamber. The perimeter of the support was covered with a 2.5-cm-wide vapour barrier. The animal holder sat securely on the vapour barrier so that all of the gas passing through the chamber went through the animal holder. Virtually none of the gas that passed through the animal holder and support plate returned to the upper portion of the chamber. Thus, the effective size of the exposure chamber was the space above the support plate or 25.45 l. In operation, with the air flow regulated at 20 l min^{-1} by the Tylan controller, the exchange rate would be 0.8 changes/per min.

A gas sample was drawn continuously at 10 l min^{-1} from immediately above the animal holder into a second Miran analyser for direct measurement of the GB concentration. The remaining 10 l min^{-1} passed through the animal holder. Six mice breathing 25 ml min^{-1} should not be subject to significant rebreathing under these conditions (Moss and Asgharian, 1994).

During exposures, the trial chemical (neat GB) was introduced to the input air at the injection port from 10- or 25-µl Hamilton syringes (series 1801 and 1802 RN) fitted with 26-gauge, 2″ blunt-tip needles (Hamilton Co., Reno, NE) driven by a Model 22 syringe pump (Harvard Apparatus, Inc., Holliston, MA).

Exposure concentrations

The exposure for each experiment was calculated as described below by integrating the recorder trace produced by the Miran sampling from the exposure chamber and then calculating the concentration using the daily calibration of the system. In each experiment, a time required for the exposure atmosphere to attain 99% of the operational value (t_{99}) for that exposure was calculated from the tracings. Both Miran analysers were running continuously during all exposures. A two-pen recorder provided a permanent record of the measured concentrations. The exposure concentration used for toxicity assessments was calculated only from the Miran sampling from the exposure chamber.

The Miran analysers were calibrated daily. For calibration, the two Miran analysers, a metal bellows pump (15 l min^{-1}), the injection port and an SS-45YF8 Swagelok high-flow four-way switching valve were arranged in a closed loop (total gas volume = 12.2 l). The valve allowed either a dynamic flow through the analysers (purge setting) or a closed recirculating loop (calibration). For calibration, a measured amount of GB was injected into the closed, recirculating loop. With the pump creating a rapid circulation of the contained air, the chemical concentration in the loop was allowed to equilibrate. When a maximum, stable absorbency value was obtained on the recorder tracings of both Miran analysers, the loop was purged. This process was repeated several times with varying amounts of GB to obtain a series of peaks on the recorder tracings from which calibration curves of absorbency vs concentration were created. To maintain linear calibrations, the Miran analysers, which are stable, single-beam spectrophotometers without linearization, were adjusted (path length, range, sensitivity) so that the maximum absorbency used in any experiment did not exceed 0.15 optical density. The recorders were adjusted so that the maximum peak height on the calibration curve was ca. 80% of the full chart width and the absorbencies of the trial exposures were between 25 and 70% of the full chart width. To simplify subsequent calculations, instead of peak height, the area under the tracing for one chart division was measured as the calibration value for each peak. These values were used to create a calibration curve for that set of experimental conditions (Fig. 1). The area measurements were made using an electronic planimeter system (Sigma-Scan version 3.0; Jandel Scientific, Corte Maderi, CA). For each experiment, the

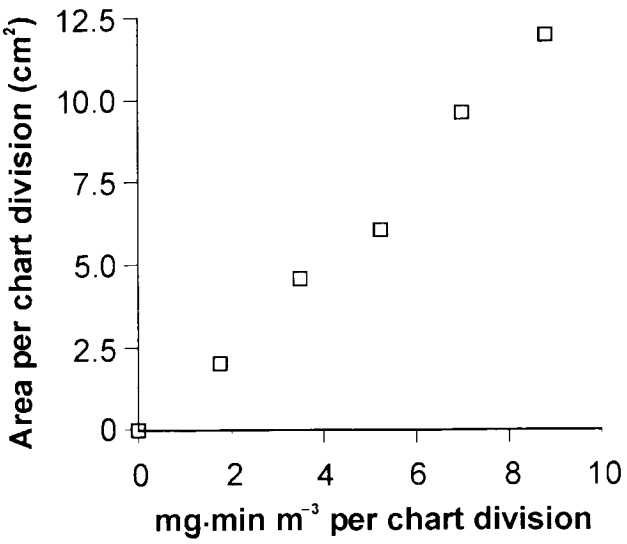

Figure 1. A typical calibration curve for GB analysis from one of the 360-min exposures. Normal spectrophotometric calibration methods were used, substituting peak area for peak height. Time is the chart division unit, hence concentration-time (mg·min m^{-3}).

chart speed was the same for both calibrations and exposures. Because the chart moved at constant speed, the chart divisions were related directly to time and the number of divisions under the tracing could be used to establish the exposure times involved.

To estimate the total exposure, the recorder tracings (which could be several feet long) were divided into convenient segments for the Sigma-Scan instrument and the area under each segment was measured. The average area for one recorder chart division within each segment was calculated, changed to concentrations using the calibration curve for that experiment (Fig. 1) and then multiplied by the number of chart divisions in the segment to obtain the total exposure represented by that segment. For the total exposure in the experiment, the values for the segments were summed. The measured exposure could be compared with a theoretical or 'nominal' exposure calculated from the amount of GB injected and the constant air flow. In all but the most concentrated atmospheres, the 'nominal' and measured exposures were very similar. In the most concentrated situations, the nominal exposure was no more than 10% higher than the measured values. The measured values were used for the toxicity estimates.

Animal exposures

The mice, in pre-weighed groups of six, were placed in the animal holder in random order and the holder was placed into the chamber. The system was sealed and the air flow started. After the Miran analyzers had stabilized and baseline traces were established, the syringe containing GB was mounted on the injection port and the syringe pump was started. The exposures were timed from the first detection of chemical by the Miran sampling from inside the chamber. After the prescribed exposure time, the syringe pump was stopped and the syringe removed from the injection port. The chamber was opened when the Miran sampling from inside the chamber indicated that the atmosphere in the chamber was clear of GB (usually 8 min after the GB supply was stopped). The animals were removed, placed in cages for observation and then examined regularly for the remainder of the working day. The groups were examined and weighed daily to the end of that working week and then twice weekly until 20–24 days after exposure.

Because of the possibility of agent ingestion from contaminated food or water, neither was provided during the animal exposures.

One group of mice was kept as controls. These were handled for marking, weighing and examinations only. For each exposure time, a group of six mice were mock-exposed in the chamber (no GB) and then examined in the same manner as the exposed groups to demonstrate the effects of stress from the exposure process. The results of all test groups were compared with both of these controls.

Calculations and statistics

The data generated (exposure concentrations and times, body weights, etc.) were assembled into tables using FRAMEWORK VI[®] software (FRAMEWORK[®] VI, which runs under Windows[®], is available from Selections & Functions, Inc., Box 505, Scituate, MA 02066) and programs written in this laboratory. Various summary tables were prepared according to the needs of the experimental work.

Calculations of the LCt_{16}, LCt_{50} and LCt_{84} values were made via the established methods of Litchfield and Wilcoxon (1949) using a commercial program (Tallarida and Murray, 1987). Mean body weights were compared with the initial body weights, first using analysis of variance (F test) and then Duncan's multiple range test. The body weights on each day were compared with the corresponding control using two-tailed Student's t-tests (Kenney and Keeping, 1957).

RESULTS

The toxic responses seen in the exposed mice were those consistent with organophosphate poisoning, such as excess salivation (ptyalism), piloerection, tremors, convulsions and flaccid paralysis preceding death. With the few exceptions noted below, the affected animals exhibited these clinical signs in sequence at progressive times during the exposures and the casualties died either within the exposure chamber or very shortly after they were removed from the chamber at the end of the exposures. No abnormal clinical signs were observed and post-mortem and histological examinations did not reveal any lesions or effects that were not directly attributable to the organophosphate poisoning.

The toxicity values (LCt_{16}, LCt_{50} and LCt_{84}) calculated and the PROBIT slopes and intercepts are summarized in Table 6.

The seven groups of mice in the 20-min experiments received exposures between 372 and 483 mg·min m^{-3} GB (Table 1). The first clinical signs of poisoning were noted at 9 min and the first death at 18 min. The t_{50} values recorded for these exposures were 6.6 min, with a SEM of 0.17. For one exposure, the results of 10 randomly selected 0.2-min chart divisions were averaged to provide an indication of the precision of the GB exposure (482 ± 15 mg·min m^{-3}; mean ± SEM). Body weights of the control

Table 1—Toxicity of GB to mice: 20-min exposure

Exposure (mg·min m^{-3})	Mortality	Body weight (g)	Percentage change in body weight by day							
			1	2	6	9	13	16	20	23
Control	0	25.9	4.6[b]	6.7[b]	15[a]	20[a]	20[a]	24[a]	27[a]	28[a]
		±0.82	±1.7	±3.4	±2.7	±2.9	±3.3	±3.2	±3.7	±5.0
Mock-exposed		26.5	−2.4	2.6	11[b]	20[a]	24[a]	25[a]	27[a]	32[a]
		±1.5	±3.6	±2.4	8.7	±10	±15	±11	±15	±15
GB trials										
483	5/6	25.7	−12	−9.9	8.2	15	23	25	27	32
		±0.77								
468*	5/6	26.2	−0.70	0.12	12	18	26	26	28	31
		±1.0								
468	3/6	25.6	−6.7[cf]	−4.8[ce]	9.0[ac]	12[bd]	16[a]	17[ad†]	22[a]	25[a]
		±1.2	±5.4	±3.3	±5.5	±7.7	±8.5	±5.8	±5.8	±4.4
451	3/5	25.2	−1.96	0.03	15.0	20	25	26	33	33
		±1.1								
372	3/6	26.2	−7.1[ace]	−4.5[bce]	9.5[ac]	14[ad]	21[a]	24[a]	29[a]	32[af]
		±0.49	±2.6	±3.2	±3.2	±5.3	±5.1	±3.6	±3.8	±4.2
372	2/6	26.4	−10.5[bce]	−6.9[ce]	7.9[c]	14[ac]	21[a]	23[a]	27[a]	30[a]
		±1.7	±3.6	±3.7	±3.1	±4.0	±4.4	±3.6	±4.4	±5.1
410	0/6	25.8	−6.8[ace]	−3.6[ce]	7.5[ac]	12[ac]	17[a]	19[ae]	22[ad]	27[a]
		±0.67	±3.9	±3.3	±1.9	±2.2	±3.1	±1.6	±3.8	±4.8

Values are means ± SD. Control mice were not cycled through the chamber system.
Mock-exposed animals were 20 min in the exposure chamber without GB exposure.
[a] Significantly different to day 0; $P < 0.01$.
[b] Significantly different to day 0; $P < 0.05$.
[c] Significantly different to control; $P < 0.05$.
[d] Significantly different to control; $P < 0.01$.
[e] Significantly different to mock-exposed; $P < 0.01$.
[f] Significantly different to mock-exposed; $P < 0.05$.

and mock-exposed mice were statistically similar throughout. However, numerically there was a drop in body weight (days 1 and 2) before the average weight recovered and gains matched those in the control group. Body weight loss was recorded in all survivors of GB exposures on day 1. When statistical calculations could be made, these were significantly different from both the control and mock-exposed groups. On day 2, only the survivor of the first 468 mg·min m^{-3} exposure (marked with an asterisk in Table 1) had a body weight greater than the starting weight. Body weights of the rest were still significantly lower. Body weight gains resumed after day 2 and continued until the end of the observation period when the average body weights of all survivors and mock-exposed groups were similar to those of the control.

The five groups of mice in the 60-min experiments received exposures between 412 and 689 mg·min m^{-3} GB (Table 2). The first clinical signs of poisoning were noted at 30 min and the first death at 50 min. The t_{50} values recorded for these exposures were 5.0 min, with an SEM of 0.38. For the 412 mg·min m^{-3} exposure, 10 randomly selected, 0.8-min chart divisions averaged 410 ± 4 mg·min m^{-3}. The 412 mg·min m^{-3} exposure was only 77% of the calculated LCt_{50} of 537 mg·min m^{-3}. When this low value was removed from the LCt_{50} calculation, the resulting LCt_{50} value was 543 mg·min m^{-3} and the LCt_{01} of 424 was higher than 412, so the calculated values reported were those obtained without the 412 value. The exposure to 412 mg·min m^{-3} did result in mild toxic signs (ptyalism and tremors but no convulsions) and the animals recovered within a few hours of the exposure. Body weights of the mock-exposed group were statistically similar to the control throughout. Unlike the 20-min mock-exposed group, there was no initial body weight loss. After 23 days, the average body weight of the mock-exposed mice was higher than the control but the difference was not statistically significant. For the GB-exposed mice, body weight losses were recorded for all survivors on day 1. The differences were significant compared with day 0, control and mock-exposed groups. Body weight gains resumed on day 2 in the groups exposed to 412 and 518 mg·min m^{-3} and continued until the end of the 23-day observation period when these mice reached the body weights of the control group. In the group exposed to 550 mg·min m^{-3}, the initial recovery was much slower and body weights below the starting values were recorded for 3 days. At day 23 the body weights were equal to those of the control and mock-exposed groups.

The six groups of mice in the 180-min experiments received exposures between 772 and 1100 mg·min m^{-3} GB (Table 3). The first clinical signs of poisoning were noted at 100 min and the first death at 140 min. The t_{50} values for these exposures were 5.1 min, with an SEM of 0.17. For the 772 mg·min m^{-3} exposure, 10 randomly selected 1.2-min chart divisions averaged 772 ± 10 mg·min m^{-3}. The mock-exposed group showed body weights on days 1 and 2 significantly below the control group. Body weights of this group remained significantly below the control for the 21 days of the experiment. For GB-exposed mice, significant body weight loss was recorded in all survivors on day 1 compared with day 0, control and mock-exposed groups. Although recovery had started by day 2, body weights above those on day 0 were first recorded on day 6. On day 21, all survivors were growing well and some had

Table 2—Toxicity of GB to mice: 60-min exposure

Exposure (mg·min m^{-3})	Mortality	Body weight (g)	Percentage change in body weight by day								
			1	2	3	6	9	13	16	20	23
Mock-exposed		25.9 ±0.96	3.0 ±1.7	5.6[a] ±2.8	8.7[a] ±2.7	13[a] ±2.8	15[a] ±2.6	20[a] ±3.8	27[a] ±8	28[a] ±8	35[a] ±11
GB trials											
689	6/6	24.9 ±1.6									
557	5/6	23.8 ±1.5	−6.4	−5.0	0.09	11	12	15	21	24	25
550	3/6	24.6 ±0.93	−16[ace] ±4.8	−15[ace] ±7.8	−6.5[ce] ±8.1	3.4[cfb] ±7.0	7.7[cdf] ±6.2	16[d] ±5.7	21[d] ±5.9	25[d] ±6.9	25[cdf] ±5.4
518	1/6	24.0 ±1.7	−3.4[cf] ±6.3	0.30[ce] ±2.6	4.7[f] ±2.4	12[cc] ±2.1	14[ac] ±2.6	22[a] ±3.5	26[a] ±4.4	31[a] ±4.6	31[a] ±5.0
412	0/5	27.8 ±1.1	−0.58[d] ±4.1	0.47[df] ±5.0	4.1[af] ±3.7	7.7[acf] ±4.2	9.4[ac] ±6.1	13[adf] ±7.3	17[adf] ±7.5	19[a] ±9.1	22[adf] ±9.2

Values are means ± SD. Data for control animals are given in Table 1.
Mock-exposed animals were 60 min in the exposure chamber without GB exposure.
[a] Significantly different to day 0; $P < 0.01$.
[b] Significantly different to day 0; $P < 0.05$.
[c] Significantly different to control; $P < 0.05$.
[d] Significantly different to control; $P < 0.01$.
[e] Significantly different to mock-exposed; $P < 0.01$.
[f] Significantly different to mock-exposed; $P < 0.05$.

Table 3—Toxicity of GB to mice: 180-min exposure

Exposure (mg·min m^{-3})	Mortality	Body weight (g)	Percentage change in body weight by day								
			1	2	3	4	6	8	14/15	16/18	21
Mock-exposed		28.1 ±0.96	−3.0[dc] ±2.4	−0.18[dc] ±2.7	1.3[c] ±3.3	0.66 ±3.2	5.0[c] ±3.7	3.5[c] ±4.0	12[ac] ±3.2	14[ac] ±3.3	15[ac] ±3.6
GB trials											
1100	6/6	24.6 ±1.9									
1017	4/6	25.1 ±1.4	−10.6	−4.3	—	—	5.2	12	17	18	24
810	2/6	25.7 ±2.2	−13[ace] ±4.5	−9.6[bce] ±3.3	−2.8[c] ±3.9	—	4.6[c] ±3.8	12[ce] ±4.4	19[bf] ±5.3	22[ae] ±4.2	27[ae] ±5.0
788	2/6	26.7 ±0.7	−10.0[ace] ±2.5	—	—	−0.85 ±1.8	2.4[c] ±2.2	7.2[ac] ±2.5	13[ac] ±3.2	16[ac] ±3.3	17[ac] ±4.5
725	2/6	26.2 ±2.6	−7.4[bce] ±2.1	—	—	−1.6 ±2.7	1.5[c] ±4.1	5.6[bc] ±5.2	14[b] ±8.7	18[a] ±9.7	17[ad] ±9.1
772	0/6	25.9 ±1.2	−9.2[bc] ±7.5	−7.4[bce] ±5.2	−1.3[c] ±5.5	—	4.4[c] ±3.0	11[ace] ±2.9	17[af] ±3.7	20[ade] ±3.5	24[ae] ±5.1

Values are given as means ± SD. Control data are given in Table 1.
Mock-exposed animals were in the exposure chamber for 180 min without GB.
[a] Significantly different to day 0; $P < 0.01$.
[b] Significantly different to day 0; $P < 0.05$.
[c] Significantly different to control; $P < 0.01$.
[d] Significantly different to control; $P < 0.05$.
[e] Significantly different to mock-exposed; $P < 0.01$.
[f] Significantly different to mock-exposed; $P < 0.05$.

gained enough so that the body weights of the exposed groups were not significantly different from the controls.

The six groups of mice in the 360-min experiments received exposures between 969 and 1493 mg·min m^{-3} GB (Table 4). The first clinical signs of poisoning were noted at 280 min and the first death at 320 min. The t_{50} values for these exposures were 5.5 min, with an SEM of 0.30. For the 1235 mg·min m^{-3} exposure, 10 randomly selected 1.53-min chart divisions averaged 1228 ± 10 mg·min m^{-3}. Clinical signs of toxicity were first evident at ca. 300 min and slowly became more pronounced as the exposure continued. With one exception, the casualties died within a short time of the end of the exposure period. The one mouse that survived the 1474 mg·min m^{-3} exposure did not thrive and was essentially the same body weight after 23 days. In the group of mock-exposed mice, initial significant body weight loss (compared with the control) was quickly reversed and growth continued for 23 days. In the

Table 4—Toxicity of GB to mice: 360-min exposures

Exposure (mg·min m^{-3})	Mortality	Body weight (g)	Percentage change in body weight by day								
			1	2	3	4	6/7	10	14	19	23
Mock-exposed		27.3	−1.8[c]	−0.28[c]	1.7[c]	3.5	7.1[acd]	14[a]	19[a]	23[a]	30[acf]
		±1.4	±1.5	±1.5	±1.0	±2.1	±2.5	±3.7	±9.7	±5.8	±5.8
GB trials											
1493	6/6	32.3									
		±1.4									
1474	5/6	31.5	−12.4	—	—	—	−8.9	−3.2	—	0.75	−0.12
		±1.6									
1235	6/6	31.9									
		±1.5									
1386	1/6	25.8	−3.9[cf]	−1.4[c]	1.4[c]	3.0	6.9[bc]	12[ac]	15[a]	24[a]	21[ac]
		±1.4	±1.5	±1.8	±1.2	±2.2	±4.2	±5.2	±5.7	±7.5	±7.4
1364	3/6	26.0	−12[ce]	−14[cf]	−7.0[d]	−2.2	−0.94[c]	6.5[c]	10.5[bd]	17[ad]	19[ac]
		±1.4	±7.9	±14	±12	±9.6	±10.7	±10.0	±10.5	±7.8	±9.1
969	1/6	32.0	−2.2[c]	−1.9[c]	—	—	2.3[c]	6.4[bc]	8.3[ace]	12[ac]	13[acf]
		±2.3	±2.6	±2.4			±4.8	±6.1	±5.8	±5.4	±5.7

Values are given as means ± SD. Control data are given in Table 1.
Mock-exposed animals were 360 min in the exposure chamber without GB.
[a] Significantly different to day 0; $P < 0.01$.
[b] Significantly different to day 0; $P < 0.05$.
[c] Significantly different to control; $P < 0.01$.
[d] Significantly different to control; $P < 0.05$.
[e] Significantly different to mock-exposed; $P < 0.01$.
[f] Significantly different to mock-exposed; $P < 0.05$.

Table 5—Toxicity of GB to mice: 720-min exposures

Exposure (mg·min m^{-3})	Mortality	Body weight (g)	Percentage change in body weight by day								
			1	2	4	5	6	7	8	13	17	20
Mock-exposed		27.1	−4.0[c]	−2.4[c]	−1.0	1.1	3.2[c]	4.5[c]	3.6[bc]	8.9[bc]	13[ac]	13[ac]
		±1.7	±4.7	±2.8	±1.6	±4.2	±3.9	±4.0	±3.7	±3.2	±3.9	±3.9
GB trials												
2303	6/6	28.6										
		±1.1										
2313	5/6	26.3	−20	−17	−4.6	−4.6	−2.0	0.07	3.2	—	17	21
		±1.4										
2081	2/6	30.1	−10.3[acf]	−7.2[cf]	−3.4[f]	−2.6[e]	−1.8[ce]	−0.8[cf]	—	7.9[ac]	11[ac]	13[ac]
		±1.2	±3.6	±3.4	±1.6	±1.6	±1.4	±1.2		±4.6	±2.4	±3.4
1625	0/6	25.7	−10.0[bcf]	−6.8[cf]	−1.1[e]	0.17	2.2[c]	3.7[c]	4.8[c]	11[bc]	—	16[ac]
		±1.8	±3.1	±2.4	±2.5	±2.2	±2.4	±2.4	±2.6	±4.9		±3.5
2200	1/6	29.0	−9.9[cf]	−4.7[c]	—	—	—	5.1[c]	—	7.6[bc]	10.1[ac]	12[ac]
		±2.6	±2.6	±3.6				±2.6		±3.3	±3.7	±4.3
2096	0/6	29.2	−10.7[acf]	−6.8[bcf]	−2.0[c]	−1.1[f]	0.50[f]	1.5[c]	2.9[c]	—	9.4[ac]	13[ac]
		±1.8	±2.9	±2.4	±2.5	±2.8	±2.3	±2.4	±3.1		±3.3	±3.9
1366	0/6	25.6	−7.2[c]	−3.6[c]	1.4	6.2	7.7[c]	8.4[bcf]	9.8[bc]	17[af]	—	25[ae]
		±2.0	±3.4	±2.4	±2.8	±3.5	±4.1	±4.2	±4.7	±7.5		±5.1

Values are given as means ± SD. Control data are given in Table 1.
Mock-exposed mice were 720 min in the exposure chamber without GB.
[a] Significantly different to day 0; $P < 0.01$.
[b] Significantly different to day 0; $P < 0.05$.
[c] Significantly different to control; $P < 0.01$.
[d] Significantly different to control; $P < 0.05$.
[e] Significantly different to mock-exposed; $P < 0.01$.
[f] Significantly different to mock-exposed; $P < 0.05$.

groups exposed to 969, 1364 and 1386 there was significant body weight loss on days 1–3 compared with the control and, in the 1364 mg·min m^{-3} group, with the mock-exposed group as well. Although all were growing, the body weights of all of the GB-exposed groups were lower than the mock-exposed and control groups on day 23.

The seven groups of mice in the 720-min experiments received exposures between 1366 and 2303 mg·min m^{-3} GB (Table 5). The first clinical signs of poisoning were noted at 380 min and the first death at 624 min. The t_{50} values recorded for these exposures were 5.5 min, with an SEM of 0.15. For the 1625 mg·min m^{-3} exposure, 10

Table 6—Summary of GB toxicity to mice

Exposure time (min)	Toxicity			PROBIT	
	LCt_{16}	LCt_{50}	LCt_{84}	Slope	Intercept
20	340 (319–355)	430 (387–478)	550 (520–579)	9.4	−19.7
60	501 (499–502)	540 (507–571)	590 (588–591)	28	−72
180	690 (678–697)	900 (795–1020)	118 (1160–1190)	8.5	−20
360	930 (838–1030)	1210 (941–1550)	1570 (1420–1740)	8.7	−22
720	2000 (1995–2007)	2210 (2140–2280)	2450 (2440–2460)	23	−73

Values are given as LCt_{xx} and 95% confidence limits.

randomly selected 2-min chart divisions averaged 1617 ± 13 mg·min m^{-3}. When the PROBIT calculations were done with all of the data, the LCt_{50} was 2226 mg·min m^{-3}, the LCt_{01} was 1445 mg·min m^{-3} and the lowest exposure was less than the LCt_{01}. When the 1366 exposure was removed from the calculation, the LCt_{50} and LCt_{01} values were 2206 and 1630 mg·min m^{-3}, respectively. Because the lowest remaining exposure value was essentially the same as the LCt_{01}, that low value was retained. There were mild clinical signs of poisoning in the low exposure groups (pytalism and mild tremors) and the animals recovered within a few hours of the insult. Compared with the controls, the body weights of the mock-exposed mice were significantly lower at all times. Body weights had begun recovery on day 2 but growth was slower, such that none were significantly increased over day 0 until day 6 and the mice did not achieve parity with the control by day 20. Body weights were significantly reduced on day 1 in all GB-exposed groups when compared with the control and, in the groups exposed to 2096–2313 mg·min m^{-3} GB, the weight loss was significantly greater than in the mock-exposed mice. Recovery had begun in these groups by day 2 but was slower than in the control and mock exposure groups so that significant differences to the mock-exposed group continued until day 7 and on day 20 all were significantly below the control.

DISCUSSION

Historically, the GB toxicity values for mice (and other species) were generated with responses to battlefield attacks in mind. The majority of the effort was directed towards peracute toxicity, i.e. high concentration, very short (1–30 s) exposures. In the literature, only one of the 10 identified historical studies involving GB toxicity to mice contained data for exposures of >10 min (Bide *et al.*, 1998, 2004a). The compilation of historical data has provided a reasonable set of toxicity data for 0.17–30 min exposures. The current study with mice is part of an ongoing effort to provide the multi-species data necessary to predict the human toxicity of the nerve agents for 0.5–12 h exposures.

All of the experiments done to establish toxicity values are reported (Tables 1–5) and the toxicity values (LCt_{16}, LCt_{50} and LCt_{84}) calculated and the PROBIT slopes and intercepts are summarized in Table 6. At the 60- and 720-min exposure times there were low value exposures outside of the LCt_{01-99} range of the remainder for which no lethal responses were recorded. These two low exposure experiments were not used to calculate the toxicity values. As demonstrated, the inclusion of zero (or 100%) values somewhat removed from the core LCt_{01}–LCt_{99} values in the PROBIT calculations used (Litchfield and Wilcoxon, 1949; Tallarida and Murray, 1987) can alter the calculated values for the PROBIT slope and intercepts, and hence the LCt_{05} and LCt_{01}, without great change in the LCt_{50}.

Neither Haber's rule nor the 'toxic load model' will predict the observed toxicity of GB in mice for 1–12 h exposures. Haber's rule — i.e. the LCt_{50} varies directly as the product of concentration and exposure time ($LCt_{50} = CT$; Haber, 1924) predicts an LCt_{50} value of 430 mg·min m^{-3} (based on the 20-min exposure value) whereas the experimental values diverge progressively to 2210 mg·min m^{-3} at the 720-min exposure (Table 7). A similar divergence occurs at shorter exposure times (Bide *et al.*, 1998, 2004a). Using Haber's rule to estimate the toxicity of GB is clearly unacceptable. From the historic data for GB toxicity to mice, an empirical, three-dimensional planar surface model — a toxic load model — was defined to relate exposure time and toxicity over the exposure range of 0.17–30 min (Bide *et al.*, 1998, 2004a). This empirical toxic load model was described by the equation

$$Y = -8.3461 + 3.6907 \log_{10} C + 2.7947 \log_{10} T$$

with the resulting n value being 1.3206. Assuming that the same relationship would continue to 720 min, LCt_{50} values were predicted for the extended exposure times (Table 7).

Table 7—Comparison of predicted and experimental toxicity

Exposure time (min)	LCt_{50} (mg·min m^{-3})		LC_{50} (mg m^{-3})	
	Pred.	Exp.	Pred.	Exp.
20	380	430	19	21.5
60	490	540	8.2	9.0
180	640	900	3.6	5.0
360	760	1210	2.1	3.4
720	900	2210	1.25	3.1

Predicted values were calculated using the equation:
$Y = -8.3461 + 3.6907 \log_{10} C + 2.7947 \log_{10} T$
developed for the toxicity of GB to mice for short exposure times (Bide *et al.*, 2004a).

All predicted LCt_{50} values are lower than the experimental numbers. Although the predicted and experimental values are close enough at 60 min for predictions to be made, as the exposure time increased to 180 min and beyond the separation increased in a non-linear fashion such that at 720 min of exposure the experimental LCt_{50} values were more than double the value predicted by the toxic load model. Similarly, the LC_{50} values separated from the predicted values in a progressive manner as the exposure time increased. To use the toxic load model generated for 0.17–30 min exposures for mice for times beyond 60 min would lead to significant overestimation of the toxicity. A parallel study in rats (Mioduszewski *et al.*, 2000, 2001) produced a similar result and, unfortunately, the two studies do not produce similar/parallel exposure time–toxicity response curves for the two species. Thus, prediction of human toxicity from either study is hazardous and studies with other species will be needed to establish a defendable human toxicity estimate for the longer, lower level exposures.

In mice, as the exposure time increased, the lethal concentration of GB (LC_{50}) appeared to be approaching a threshold value of ca. 3 mg m^{-3}. Unfortunately, the PROBIT slope for the toxicity calculations did not follow a similar pattern to the LCt_{50} values (Table 6) so that a confident or rigorous prediction of the LCt_{05} or LCt_{01} threshold values was not possible. However, as a first approximation (and being conservative), a value of 8 may be used for the PROBIT slope. This is the rounded-down value of the shallowest experimental PROBIT slope (8.5; 180 min).

Two other measures of toxicity are reported: the time for the first appearance of clinical signs and the time for the first recorded death. Both of these follow a similar exposure-time response pattern to that seen with the LCt_{50}. Despite the higher scatter of the data, it is clear that neither follow either Haber's rule or the toxic load model. However, in both cases, the ratios of the times recorded to the set exposure times provide relatively constant values, indicating that the toxic effects appear in a cascade rather than a slow progression. This is consistent with the concept of a threshold but does not provide proof.

The reasons for the observed exposure time–toxicity responses are not clear from our current studies and knowledge. There may be several factors acting alone or in conjunction that limit the long-term toxic responses and provide 'protection' to the animal. These could include excretion of the GB, enzymatic destruction or modification, *de novo* synthesis of the affected acetylcholinesterases or synthesis of other substances that act as agent 'sponges'.

The caveats for both the LCt_{50} values and the PROBIT slopes are extensive. The data were generated using mice of a single source, strain, sex and body size, fed a uniform diet and known to be essentially healthy and specific pathogen free, as well as having had no known exposure to toxic materials. This is very different from the situation encountered in either wild mice or a human population (Dixon, 1976; Krasovskii, 1976; Blackburn, 1984). A much lower PROBIT slope would be expected for the wider mouse population, reflecting the greater variability in the subjects, and indeed this was seen in combined data from several mouse experiments (Bide *et al.*, 1998, 2004a).

The extended exposure periods were clearly stressful to the mice, as indicated by the body weight loss in the mock-exposed groups. Significant weight loss was recorded in the mock-exposed animals, which was more severe as the exposure times were extended. Similar weight loss was observed in the GB-exposed animals but there were no consistent or significant differences between the mock and GB-exposed groups after the mice recovered from the initial insult. This observation may have resulted from the small numbers of animals involved, and certainly the standard deviations observed were larger following the GB insult. The results of this study do not preclude the appearance of clinical signs and symptoms after longer periods. One mouse exposed to GB for 720 min did not thrive but did not show evidence of continued wasting within the experimental period. With this one exception, none of the survivors showed debilitating effects that would cause continuing weight loss. There was no evidence of brain damage of the type reported in rats (Baron, 1981; Koelle, 1981; McLeod *et al.*, 1984; Abou-Donia *et al.*, 2002) or of the limb paralysis recently reported in guinea pigs (Bide *et al.*, 2002, 2004b). However, care must be exercised in applying these comparisons because, with the small numbers of mice involved, changes in a small proportion of the population might not be manifest.

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