THE GERMICIDAL AND SPORICIDAL EFFICACY OF METHYL BROMIDE FOR BACILLUS ANTHRACIS[1]

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During recent years there has been a significant increase in industrial anthrax among workers handling imported wool and hair. In some areas this increase has assumed the proportions of a minor epidemic. This trend is reflected in the statistics from one state alone in which a total of 24 cases of industrial anthrax were reported from January 1 to September 30, 1949. Although all of these cases occurred in the carpet-manufacturing industry, 22 of them were confined to a single plant. An urgent need has thus been demonstrated for the development of effective, nondeleterious methods for the disinfection of imported animal products.

In a 20-year survey on the incidence of anthrax in the United States for the period from 1919 to 1938, Smyth (1939) reported a total of 1,683 recorded cases. Of this number, hides, skins, wool, and hair were responsible for 754 cases. Furthermore, he indicated that infected hides and skins were responsible for the largest number of individual cases. In a later 5-year survey from 1939 to 1943, Smyth and Higgins (1945) reported a decrease in the total number of anthrax cases resulting from imported hides and skins and a 365 per cent increase in cases from imported wool and hair. As a result of these findings, Smyth and Higgins advocated that steps be taken to protect industrial workers through the development of adequate disinfection methods for processing imported, contaminated materials at ports of entry.

The authors (1948, 1949) have already completed studies on the heat resistance of *Bacillus anthracis* spores in hair and bristles used in the manufacture of lather brushes and have recommended heat-processing methods that are nondeleterious for these materials. However, such processing methods would obviously be harmful to hides, skins, and wool. In view of the foregoing facts, it was decided that further exploration of chemical disinfection methods should be undertaken.

A survey of the literature revealed many references to studies on the efficacy of chemical agents for the destruction of *B. anthracis* under both laboratory and field conditions. Among the many compounds tested, only a few have been reported as satisfactory germicidal agents, whereas others either have demonstrated partial disinfection or have been completely ineffective. Some of the chemicals reported to be effective for the destruction of *B. anthracis* spores or vegetative cells or both are hydrogen sulfide (Andrjevski, 1928); chlorine and

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N-chloro derivatives of ammonia, methylamine, and glycine (Tilley and Chapin, 1930; Hailer and Von Bockelberg, 1939); cod-liver oil (Nélis, 1939); hydrogen peroxide (Pagnini, 1939; Jordan and Burrows, 1945); commercial disinfectants, e.g., "amuchin" and "antisapril" (Liddo, 1940); 2 per cent hydrochloric acid and 2.7 per cent sulfuric acid, plus 20 per cent sodium chloride (Hailer and Heicken, 1941); formaldehyde gas and 10 per cent formalin (Salle and Korzenovsky, 1942; Kelser and Schoening, 1943); ethylene oxide (Velu *et al.*, 1942); and potassium permanganate (Jordan and Burrows, 1945).

Although these compounds may be effective for the destruction of anthrax spores and vegetative cells, some of them would be entirely unsatisfactory for general disinfection purposes, as well as deleterious for certain industrial materials. Therefore, it seemed advisable to investigate the practicability of other chemical agents, especially those compounds that are highly penetrating gases at ordinary temperatures.

Since 1932 methyl bromide (CH_3Br) has received wide acclaim as an effective disinfesting agent and to a limited extent as a disinfectant. Its versatility has been demonstrated by its extensive use as an insecticide (Le Goupil, 1932; Mackie and Carter, 1937; Fisk and Shepard, 1938; Mackie, 1938, 1941; Munro and Delisle, 1941; Donohoe and Gaddis, 1942; Sherrard, 1942; Yust *et al.*, 1942; Roehm *et al.*, 1943; Stafford and Steinweden, 1943; Tarleton and Dworsky, 1943; Latta, 1944; Mrak and Phaff, 1947); as a nematocide (Taylor and McBeth, 1940, 1941; Andrews *et al.*, 1943; Swanson and Taylor, 1943; Harris and Carter, 1945); and as an inhibitory and germicidal agent for microorganisms (bacteria, viruses, and fungi) responsible for spoilage or damage to food products and plants (Mrak, 1941; Baerwald, 1945; Johnson, 1945; Whelton *et al.*, 1946). Furthermore, the potentialities of this compound have been emphasized by its ability to penetrate tightly packed materials, its effectiveness at various temperatures, its low rate of moisture absorption, and its noninjurious effect on a wide range of products.

Conversely, however, Salle and Korzenovsky (1942) and Whelton *et al.* (1946) have reported that methyl bromide is ineffective for the killing of sporeforming bacteria after exposures of 2 and 24 hours, respectively.

Because of these characteristics of methyl bromide, the studies to be reported were undertaken in order to determine the germicidal and sporicidal efficacy of this compound for *B. anthracis*.

METHODS AND RESULTS

Materials. The methyl bromide used in this investigation was the standard commerical grade, 99.5 per cent pure, obtained as a liquid in 1-pound cans. The chemical properties of this compound were described by Dow Chemical Company (1940). A few of its more important characteristics are listed as follows: molecular weight, 94.94; boiling point, 4.5 C; freezing point, −93 C; and specific gravity of liquid, 1.732 at 0 C.

Extreme precautionary measures were exercised in handling methyl bromide because of its known toxicity (threshold limit, 20 ppm). All exposure tests were

conducted under a chemical fume hood. The general precautions followed in these studies were those recommended by the Division of Industrial Hygiene, United States Public Health Service (1938), Dudley *et al.* (1940), and Von Oettingen (1946).

Six representative virulent cultures of *B. anthracis*, including strains of canine (A1), bovine (A2, A3, A4), human (A5), and ovine (A6) origin, were employed in these studies. Spore suspensions from cultures of each strain were prepared and standardized according to methods previously reported by the authors (1948). These spore suspensions were tested for viability and pathogenicity by serial dilution culture methods and animal inoculation tests prior to each series of experiments.

The test materials employed in these studies were (a) sterile filter paper strips (7.0 cm by 1.5 cm) inoculated with 100,000 to 50,000,000 spores per strip; (b) suspensions of spores in physiological saline standardized within the same range; and (c) 1- to 24-hour meat infusion agar[2] slant cultures from the various representative strains of *B. anthracis*. The filter paper strips were inoculated with spores and dried at 65 to 75 C for 3 to 4 hours prior to exposure or were inoculated with spore suspensions immediately before exposure. All test materials and uninoculated controls were exposed to methyl bromide by (a) sealed chamber, (b) vacuum chamber, (c) bubbling, and (d) vaporization methods that will be described later.

Following exposure, all test and control materials were cultured in Difco A. C. medium.[3] One-half of all exposed filter paper strips were inoculated directly into the medium, whereas duplicate sets of strips were suspended in sterile distilled water for 24 hours prior to culturing for dilution and the removal of adsorbed bromides. Positive cultures were examined microscopically and confirmed by animal inoculation tests. After a 2-week incubation period at 37 C, all negative cultures were inoculated with a standard loop from each respective viable spore suspension to determine whether failure to obtain growth was due to inhibition by bromides carried over with the inoculum. Other uninoculated control materials were examined before and after exposure for (a) inorganic bromides by the titration method of Friedman (1942) and (b) changes in hydrogen ion concentration by electrometric titration.

Exposure of B. anthracis to methyl bromide: Sealed chamber method. Preliminary studies were conducted in order to ascertain whether gaseous methyl bromide possessed any lethal activity for *B. anthracis* spores. In these initial experiments, wet and dry filter paper strips contaminated with spores from canine strain (A1) were exposed to an air and methyl bromide mixture for 3, 7, and 26 hours under an inverted 3.5-liter glass jar sealed to a glass plate with vaseline. Before the sealing, open petri dishes containing the inoculated strips and a beaker containing 9 ml of liquid methyl bromide were placed under the jar.

[2] Meat infusion, 1,000 ml (from 500 g of meat); Difco peptone, 5 g; sodium chloride, 5 g; Difco agar, 20 g; pH, 7.3.

[3] Proteose peptone no. 3, 20 g; beef extract, 3 g; yeast extract, 3 g; malt extract, 3 g; glucose, 5 g; agar, 1 g; distilled water, 1,000 ml; pH, 7.3.

In these initial tests, all anthrax spores were destroyed by exposure to methyl bromide for 26 hours, but were able to survive exposures of 3 or 7 hours. Since these tests indicated that methyl bromide possessed potential sporicidal properties, a vacuum chamber apparatus, which provided for more adequate control of the pressure and concentration of the gas, was devised for subsequent exposure studies. This apparatus is shown in figure 1.

Vacuum chamber exposure tests. In tests with this apparatus, filter paper strips, inoculated with 100,000 to 50,000,000 moist or dried spores from each strain of *B. anthracis*, were placed across glass rods in a vacuum desiccator (*E*). The system was then evacuated one to three times with a filter pump (*A*) and flushed after each evacuation with methyl bromide vapor admitted from a tube (*H*). Although CH_3Br is a gas at ordinary room temperatures and normal atmospheric

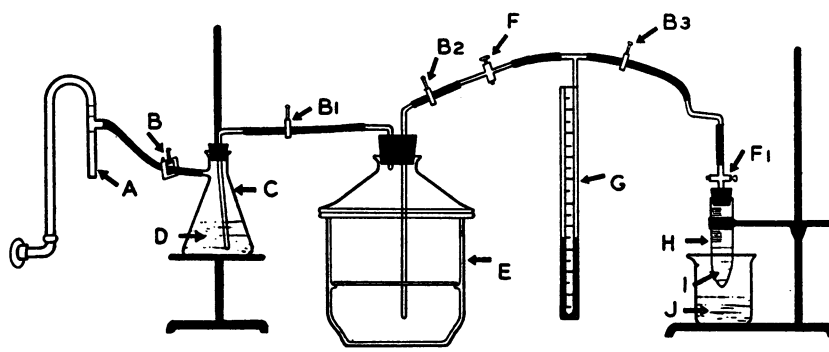

Figure 1. Methyl bromide exposure apparatus (vacuum desiccator method).

A, filter pump.	*F*, F_1, stopcocks.
B, B_1, B_2, B_3, pinch clamps.	*G*, mercury manometer.
C, 500-ml filtration flask.	*H*, 50-ml centrifuge tube.
D, KOH (1% alcoholic).	*I*, methyl bromide.
E, vacuum desiccator.	*J*, hot water bath.

pressure, the rate of vaporization was increased when desired by a hot water bath (*J*). The evacuated air and CH_3Br gas mixtures were bubbled through a 1 per cent alcoholic potassium hydroxide solution[4] (in flask C), which neutralized the CH_3Br by hydrolysis to KBr and CH_3OH, as suggested by Dudley (1939). This evacuation and flushing procedure ensured constant concentrated atmospheres of 3.4 to 3.9 g of methyl bromide per liter for each processing experiment. These concentrations were calculated by the general law for the expansion of gases, which is expressed as $pv = RmT$, in which m is the mass of gas at absolute temperature T and R is the gas constant.

After final flushing, the desiccator was closed by pinch clamps (B_1 and B_2), and all connections and openings were further sealed by a solution of vaspar (50 per cent each of vaseline and paraffin). The test materials remained in contact with methyl bromide, at room temperature, for periods ranging from 1 to 72 hours.

[4] One g KOH in 100 ml 95 per cent C_2H_5OH.

The results of 402 tests by this method of exposure are summarized in table 1. It is apparent from this table that methyl bromide completely destroyed all spores exposed in the presence of moisture for 24 to 72 hours, whereas some spores survived when exposures were made in the absence of moisture for the same periods of time. Furthermore, spores survived in some tests, in both the presence and absence of moisture, when exposed for only 1 hour. Therefore these results indicate that the sporicidal action of methyl bromide depends upon prolonged contact of the spores with the gas in the presence of moisture.

Although spores from each of the six strains of *B. anthracis* were readily destroyed by methyl bromide under the conditions described above, it seems worthy of note that one bovine strain (A3) showed a tendency to be more resistant to methyl bromide than any of the other strains investigated. In studies previously reported by the authors (1948) this same strain was also found to be more resistant to heat.

TABLE 1

Efficacy of methyl bromide for the destruction of B. anthracis spores in the presence or absence of moisture

EXPOSURES	ABSENCE OF MOISTURE				PRESENCE OF MOISTURE			
Exposure period (hr)...	1	24	48	72	1	24	48	72
Total number of tests..	24	54	92	43	24	50	77	38
Number of tests all spores killed.....	16	52	80	38	20	50	77	38
Percentage of tests all spores killed.....	66.67	96.30	86.96	88.37	83.33	100.00	100.00	100.00

Since excessively dehydrated spores are considered to be more resistant than moist spores, it seemed advisable to determine whether such spores were susceptible to the action of CH_3Br. Spore-inoculated (strains A1, A2, A3, A4, A5, A6) filter paper strips were dried at 65 to 75 C for 4 hours, then stored over $CaCl_2$ in a desiccator at room temperature or in an oven at 45 C for $2\frac{1}{2}$ months. In parallel tests these spore-inoculated strips were exposed to methyl bromide for 48 hours in vacuum chambers containing either $CaCl_2$ or sterile distilled water. These experiments (90 tests) revealed that excessively dehydrated spores from all strains were completely destroyed when exposed to CH_3Br in the presence of moisture, whereas such spores exposed in the absence of moisture survived in 16 out of 45 tests (36.4 per cent). Therefore it is apparent that desiccated spores exposed in the presence of moisture are no more resistant than nondesiccated spores.

Bubbling and vaporization exposure tests. Additional studies were conducted with bubbling and vaporization exposure methods in order to determine whether (a) gaseous CH_3Br was effective for killing high concentrations of anthrax spores suspended in physiological saline or (b) this gas was germicidal for vegetative cells of *B. anthracis*. The apparatus devised for these exposure studies is shown in figure 2.

In the bubbling exposure tests, saline suspensions containing 1,000,000 to 50,000,000 anthrax spores from cultures of strains A1, A2, A3, and A4 were placed in a modified Folin tube (*F*). Methyl bromide vapor from a tube (*J*) was introduced into the suspension for 1 hour through the sintered glass bubbler tube (*G*). The Folin tube was then sealed by closing pinch clamps (D_1 and D_2), and the suspensions were incubated 24 to 72 hours at room temperature. By replacement of the Folin tube in the system with 200-by-35-mm test tubes containing young agar slant cultures from resistant strain A3, methyl bromide was vaporized over the surface of the cultures.

The results of the bubbling and vaporization tests are summarized in table 2. It is apparent from this table that all spores suspended in saline were killed after exposure to CH_3Br for 24 to 72 hours (12 tests) and that vegetative cells in young agar slant cultures were likewise destroyed after exposures of 24 and 48 hours

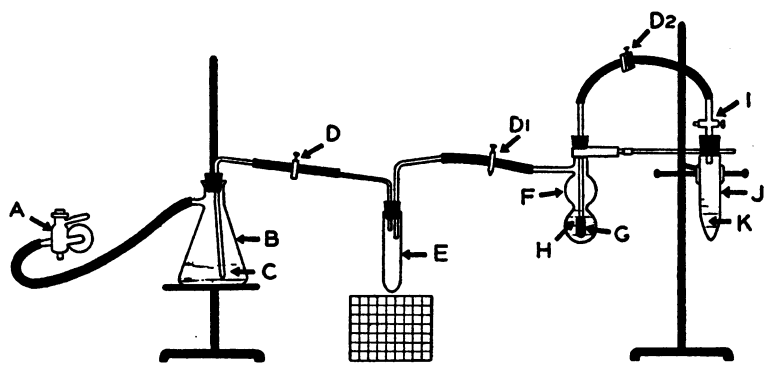

Figure 2. Bubbling apparatus for continuous methyl bromide exposure.

A, vacuum system.
B, 500-ml filtration flask.
C, KOH (1% alcoholic).
D, D_1, D_2, pinch clamps.
E, suction trap (for overflow).
F, modified Folin tube.
G, sintered glass bubbler tube.
H, spore suspension.
I, stopcock.
J, 50-ml centrifuge tube.
K, methyl bromide.

(7 tests). Furthermore, it was demonstrated that prolonged contact with the gas was essential for spores and cells alike, as evidenced by their survival following exposures of only 1 hour.

Exposure of spores to liquid methyl bromide. A final series of 30 tests was conducted in order to ascertain whether liquid CH_3Br per se possessed any sporicidal activity for *B. anthracis*. In these experiments spore-inoculated filter paper strips and concentrated spore suspensions (cultures A1, A2, A3, A4, A5, A6) were introduced into sealed test tubes containing 20 to 25 ml of liquid CH_3Br. The compound was maintained in a liquid state by packing the tubes in an insulated cabinet filled with dry ice. Tubes containing untreated spore suspensions were packed in dry ice along with the test materials to serve as controls. After exposure periods of 1 and 48 hours, culture tests demonstrated that liquid CH_3Br was ineffective for the destruction of *B. anthracis* spores in any of these tests.

Since our studies have demonstrated that gaseous CH_3Br, in the presence of moisture, is germicidal and sporicidal for *B. anthracis* after exposures of 24 hours,

TABLE 2

The killing of spores and vegetative cells of B. anthracis during exposure to CH_3Br by bubbling and vaporization methods

METHOD OF EXPOSURE	QUANTITY OF LIQUID CH_3Br USED	TOTAL NO. TESTS	VIABILITY OF B. ANTHRACIS SPORES AND CULTURES AFTER EXPOSURE TO CH_3Br							
			Length of exposure (hr)							
			1		24		48		72	
			Tests killed	Tests survived	Tests killed	Tests survived	Tests killed	Tests survived	Tests killed	Tests survived
	ml									
Bubbling method Saline spore suspensions (4 strains)	13–88* Avg = 21.1	16	0	4	4	0	4	0	4	0
Vaporization method 1-, 6-, 24-hr agar slant cultures (bovine strain A3)	14–19 Avg = 16.0	8	0	1	4	0	3	0	No tests	No tests

* In the first 72-hr exposure test 88 ml of CH_3Br were bubbled through the spore suspension at a rapid rate. In all subsequent tests 13 ml to 27 ml (average 16.8 ml) of CH_3Br were bubbled through the suspensions at a lower rate of flow.

TABLE 3

Results of Friedman tests for inorganic bromides absorbed by test materials during exposure to CH_3Br

METHOD OF EXPOSURE	TEST MATERIALS EXPOSED TO CH_3Br	QUANTITY SAMPLE TESTED	TOTAL NO. TESTS	AVERAGE NO. MG BROMIDE ABSORBED PER SAMPLE			
				LENGTH OF EXPOSURE (HR)			
				1	24	48	72
		ml					
Vacuum desiccator	* Filter paper strips (exposed dry)	30	12	0.103	0.192	0.315	0.497
	* Filter paper strips (exposed wet)	30	10	0.146	0.702	0.768	No tests
Bubbling	Physiological saline	21	4	No tests	0.965	5.290	3.075

* Each filter paper strip macerated in 30 ml distilled water for analysis.

regardless of the method employed, it is believed that the destructive action exhibited was probably due to hydrobromic acid rather than methyl alcohol, the hydrolytic products of this compound. This is further indicated by numerous

references in the literature reporting the low germicidal and sporicidal efficiency of the alcohols. Additional evidence in support of this view was provided by pH determinations and chemical titration tests with moist filter paper strips and physiological saline blanks, prior to and after exposure to methyl bromide.

The average pH values of filter paper strips and physiological saline control blanks were found to decrease from pH 7.17 to pH 6.84 and pH 5.91 to pH 2.45, respectively, during exposure to methyl bromide. The greater increase in hydrogen ion concentration exhibited by the latter (saline blanks) was attributed to the larger amount of moisture present for the absorption of CH_3Br. Chemical titration tests by the method of Friedman (1942) indicated that inorganic bromides were formed on or in the control materials during exposure to methyl bromide. The increase in the milligrams of inorganic bromides formed was proportional to the length of exposure and the amount of moisture present as indicated in table 3.

DISCUSSION

Since methyl bromide has not been extensively investigated as a germicidal agent, no information is available on the mechanism of its action. Therefore it is necessary to refer to previous disinfestation and toxicological studies with the compound in order to obtain a better understanding of its possible reactions. Reports of numerous fumigation studies indicate that CH_3Br is readily hydrolyzed to hydrobromic acid and methanol in the presence of moisture (De Francolini, 1935; Mackie, 1938; Dudley *et al.*, 1940; Shrader *et al.*, 1942). Furthermore, it is a more effective lethal agent for insects when exposure is accomplished at high relative humidities (Fisk and Shepard, 1938; Steinweden, 1945).

This experimental evidence provides further corroboration of our conclusion that the sporicidal action of gaseous methyl bromide is due to hydrobromic acid, one of its hydrolytic products. However, toxicological studies with animals exposed to CH_3Br vapors have given rise to several other trends of thought concerning the breakdown of the compounds and the mechanism of the toxic effects produced. The theories that have been propounded fall into three general categories as follows: (1) that the alkyl halide molecule itself is directly responsible by penetrating the tissues, thus causing toxic responses (Irish *et al.*, 1941); (2) that an intracellular hydrolysis of CH_3Br takes place, resulting in the formation of hydrobromic acid and methanol which may act as toxic agents (Miller and Haggard, 1943); or (3) that further intracellular breakdown products, such as formaldehyde, halide ions, or quaternary ammonium compounds, might be responsible for toxicity (Von Oettingen, 1946; Heppel and Porterfield, 1948).

Although the specific mechanism of the action of methyl bromide is still a controversial issue, our conclusion that hydrobromic acid is probably the sporicidal and germicidal agent for *B. anthracis* seems to be plausible in view of the experimental data presented above. However, studies in support of this hypothesis and further investigations on the potentialities of CH_3Br for the disinfection of contaminated animal products are being conducted.

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SUMMARY AND CONCLUSIONS

The results of studies on the germicidal and sporicidal efficacy of CH_3Br for *Bacillus anthracis* are presented. Six virulent cultures of *B. anthracis*, including strains of canine, bovine, ovine, and human origin, were employed in these studies.

Preliminary experiments demonstrated that spores were able to survive in a mixed atmosphere of air and methyl bromide for 3 or 7 hours, but were destroyed after exposure for 26 hours.

In 165 tests with a vacuum chamber apparatus, methyl bromide, in concentrations of 3.4 to 3.9 grams per liter, completely destroyed anthrax spores on filter paper strips following exposure for 24 to 72 hours in the presence of moisture, whereas spores survived in 4 out of 24 (16.67 per cent) such tests during exposures of 1 hour. In the absence of moisture, however, some spores survived in 27 out of 213 tests (12.68 per cent) regardless of the exposure period.

In other experiments (24 tests) CH_3Br was either bubbled through saline spore suspensions or vaporized over the surface of young agar slant cultures of *B. anthracis* for 1 hour; this was followed by incubation in the sealed exposure tubes for 24 to 72 hours at room temperature. All spores and vegetative cells were killed during exposure to CH_3Br under the conditions of these tests.

Excessively dehydrated spores (i.e., spores dried over $CaCl_2$ at room temperature or at 45 C for 60 to 75 days) were found to be no more resistent to the action of methyl bromide than moist nondesiccated spores.

Liquid methyl bromide per se was ineffective for the destruction of spores from any of the six test strains of *B. anthracis* during exposure periods of 1 and 48 hours.

Since these studies demonstrated that gaseous CH_3Br, in the presence of moisture, is germicidal and sporicidal for *B. anthracis* after exposures of 24 hours, regardless of the method employed, it was concluded that this destructive action was probably due to hydrobromic acid, a hydrolytic product of this compound. Chemical titration tests for inorganic bromides and pH determinations on

control materials exposed to CH_3Br provided further evidence in support of this theory.

Finally it is suggested that the inability of other workers to demonstrate any significant bactericidal activity with methyl bromide may be due in part at least to an insufficient length of exposure and failure to provide adequate moisture during exposure tests.

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