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Paraformaldehyde for Surface Sterilization and Detoxification

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Paraformaldehyde powder, depolymerized by heat, produced formaldehyde gas that was effective in sterilizing laboratory rooms, a mobile laboratory trailer, various surfaces, fiberglass filter medium, and specialized laboratory equipment. A sporulating and nonsporulating strain of bacteria, a virus, and a bacterial toxin were used as test agents. The dissemination procedure and equipment, the assay techniques, and the facilities and equipment sterilized were described.

Sterilization by vapors and gases has become an accepted procedure in many infectious disease laboratories. Equipment that cannot withstand the application of heat or liquid chemicals can usually be decontaminated with ethylene oxide gas, vaporized Formalin, or β-propiolactone. However, one aspect of decontamination that is not adequately covered is one involving highly sophisticated electronic equipment that has been contaminated with bacterial toxins.

Recently a requirement arose for decontamination of such equipment and of other surfaces contaminated with infectious microorganisms or dangerous toxins, of which *Clostridium botulinum* toxin is typical. Ethylene oxide gas is ineffective against this toxin, and it was feared that vaporized Formalin or β-propiolactone might result in some unforeseen or accidental damage to equipment. This led to the investigation of formaldehyde gas, released from dry paraformaldehyde, as a sterilizing and detoxifying agent.

The name paraformaldehyde was first employed in 1888 by Tollens and Mayer. In 1890, Losekann discovered that this polymer contained combined water and reported that it was a polymer hydrate having the formula $(CH_2O)_6 \cdot H_2O$. In 1897, Delepine concluded that paraformaldehyde was a mixture of polymeric hydrates with the average formula $(CH_2O)_6 \cdot H_2O$ and that it was formed by condensation of methylene glycol, as indicated by $n\ CH_2(OH)_2 \rightarrow (CH_2O)_n \cdot H_2O + (n - 1)H_2O$. The chemical composition of paraformaldehyde is best expressed by the formula $HO \cdot (CH_2O)_n \cdot H$ (11).

Paraformaldehyde polymer, produced by evaporating aqueous Formalin, is either a flake or a coarse or fine powder. The particle size of the fine powder is such that a minimal 90% by volume will pass through a 200-mesh screen; only about 60% of a coarse powder will pass. Paraformaldehyde is combustible, with a flash point (Tag Open Cup) of approximately 93 C and an ignition temperature of about 302 C. The explosive range for formaldehyde gas (per cent by volume in air) is 7 to 73%, and the vapor pressure is greatest at the dew point and varies with the humidity. At 30 C, the vapor pressure is 1 mm of mercury in dry air and 3 mm of mercury at the dew point (4). Paraformaldehyde powder, when placed on a heated surface, depolymerizes to formaldehyde gas. The formaldehyde gas is dry, free of contaminants such as methanol and formic acid, and has the same chemical properties as formaldehyde gas obtained from the chemical reaction between potassium permanganate and aqueous Formalin.

The history and use of formaldehyde as a sterilizing agent are well reviewed by Walker (11) and Phillips (8). A new process for disseminating formaldehyde gas from paraformaldehyde was patented in 1961 by Charles Kaitz (U. S. Patent 2,993,832). His process was to heat a fine white powder containing 91% formaldehyde. Today this method has widespread use in the poultry industry (10).

Because of the favorable experience of the poultry industry, the fine, powdered paraformaldehyde was selected for this study. The paraformaldehyde was 91 to 95% formaldehyde with 9 to 5% water and a molecular weight of approximately 600. Paraformaldehyde powder contains both free and combined forms of water. "The free water is merely attracted to the polymer by polar forces at points along its length. Water that is chemically combined with paraformaldehyde acts more or less as a terminating agent for the polymer chains. The fact that the two types of water are present in approximately equal amounts indicates that the polymer must contain

about 20 polyoxymethylene units, from which the molecular weight of 600 is estimated" (4).

MATERIALS AND METHODS

Test microorganisms and toxin. The microorganisms used to determine the sterilization effectiveness of paraformaldehyde were (i) suspensions of *Bacillus subtilis* var. *niger* spores at concentrations of 10^4, 10^5, 10^6, and 10^7 spores/ml, (ii) suspensions of *Serratia marcescens* at concentrations of 10^4 and 2×10^{10} organisms/ml, and (iii) a suspension of Newcastle disease virus at a concentration of $10^{8.5}$ embryo lethal $dose_{50}$ (ELD_{50})/ml. To assess paraformaldehyde as a detoxifying agent, *C. botulinum* toxin type A was used both as a suspension at a concentration of 5×10^5 mouse intraperitoneal lethal $dose_{50}$ ($MIPLD_{50}$)/ml and as a dry powder at a concentration of 2×10^9 $MIPLD_{50}$/g.

Disseminators. The fine, powdered paraformaldehyde was depolymerized by heating the powder on a modified hot plate or in household electric frying pans at a controlled temperature of 232 C. At 232 C, these disseminators depolymerize approximately 20 g/min of paraformaldehyde to the gaseous state. The capacity of the electric frying pans (11.5 by 11.5 by 2 in) is about 2 to 3 lb. When hot plates are used, a suitable container for the powder must be placed on the surface of the hot plate. Erlenmeyer flasks (500 or 1,000 ml) were used as generators in test chamber experiments. The flask with paraformaldehyde was placed on the hot-plate surface with a glass tube connected to the test chamber (Fig. 1).

Inhibiting activity of formaldehyde gas. In the assay of bactericidal chemicals, the problem of distinguishing bactericidal from bacteriostatic action is often encountered. The techniques described have been used here for many years to determine the effectiveness of sterilization procedures that involve vaporized Formalin. It has been our experience that these techniques do not result in sufficient carry-over to dilution blanks and plates to inhibit the growth of the test organisms. Harry (6) and Willard and Alexander (12) have reported similar findings from the use of similar techniques.

To test the possibility that carry-over from depolymerized paraformaldehyde might differ from that of vaporized Formalin, one of the early tests, described in the next paragraph, utilized duplicate sets of cotton swabs. One set was moistened with physiological saline, the other with a noninhibitory formaldehyde neutralizing solution (four parts Na_2SO_3 to one part NH_4Cl). Duplicate assay samples were taken and plated as described, with no difference in recovery. This confirmed our previous experience that the formaldehyde concentration used (0.8% by volume in air) did not result in a significant carry-over to the growth media.

Bacterial test challenge. Two microbiological laboratory rooms (4,598 and 2,250 ft^3) and a mobile laboratory trailer (2,200 ft^3) were seeded with *B. subtilis* var. *niger* spores and *S. marcescens* organisms by placing suspensions of the organisms at delineated sites. During these tests, a static air condition was achieved by turning off the air supply and exhaust. The relative humidity was adjusted to 60%, and the room temperature to 23.3 C. Fifteen test locations were seeded with *B. subtilis* and 15 with *S. marcescens* in each room area, and 20 test locations were seeded with *B. subtilis* in the mobile trailer. Before each test, control swabs were taken from the seeded locations to substantiate the viability of the microorganisms. The paraformaldehyde powder (1,379 g in the 4,598-ft^3 room, 675 g in the 2,250-ft^3 room, and 330 g for the mobile trailer) was placed in the disseminators, the entrance door was sealed, and dissemination was begun. After dissemination, 1-hr contact time was maintained for all tests. The areas were aerated by restarting the air supply and exhaust systems for 1 to 2 hr before the test areas were entered. The seeded locations were then sampled with sterile, moistened cotton swabs and streaked on corn steep-agar plates (2). The plates were incubated for 48 hr at 37 C.

Two adjacent and interconnected rooms housing aerosol facilities were used to evaluate the feasibility of sterilizing large enclosures. The total free volume of the two rooms, exclusive of equipment present, is 67,216 ft^3. One room is a four-storied enclosure, the other a five-storied enclosure. Expanded metal grat-

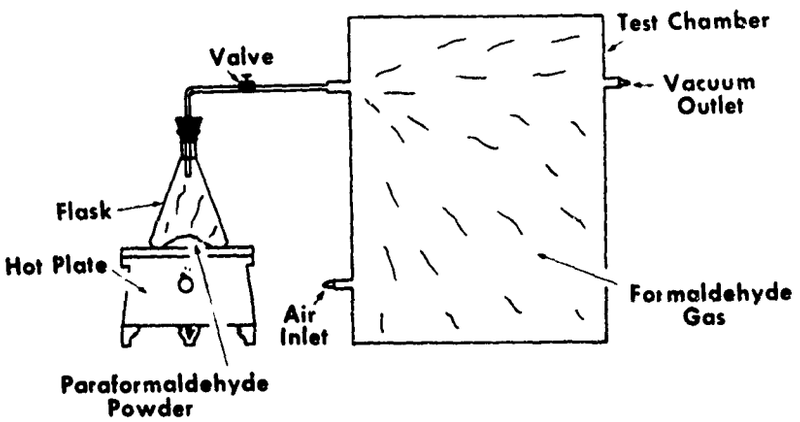

FIG. 1. *Paraformaldehyde depolymerization disseminator.*

ings divide the floor levels in each room. There are connecting doors at the first, second, and third floors and at the elevator shafts.

Two-hundred clearly delineated sites throughout the rooms were seeded with *B. subtilis* spores at a concentration of 10^5 spores/ml to assess the effectiveness of the formaldehyde gas. A total of 44.4 lb of paraformaldehyde was placed in 18 frying pans strategically spaced throughout the enclosures.

During this test, the room temperature was 31 C. This was higher than the 24 C desired and was caused by the heat from a previous steam formaldehyde sterilization of the test facilities (1). The relative humidity was 50 to 55%. The test procedure was as follows. Place 2.5 lb of powder in each fry pan; turn off all supply and exhaust ventilation; turn on all internal air circulating fans; turn on all fry pans with automatic control timers; disseminate for approximately 1 hr; maintain a 2-hr contact period; and turn on all exhaust and supply ventilation to the rooms for an overnight aeration period.

By the following morning, the formaldehyde gas had completely dissipated, and personnel entered the facilities without need for respiratory protection. Surface samples for residual *B. subtilis* spores were taken from the 200 sites by the technique previously described.

Upon entering the room, it was found that two electric circuits had tripped from overload, which turned off four fry pans shortly after heat-up time. As a result, 8.3 lb of paraformaldehyde powder was not depolymerized. The remainder of the pans operated satisfactorily, and a total of 35.7 lb of powder (0.24 g/ft³ of air space) was actually disseminated during this sterilization test procedure.

Samples of 15 types of surfaces commonly found in a laboratory facility were placed in a 25 ft³ plastic test chamber. The surfaces used were glass, rubber, plastic, stainless steel, galvanized metal, wood, paper, sponge, filter paper, painted surface (oil base), 0.25-inch rigid plastic, copper, aluminum, vinyl sheeting, and mild steel. A 1-ml amount of *B. subtilis* with a concentration of 10^7 spores/ml was nebulized into the chamber with a Chicago nebulizer (9). Positive-control swabs were taken in the chamber area to assure contamination of the different surfaces. After the humidity was adjusted to 60% and the temperature to 24 C, the formaldehyde gas was released into the chamber by the use of 0.3 g of paraformaldehyde/ft³ of space. After a specified contact time (2 hr for three tests, and 1 hr for two tests), the test chamber was aerated, and each test surface was sampled as before.

Twelve dry, filter-paper patches seeded with *B. subtilis* spores (10^6 spores/patch) were placed on each side and between the layers of the 50 FG filter medium (Pittsburgh Plate Glass Co., Pittsburgh, Pa.) in the filter housing of a class I cabinet (5). After reassembly of the filter, the cabinet exhaust blower was turned off, and then paraformaldehyde was disseminated in the cabinet work area. During depolymerization of the powdered paraformaldehyde, the exhaust blower of the cabinet was turned on for 30 sec, and then turned off; this was repeated three more times. The temperature and the relative humidity in the cabinet were 24 C and 60%, respectively. After a 1-hr contact time, the filter was disassembled, and the 24 patches were aseptically removed, placed in nutrient broth, and incubated for 24 hr at 37 C. After incubation, duplicate 0.1-ml amounts of the broth were plated on corn steep-agar and incubated.

Plastic vaccine storage tubes were placed in a 2-ft³ rigid plastic chamber by means of three packaging conditions: loose, in an open plastic bag, and in a closed plastic bag. The vaccine tubes are 57.7 mm long by 8.5 mm in diameter (outside dimension) and have a volume of 1 ml. The tubes have a 7.5-mm opening (inside dimension) on one end; the other end (22 mm in length) tapers down to a 2-mm opening (inside dimension). The tubes were contaminated by nebulization (as in the previous test) with a liquid suspension of *B. subtilis* spores and *S. marcescens* organisms, each with a concentration of 104 organisms/ml. Replicate tests were run with each bacterium. Before each test, the relative humidity in the chamber was adjusted to 60%, and then 2 ml of each test organism was aerosolized into the chamber with a Chicago nebulizer. The formaldehyde gas was disseminated into the chamber by means of a hot plate and a flask containing paraformaldehyde. After a 1-hr contact period, the chamber was opened, and six vaccine tubes were selected at random with sterile forceps, placed in nutrient broth, and incubated for 24 hr. Then duplicate 0.1-ml amounts of the broth were plated on corn steep-agar, incubated for 24 hr at 37 C, and observed for growth.

Various types of laboratory equipment, such as large-volume air samplers (LVAS), analytical balances, clinical centrifuges, *p*H meters, and other complex electrical equipment were contaminated with *B. subtilis* spores at a concentration of 10^6 spores/ml. In all cases, the relative humidity was no lower than 50% at a temperature of approximately 24 C. The instruments were exposed to formaldehyde gas at a concentration of 0.3 g of paraformaldehyde/ft³ in appropriate enclosures with varying contact times (from 1 to 2 hr), aerated, and then sampled with moistened cotton swabs as before.

Virus test challenge. Newcastle disease virus (NDV) GB strain at a concentration of $10^{8.5}$ ELD_{50}/ml [calculated by the Reed-Muench method (3)] was used both as an aerosol and as a suspension inoculated on filter patches. A 2-ml amount of the virus suspension was nebulized into a 1-ft³ chamber and allowed to dry for 15 min; positive control samples were then taken from the interior surfaces of the chamber with moistened cotton swabs to assure the presence of viable NDV. The temperature and relative humidity in the 1-ft³ chamber cabinet were 24 C and 60%, respectively. Subsequently, formaldehyde gas (0.3 g of paraformaldehyde) was released into the chamber and, after a 1-hr contact, the not previously sampled surfaces within the chamber were streaked with six moistened cotton swabs to recover residual NDV. The cotton swabs were placed in sterile nutrient broth.

Six filter pads containing NDV were randomly spaced within a class I cabinet (5) without air-exhaust ventilation. The temperature and relative humidity in the class I cabinet were 24 C and 60%, respectively. After dissemination of formaldehyde gas in the cabinet (approximately 42 ft³) and maintenance of min

TABLE 1. *Formaldehyde gas sterilization of facilities, materials, and equipment*

Test condition	Vol	Amt of paraformaldehyde	Organism	Concn/ml	Viable recoveries[a]
	ft^3	g			
Laboratory rooms	2,250	675	*B. subtilis*	10^6	0/5
	4,598	1,379	*S. marcescens*	2×10^{10}	0/5
Large room	67,216	20,165	*B. subtilis*	10^5	0/1
Mobile laboratory	2,200	330	*B. subtilis*	10^6	0/5
Surfaces	25	7.5	*B. subtilis*	10^7	0/5
Filter media class I cabinet	42	12.6	*B. subtilis*	10^6 [b]	0/5
Vaccine tubes	2	.6	*B. subtilis*	10^4	0/2
			S. marcescens	10^4	0/2
Laboratory equipment	100 to 500	30 to 150	*B. subtilis*	10^6	0/10

[a] Number of viable recoveries/total tests conducted.
[b] 10^6 per patch.

contact period, the filter patches were aseptically transferred to sterile nutrient broth. The assay procedure used for recovery, identification, and confirmation of NDV is the same as that described by Barbeito et al. (3).

Toxin test challenge. A large-volume air sampler was placed in a 3 ft^3 plastic chamber inside a gastight ventilated cabinet (5). A 1-ml amount of a liquid suspension of *C. botulinum* toxin (type A) at a concentration of 5×10^5 MIPLD$_{50}$/ml was aerosolized with a Chicago nebulizer into the test chamber. The temperature and relative humidity were maintained at 24 C and 75 to 80%, respectively. The air sampler was run for 5 min; then the sampler was turned off, the collecting grid was dismantled, and the collecting fluid was drained. The fluid was passed out of the cabinet through an autoclave and sterilized.

Positive-control swabs taken before decontamination showed that the LVAS was contaminated with toxin when swab diluent was injected into mice.

Formaldehyde gas was then liberated into the chamber. After a 2-hr contact period, surface samples were taken from 10 locations on the LVAS with sterile, moistened cotton swabs. The swabs were immersed in 2 ml of sterile, distilled water and shaken on a Vortex mixer. To determine whether detoxification was achieved, 0.5 ml of the suspension was inoculated intraperitoneally (ip) into each of three 16- to 22-g mice, and the mice were caged for 5 days. Their survival indicated that detoxification had been achieved.

Three tests were performed with dry powder of *C. botulinum* type A toxin with a 3% moisture content in 20-mg amounts (spread on a flat surface) inside a 1-ft^3 plastic chamber. The temperature was 23 C with a relative humidity of 60%. Contact time for this test was 4 hr. To further test formaldehyde gas against the toxin, one test was conducted with 10 g of dry powder spread on paper inside the chamber. This test was conducted at 24 C and at a relative humidity of 45% with a contact time of 48 hr. The minimum contact period for bulk detoxification was not established.

The assay procedures for these tests were as follows: for the 20-mg sample, 10 mg was removed and put into 6 ml of sterile distilled water; for the 10-g sample, samples of the toxin were taken from different positions or levels within the powder (bottom side, center level, two top sides, and three different mixed samples), and each of these samples was dissolved in 6 ml of sterile distilled water. Subsequently, 0.5 ml of the diluent from the samples of both the 20-mg tests and the 10-g test were inoculated ip into each of 10 mice weighing 16 to 22 g. The detoxification end point was the same as described previously.

TABLE 2. *Formaldehyde gas sterilization of Newcastle disease virus*

Test condition	Vol	Amt of paraformaldehyde	Organism concn[a]	Viable recoveries[b]
	ft^3	g		
Test chamber	1	.3	$10^{8.5}$	0/1
Class I cabinet	42	12.6	$10^{8.5}$	0/1

[a] Expressed as ELD$_{50}$/ml.
[b] Number of viable recoveries/total tests conducted.

TABLE 3. *Formaldehyde gas detoxification of C. botulinum toxin type A*

Test condition	Vol	Amt of paraformaldehyde	Toxin concn	Toxin recovered[a]
	ft^3	g		
Air sampler[b]	3	0.9	5×10^5	0/3
Powder samples (20 mg)[c]	1	0.3	2×10^9	0/3
Powder sample (10 g)[c]	1	0.2	2×10^9	0/1

[a] Toxin recovered/total tests conducted.
[b] Expressed as MIPLD$_{50}$/ml.
[c] Expressed as MIPLD$_{50}$/g.

RESULTS

The sterilizing and detoxifying effectiveness of formaldehyde gas produced by depolymerization of dry paraformaldehyde is shown in Tables 1–3. In all the tests, the microorganisms were killed and the toxin was detoxified. Various surfaces

contaminated with liquid suspensions of bacterial spores of *B. subtilis* var. *niger* at a concentration of 10^7 spores/ml and *S. marcescens* organisms at the highest concentrations of 2×10^{10} organisms/ml were sterilized after cepolymerization of 0.3 g of paraformaldehyde/ft³ of space for a 1-hr contact period. Under the same conditions, *C. botulinum* type A toxin was detoxified both as a liquid suspension at a concentration of 5×10^5 $MIPLD_{50}$/ml and as dry material (spread in various amounts in thin layers on flat surfaces) with a 3% moisture content at a concentration of 2×10^9 $MIPLD_{50}$/g. Surfaces contaminated with NDV at a concentration of $10^{8.5}$ ELD_{50}/ml were sterilized after depolymerization of 0.3 g of paraformaldehyde/ft³ of space and only a 30-min contact period. The high concentrations of bacteria, virus, and toxins challenged in these tests show that formaldehyde gas is capable of killing microorganisms and detoxifying *C. botulinum* toxin.

The amount of paraformaldehyde powder used was 0.3 g/ft³ of space in all tests with three exceptions: (i) the mobile laboratory, 0.15 g/ft³ of space; (ii) the 10-g *C. botulinum* toxin sample, 0.6 g/ft³ of space; and (iii) the rooms housing aerosol facilities, 0.24 g ft³ of space.

No visible residual was observed during these tests.

DISCUSSION

The test results indicate the potential for use of paraformaldehyde in the infectious disease laboratory. The range of its use appears to be equal to that of Formalin. The absence of any failure in the tests conducted to date indicates that the dry gas liberated from depolymerization of paraformaldehyde may be a more effective sterilizing agent than vaporized Formalin. The gas also disseminates more readily throughout the area being treated. After treatment it is more rapidly dissipated by aeration. During the work done so far, there have been no problems of repolymerization or other residual when the concentration and humidity were controlled. Nor has there been a visible or operational effect on any of the mechanical, electronic, or optical equipment treated.

On one occasion, a residual surface precipitate was obtained by deliberately increasing the relative humidity to 100% in a laboratory room. As moisture condensed on surfaces, paraformaldehyde was heated to release the formaldehyde gas. After treatment and aeration, a white precipitate, assumed to be paraformaldehyde, was seen on many surfaces in the room.

The toxicity of formaldehyde is well known, and its effects on body tissues are well documented (7). Its irritating vapors generally render an area uninhabitable before a toxic concentration is present. However, the flammability of formaldehyde gas and paraformaldehyde is not so well understood and should be considered whenever they are disseminated.

When exposed to an open flame, paraformaldehyde ignites and decomposes to carbon dioxide and water, rendering it ineffective as a sterilizing agent. Its ignition temperature is 302 C.

Formaldehyde gas is flammable in air at concentrations of 7 to 73% by volume. The concentration resulting from depolymerization of 0.3 g of paraformaldehyde/ft³ of space does not approach this range. However, it is possible to achieve this by liberating excessive amounts of the gas into an enclosed space. Our tests show that 0.3 g/ft³ is ample for sterilization, and this quantity should not be exceeded.

The application of paraformaldehyde to large-scale space sterilization is presently limited by lack of a suitable, commercially available generator for large-quantity depolymerization. Developmental efforts are under way in this direction. However, the use of generators such as those described is easy and practical for small-volume space sterilization.

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LITERATURE CITED

1. Alg, R. L., G. J. Harris, and M. S. Barbeito. 1964. Disinfection with BPL Soap Chem. Spec. 40:97–100.
2. Barbeito, M. S. 1966. Emergency disinfection of operating room and patient ward with beta-propiolactone. J. Am. Hosp. Assoc. 49:100–106.
3. Barbeito, M. S., C. T. Mathews, and L. A. Taylor. 1967. Microbiological laboratory hazard of bearded men. Appl. Microbiol. 15:899–906.
4. Celanese Chemical Company Division. 1961. Celanese paraformaldehyde, p. 4 (product bulletin S-165). Celanese Corp. of America, New York.
5. Gremillion, G. G. 1960. The use of bacteria-tight cabinets in the infectious disease laboratory. Proc. Symp. Gnotobiotic Technol. 2nd, p. 171–182.
6. Harry, E. G. 1961. Surface disinfection of poultry buildings by formaldehyde and sulphur dioxide. Vet. Rec. 73:522–526.
7. Industrial Hygiene Research Laboratory, National Institute of Health. 1945. Formaldehyde: its toxicity and potential dangers. Public Health Rept. Suppl. 181.
8. Phillips, C. R. 1968. Gaseous sterilization, p. 669–685. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization, and preservation. Lea and Febiger, Philadelphia, Pa.
9. Rosebury, T. 1947. Experimental air-borne infection. Williams & Wilkins Co., Baltimore, Md.
10. Vineland Poultry Laboratories. Fumigation with Formaldegen (Bulletin R5-767030). Vineland, New Jersey.
11. Walker, J. F. 1953. Formaldehyde. Reinhold Publishing Corp., New York.
12. Willard, M., and A. Alexander. 1964. Comparison of sterilizing properties of formaldehyde-methanol solutions with formaldehyde-water solutions. Appl. Microbiol. 12:229–233.