New Formaldehyde Base Disinfectants

RALPH TRUJILLO AND KERMIT F. LINDELL

Biosystems Research Department, Sandia Laboratories, Albuquerque, New Mexico 87115

Received for publication 12 March 1973

Preparations of formaldehyde in various organic liquids—ethylene glycol, glycerol, and propylene glycol—serve as effective disinfectants towards microbial vegetative cells and spores. This disinfection is a temperature-dependent process and is manifest when these formaldehyde base disinfectants are dissolved in water. The irritating vapors associated with formaldehyde disinfection are not present in either of these new formaldehyde base disinfectants or in aqueous solutions of them.

Formaldehyde is considered to be an extremely effective disinfectant and has been used to inactivate bacteria, fungi, yeasts, and molds. However, the disinfectant use of formaldehyde has been limited because of the penetrating odor of formaldehyde and associated irritation to eyes and nose.

In the course of studying the sporocidal and sporostatic properties of aqueous formaldehyde (3) this laboratory developed a means of preparing a liquid form of formaldehyde which possesses the disinfectant properties of formaldehyde without the objectionable properties of odor and eye and nose irritation. We report on the preparation and properties of these new formaldehyde base disinfectants.

MATERIALS AND METHODS

Organisms. Stock preparations of Bacillus subtilis, Bacillus brevis, Bacillus megaterium, Escherichia coli, Staphylococcus aureus, and Streptococcus fecalis were prepared by inoculating each of these organisms into separate flasks containing Trypticase soy broth (4% wt/vol, BBL) and allowing the inoculum to shake at 23°C for 20 to 26 h. The resulting bacterial suspensions were diluted 1:100 in 4% Trypticase soy broth, and the microbial concentration was determined by plating out with Trypticase soy agar (4% wt/vol, BBL) after appropriate serial dilutions. Serial dilutions involving vegetative cells were accomplished using dilution bottles containing 0.9% sodium chloride and 0.01% nutrient broth (BBL). These vegetative cells were used in the minimal inhibitory concentration-minimal bacteriocidal concentration (MIC-MBC) studies.

Bacillus subtilis var. niger spores were prepared by an active culture technique previously described (4). The spores were suspended in 95% ethanol at a concentration of $6 \times 10^9$ spores/ml and were stored at $-10^\circ$ C.

MIC-MBC studies. Studies on the MIC and MBC of the various formaldehyde base disinfectants were determined using accepted protocols and techniques (1). Basically, the MIC studies were carried out by depositing varying concentrations of a given disinfectant into a series of test tubes and adding a sample of a bacterial suspension to each of the tubes. The bacterial suspension consisted of bacteria suspended in a complete and complex growth medium. The tubes were observed for turbidity as a sign of microbial growth after 24 and 48 h of incubation at 35°C. The lowest concentration of disinfectant which yields a clear, nonturbid solution is denoted the MIC. A 0.1-ml sample from each clear tube was plated out with Trypticase soy agar, and the extent of colony development was noted after incubation for 48 h. The concentration which yields no colonies is defined as the MBC.

Sporocidal studies. Studies on the sporocidal properties of the various formaldehyde base disinfectants were carried out by pipetting 0.1 ml of an ethanol spore suspension into screw-cap test tubes and removing the ethanol under vacuum. Each study consisted of two such vials to which was added 10 ml of either a control solution or a test solution. The test solution consisted of the formaldehyde base disinfectant, while the composition of the control solution was identical to the test solution except that it contained no formaldehyde. After addition of the appropriate solutions, the test tubes were insonated for 1 min in an ultrasonic bath (Turco Products, Inc., 20 A, 250 V) and subsequently shaken vigorously to achieve a uniform spore suspension. The tubes were then placed in a Blue M constant-temperature water bath at a given temperature controlled to $\pm 0.1^\circ$ C. Samples were withdrawn periodically, serially diluted, and plated out using Trypticase soy agar. Plate counts were made after incubation at 31°C for 4 to 5 days. This incubation period insured sufficient time for colony development.

Disinfectants. The four different formaldehyde base disinfectants studied were formaldehyde in water (FW), formaldehyde in ethylene glycol (FEG), formaldehyde in glycerol (FG), and formaldehyde in...
propylene glycol (FP). Each of these disinfectants was prepared by refluxing 5.0 g of paraformaldehyde (Matheson, Coleman & Bell) in 50 ml of the appropriate solvent until a clear solution was obtained. All solutions were clear after about 5 min of heating except for FW which required approximately 2 h of refluxing for a clear solution to be obtained. The formaldehyde concentration was determined for each solution by using the phenylhydrazine hydrochloride-potassium ferricyanide method (2) and reading the absorption at 515 nm. The resulting 10% FW solution exhibited the acrid and penetrating odor associated with formaldehyde solutions, while the FEG, FG, and FP solutions were free of such obnoxious properties.

The stock 10% formaldehyde solutions were diluted 1:100 in 4% Trypticase soy broth in the MIC-MBC studies, while 1:10 dilutions of the stock 10% formaldehyde solutions were employed in studying the sporocidal properties of these formaldehyde base disinfectants. The 1:10 dilutions were effected using a solvent appropriate for a given disinfectant, e.g., water for FW, glycerol for FG, etc. In some studies the 1:10 dilution was achieved using 1 part FG, FP, or FEG and 9 parts water to yield solutions identified respectively as FG-W, FP-W, and FEG-W. The 1:10 dilution of FW with water yields a solution which still exhibited the irritating vapors associated with formaldehyde solutions, while formaldehyde vapors were not discernible in the FG-W, FP-W, and FEG-W solutions even after heating at 40°C for 3 h.

RESULTS

Tables 1 and 2 summarize the MIC and MBC data for each of the formaldehyde base disinfectants studied. Table 3 compares the disinfectant values, i.e., the microbial concentration (cells per milliliter) per assay tube divided by the MIC value (milligram per milliliter) per assay tube, for each solution described in Tables 1 and 2 and emphasizes the similar disinfectant properties of these solutions. The MIC, MBC, and disinfectant values for all disinfectants are remarkably uniform, and this suggests that it is the formaldehyde present in each system that is the agent responsible for the disinfectant properties of the various solutions. Support for this hypothesis comes from the fact that additional MIC studies employing ten times the concentration of ethylene glycol, glycerol, and propylene present in the MIC studies documented in Tables 1 and 2 did not visibly affect bacterial growth, i.e., 1% (vol/vol) solutions of these solvents in water were not bacteriocidal.

Thus, the disinfectant properties of all formaldehyde base disinfectants studied were very similar. The difference between the disinfectants being that FW possessed the strong and characteristic odor of a formaldehyde solution while the FEG, FG, and FP solutions did not.

### Table 1. Formaldehyde-water (FW) and formaldehyde-ethyleneglycol (FEG) disinfectant properties

| Organism      | FW    | FEG   |
|---------------|-------|-------|
|               | MIC  | MBC  | MIC  | MBC  |
| B. subtilis   | 0.123| 0.245| 0.133| 0.265|
| B. megaterium | 0.245| 0.245| 0.265| 0.265|
| B. brevis     | 0.123| 0.123| 0.133| 0.133|
| E. coli       | 0.123| 0.245| 0.133| 0.265|
| S. aureus     | 0.245| 0.245| 0.265| 0.265|
| S. faecalis   | 0.123| 0.245| 0.133| 0.265|

* MIC is the minimal inhibitory concentration and is measured in milligrams per milliliter.

* MBC is the minimal bacteriocidal concentration and is measured in milligrams per milliliter.

### Table 2. Formaldehyde-glycerol (FG) and formaldehyde-propylene glycol (FP) disinfectant properties

| Organism      | FG    | FP    |
|---------------|-------|-------|
|               | MIC  | MBC  | MIC  | MBC  |
| B. subtilis   | 0.245| 0.245| 0.27 | 0.27 |
| B. megaterium | 0.245| 0.245| 0.27 | 0.27 |
| B. brevis     | 0.245| 0.245| 0.27 | 0.27 |
| E. coli       | 0.245| 0.245| 0.27 | 0.27 |
| S. aureus     | 0.245| 0.245| 0.27 | 0.54 |
| S. faecalis   | 0.245| 0.245| 0.27 | 0.54 |

* MIC is the minimal inhibitory concentration and is measured in milligrams per milliliter.

* MBC is the minimal bacteriocidal concentration and is measured in milligrams per milliliter.

exhibit this property. Therefore, a new class of formaldehyde base disinfectants has been prepared which possesses the disinfectant properties of formaldehyde without the strong and pungent odor associated with formaldehyde disinfection.

The MIC and MBC values for the FEG disinfectant were re-assayed after 40 days, and the MIC and MBC values obtained were found to be essentially the same as those presented in Table 1. It appears that the new formaldehyde base disinfectants do not lose their effectiveness during short-term storage. Studies on the effects of long-term storage, i.e., approximately 1 year, are currently underway.

Because of the nature of these MIC studies, i.e., 1:100 dilution of the formaldehyde solutions in 4% Trypticase soy broth and subsequent mixing with a bacterial suspension, the disinfectant properties of these disinfectants were determined in an essentially aqueous system. The FP, FEG, and FG solutions are
nonaqueous systems, and it was of interest to study the intrinsic disinfectant properties of these solutions in a nonaqueous environment. Attempts at defining the intrinsic disinfectant properties of the new formaldehyde base disinfectants by using vegetative cells were not successful because ethylene glycol, glycerol, and propylene glycol proved to be bacteriocidal to vegetative cells and/or the viscosity of ethylene glycol, glycerol, and propylene glycol solutions did not allow a uniform distribution of vegetative cells in these media. The latter point made accurate, quantitative studies on microbial inactivation extremely difficult. Therefore, the intrinsic disinfectant properties were studied using B. subtilis var. niger spores.

Exposure of B. subtilis spores to FW proved to be sporocidal, and the sporocidal property is highly temperature dependent (Fig. 1). Figure 1 also shows that FP was not sporocidal over the temperature range studied. Suspension of spores in propylene glycol or water for 3 h at 22 to 40 °C also was not sporocidal to B. subtilis spores. However, FP dissolved in water (FP-W) became sporocidal and exhibited the characteristic temperature dependent sporocidal property of formaldehyde disinfection (Fig. 2) (1). Spores suspended in solutions made up with 9 parts water and 1 part propylene glycol were not inactivated on heating from 22 to 40 °C for 3 h. FG and FEG were not sporocidal per se, but became sporocidal on dissolution in water (Fig. 3). The sporocidal properties of FG-W and FEG-W were determined at 40 °C, and at this

| Organism          | FW     | FEG    | FG     | FP     |
|-------------------|--------|--------|--------|--------|
| B. subtilis       | $2.11 \times 10^6$ | $9.75 \times 10^5$ | $7.35 \times 10^6$ | $1.58 \times 10^6$ |
| B. megaterium     | $2.28 \times 10^6$ | $7.96 \times 10^5$ | $1 \times 10^6$ | $1.02 \times 10^6$ |
| B. brevis         | $3.83 \times 10^6$ | $3.77 \times 10^5$ | $3.95 \times 10^6$ | $5.0 \times 10^6$ |
| E. coli           | $6.81 \times 10^6$ | $3.55 \times 10^7$ | $3.93 \times 10^6$ | $4.51 \times 10^6$ |
| S. aureus         | $5.96 \times 10^6$ | $2.62 \times 10^7$ | $1.51 \times 10^7$ | $7 \times 10^7$ |
| S. faecalis       | $1.55 \times 10^6$ | $2.04 \times 10^6$ | $4.0 \times 10^6$ | $1.85 \times 10^6$ |

* Disinfectant value is the microbial concentration (cells per milliliter) per assay tube divided by the MIC value (milligrams per milliliter) per assay tube.

**Fig. 1.** Sporocidal activity of F-W (●) solutions at various temperatures. FP (■) solutions were not sporocidal when heated from 22 to 40 °C. Surviving fraction refers to the ratio of survivors at some time (N) to the number of viable spores at zero time (N₀).

**Fig. 2.** Sporocidal activity of FP-W (●) solutions at various temperatures. Appropriate control solutions not containing formaldehyde were not sporocidal.
temperature their sporocidal activity was comparable to FP-W at this same temperature (Fig. 2). Control solution of glycerol, ethylene glycol, aqueous glycerol (a 1:10 dilution), and aqueous ethylene glycol (a 1:10 dilution) do not inactivate spores on exposure for 3 h at 40 C.

DISCUSSION

This investigation describes the preparation and properties of somewhat novel formaldehyde base disinfectants. These disinfectants in aqueous solution possess the disinfectant properties of formaldehyde and yet do not exhibit the disagreeable properties of odor and irritation associated with formaldehyde disinfection. The potential utility of such disinfectants has determined that material presented in this communication be covered in a pending patent application.

The essential equivalence of the MIC-MBC values for FW, FG, FEG, and FP towards a wide variety of bacteria suggests that it is the formaldehyde present in these formaldehyde base disinfectants which is causing the microbial inactivation. It should be emphasized that the 10% stock FW, FEG, FG, and FP solutions differed in that the FW solutions exhibited the characteristic irritating vapors associated with formaldehyde solutions while the FEG, FG, and FP did not. In the MIC-MBC studies, 1:100 dilutions of the stock formaldehyde base disinfectant solutions was effected using 4% Trypticase soy broth, and therefore all disinfectant solutions used in the MIC-MBC studies were essentially aqueous solutions. As previously noted, in such aqueous solutions, FW, FEG, FG, and FP all exhibited essentially the same disinfectant properties (Tables 1, 2, and 3). The disinfectant value parameter (Table 3) was adopted as a means of comparing the disinfectant activity of the various formaldehyde solutions on a weight formaldehyde basis. The disinfectant values listed in Table 3 serve as an approximate measure of the number of microorganisms inactivated per milligram of formaldehyde present in the assay system.

It was necessary to employ B. subtilis spores in an attempt to determine the intrinsic disinfectant properties of FEG, FG, and FP solutions. Figures 1 and 3 show that these solutions per se, in a nonaqueous environment, are not sporocidal over the temperature range 22 to 40 C. These sporocidal experiments are in apparent variance with the MIC-MBC experimentation and suggest the hypothesis that the nonaqueous disinfectant solutions have the formaldehyde bound in some manner which does not permit the disinfectant properties of formaldehyde to be expressed. However, dissolution of FG, FEG, and FP in water produces an essentially aqueous system which apparently releases the bound formaldehyde with subsequent disinfection. Figures 2 and 3 confirm that such a release of bound formaldehyde is in fact observed when FEG, FG, and FP solutions are dissolved in water yielding the disinfectant solutions FEG-W, FG-W, and FP-W. These aqueous solutions are sporocidal and exhibit the characteristic temperature-dependent sporocidal property associated with formaldehyde disinfection. The 1:10 dilution of FW in water yielded a solution which still exhibited a discernible level of irritating formaldehyde vapors, while such vapors were not detectable in the FEG-W, FG-W, and FP-W solutions.

The mechanism for the proposed binding of formaldehyde and the release of bound formaldehyde in various solutions is not completely understood. However, the existence of disinfectants which possess the disinfectant property of formaldehyde without its concomitant irritating properties suggests that further studies on these new formaldehyde base disinfectants can be profitably undertaken.

ACKNOWLEDGMENTS

The assistance of Charlene Dutchman is gratefully acknowledged.
This work was conducted under Contract No. W-12,883, Planetary Programs, Office of Space Science and Applications, National Aeronautics and Space Administration Headquarters, Washington, D.C.

LITERATURE CITED

1. Anderson, T. G. 1970. Testing of susceptibility to antimicrobial agents and assays of antimicrobial agents in body fluids, p. 299-310. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. The American Society for Microbiology, Bethesda, Md.

2. Hanson, N. W., D. A. Reilly, and H. E. Stagg (ed.). 1965. The detection of toxic substances in air—a manual of ICI practice, p. 131-134. W. Heffer and Sons, Cambridge.

3. Trujillo, R., and T. David. 1972. Sperostatic and sporocidal properties of aqueous formaldehyde. Appl. Microbiol. 23:618-622.

4. Trujillo, R., and N. Laible. 1970. Reversible inhibition of spore germination by alcohols. Appl. Microbiol. 20:620-623.