

Sporostatic and Sporocidal Properties of Aqueous Formaldehyde

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Aqueous formaldehyde is shown to exert both sporostatic and sporocidal effects on *Bacillus subtilis* spores. The sporostatic effect is a result of the reversible inhibition of spore germination occasioned by aqueous formaldehyde; the sporocidal effect is due to the temperature-dependent inactivation of these spores in aqueous formaldehyde. The physicochemical state of formaldehyde in solution provides a framework with which to interpret both the sporostatic and sporocidal properties of aqueous formaldehyde.

Although the bacteriostatic effects of aqueous formaldehyde appear to be well recognized (2), the effect of aqueous formaldehyde on spore germination does not appear to have been investigated. Such a study would seem necessary to distinguish between the sporostatic and sporocidal properties of aqueous formaldehyde for disinfectant purposes, especially in light of a recent study (5) on the reversible inhibition of spore germination by low levels of aliphatic or aromatic alcohols, which suggested that aldehydes also may reversibly inhibit the spore germination process. Furthermore, the temperature-dependent nature of the bacteriocidal activity of formaldehyde in aqueous solution has been described (2, 4); yet a complimentary study on the temperature-dependent nature of the sporocidal activity of aqueous formaldehyde has not been undertaken. This communication investigates the capacity of aqueous formaldehyde to inhibit the germination of *Bacillus subtilis* spores and establishes that the inactivation of these spores by aqueous formaldehyde is a highly temperature-dependent process.

MATERIALS AND METHODS

The *B. subtilis* var. *niger* spores used in this investigation were prepared by an active-culture technique described previously (5). The spores were suspended in 95% ethanol at a concentration of 3×10^9 spores per ml and were stored at -10°C .

The aqueous formaldehyde was prepared by refluxing 5 g of para-formaldehyde (Matheson, Coleman & Bell) in 80 ml of deionized water until a clear solution was obtained (approximately 1.5 hr). The aqueous formaldehyde solution was then made up to 100 ml in a volumetric flask and stored at 31°C . The formaldehyde concentration was determined

by using the phenylhydrazine hydrochloride-potassium ferricyanide method (1) and reading the absorption at 515 nm.

The effect of aqueous formaldehyde on spore germination was studied by pipetting the ethanol suspension of spores into calibrated Bausch & Lomb Spectronic 20 tubes and removing the ethanol under vacuum. A 5-ml amount of Trypticase soy broth (4% w/v, BBL), containing the aqueous formaldehyde at various concentrations, then was added to the tubes containing the spores. Insonation of these tubes for 20 sec in an ultrasonic bath (Turco Products, Inc., 20 amp, 250 v) resulted in complete suspension of these spores in the germinating medium. The spore suspensions then were incubated at 30°C . Periodically, the cultures were shaken, and optical density determinations were made with a Bausch & Lomb Spectronic 20 colorimeter. The viable-spore concentration in each suspension was determined after each experiment by serially diluting a given spore suspension and plating out on Trypticase soy agar (4% w/v). The plates were incubated at 31°C for 4 to 5 days to ensure sufficient time for colony development.

The inhibition of spore germination was shown to be reversible by removing the inhibiting aqueous formaldehyde from the spore environment via membrane filtration (Millipore Corp., type HA, $0.45 \mu\text{m}$) and resuspending the spores in germinating media free from inhibiting additive. The resuspended spores germinated. A similar study was carried out using ethylene glycol (Fisher Scientific Co.) as the inhibiting additive. In all studies, the spore suspensions were incubated at 30°C .

The effect of temperature on spore viability in aqueous formaldehyde was determined by pipetting the ethanol spore suspension into screw cap vials (25 mm inside diameter by 95 mm high) and removing the ethanol under vacuum. Each temperature study consisted of three such vials, to which was added 8 ml of either sterile, deionized water or a 1% (w/v) formaldehyde solution. After addition of the appropriate solutions, the vials were insonated in an ultra-

sonic bath for 30 sec to achieve complete suspension of the spores. One vial containing spores in 1% aqueous formaldehyde was allowed to remain at room temperature (24 C), while the remaining two vials, containing the spores suspended in either 1% aqueous formaldehyde or sterile, deionized water, were placed in a Blue M constant-temperature water bath at a given temperature controlled to ± 0.1 C. The temperatures studied were 24, 30, 40, 50, 55, and 60 C. Samples were withdrawn periodically, serially diluted, and plated out using Trypticase soy agar (4% w/v, BBL). The plates were incubated at 31 C for 4 to 5 days to ensure sufficient time for outgrowth. Dilution bottles containing either 0.5% sodium sulfite or ammonium chloride were used to serially dilute spore suspensions in two different experiments in an effort to neutralize the formaldehyde and, perhaps, reverse the formaldehyde-induced inactivation.

Those spore suspensions subjected to heating in 1% formaldehyde at 50, 55, and 60 C were tested for sterility by pipetting 0.1 ml from each suspension into separate dilution bottles containing 100 ml of sterile Trypticase soy broth (4% w/v). After 5 days of shaking at 24 C, a 1-ml sample was removed from each solution and pipetted into separate dilution bottles containing 100 ml of sterile Trypticase soy broth (4% w/v). All broth solutions were gently shaken for a total of 10 days and were visually inspected for turbidity daily.

RESULTS

The process of spore germination can be followed by observing the changes in optical density for a spore suspension as a function of time (3). Fig. 1 illustrates the data obtained when spores of *B. subtilis* var. *niger* were exposed to germinating media in the absence and presence of aqueous formaldehyde at 30 C. Increasing the formaldehyde concentration in

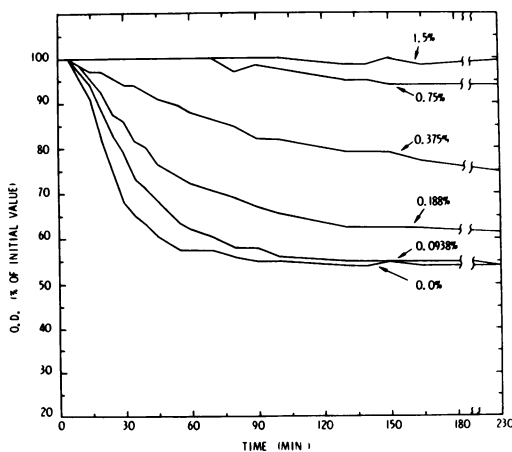


FIG. 1. Effect of various concentrations of aqueous formaldehyde on the germination of *Bacillus subtilis* spores.

the germinating media caused a decrease in the extent of spore germination. A plot of the extent of germination as a function of formaldehyde concentration is presented in Fig. 2. From such data it was possible to obtain an extrapolated value of approximately 0.8% as the level of aqueous formaldehyde required to completely inhibit germination of *B. subtilis* var. *niger* spores.

The spore suspensions containing the various formaldehyde concentrations (Fig. 1) were serially diluted and plated on Trypticase soy agar. The colony counts obtained from the spore suspensions exposed to aqueous formaldehyde were nearly a log lower than those obtained from the control spore suspension (12×10^7 spores per ml) not exposed to formaldehyde. For example, the 0.0938% formaldehyde solution, which allowed complete germination, yielded a spore concentration of 3.6×10^7 spores per ml, while the 1.5% formaldehyde solution, which caused complete inhibition of germination, yielded a spore concentration of 1.6×10^7 spores per ml. The remaining formaldehyde solutions had spore concentrations between these two values. The initial optical density of both the control and 0.0938% formaldehyde solution was 0.66 at 625 nm.

These results suggested that the inhibition of spore germination by aqueous formaldehyde was reversible, and a study to test this possibility was undertaken. *B. subtilis* spores were suspended in germinating medium containing no added formaldehyde (solution A) and 2.5% formaldehyde (solution B). After 1 hr, spore suspension B was passed through a membrane filter and suspended in germinating medium containing either a 2.5% formalde-

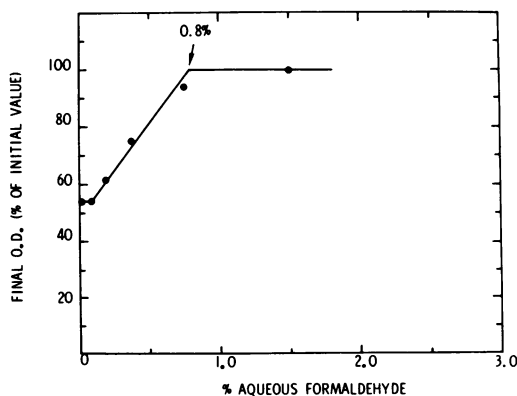


FIG. 2. Inhibition of spore germination by aqueous formaldehyde. Arrow indicates the extrapolated aqueous-formaldehyde concentration required for 100% inhibition of spore germination.

hyde (solution C) or no added formaldehyde (solution D). Fig. 3 shows that the inhibition of spore germination by aqueous formaldehyde is reversible, since the resuspended spores germinated in media containing no formaldehyde, whereas the spores resuspended in media containing formaldehyde did not germinate. The initial optical density of both solutions A and D was 0.61, whereas the viable spore concentration in solution A was determined to be 9.4×10^7 spores per ml and that of solution D was 4.5×10^5 spores per ml. These results indicate that formaldehyde can exhibit both sporostatic and sporocidal properties since aqueous formaldehyde can reversibly inhibit spore germination (sporostatic property) and decrease survival levels (sporocidal property).

The sporocidal property of aqueous formaldehyde was observed to be strongly dependent on temperature; in fact, a synergistic response was observed for the inactivation of *B. subtilis* spores on exposure to aqueous formaldehyde at elevated temperatures. Heating an aqueous suspension of *B. subtilis* spores from 30 C to 60 C for 4 hr did not result in spore inactivation, whereas spores suspended in a 1% formaldehyde solution exhibited less than a log reduction in population after 4 hr at 24 C. However, the combination of 1% formaldehyde and heat (30 to 60 C) resulted in the extensive spore inactivation illustrated in Fig. 4. The inactivation of spores by heat and dilute formaldehyde may be characterized as a synergistic

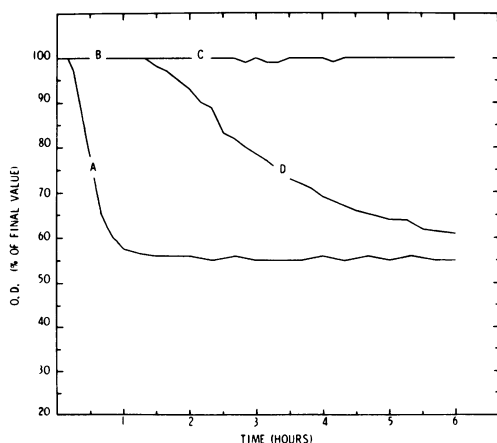


FIG. 3. Reversibility of the aqueous formaldehyde-induced inhibition of spore germination. Spores were suspended in Trypticase soy broth with (A) no additive and (B) 2.5% aqueous formaldehyde. After 70 min, spore suspension B was passed through a membrane filter and resuspended in Trypticase soy broth containing (C) 2.5% aqueous formaldehyde and (D) no additive.

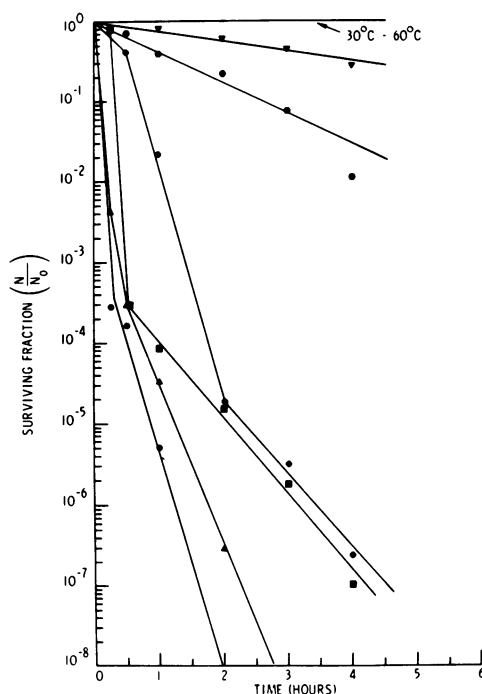


FIG. 4. Inactivation of *B. subtilis* spores suspended in 1% formaldehyde and heated at 24 C (▼), 30 C (●), 40 C (◆), 50 C (■), 55 C (▲), and 60 C (●). Heating aqueous spore suspensions from 30 C to 60 C for four hours did not inactivate the *B. subtilis* spores. N/N_0 refers to the ratio of viable spores at a given time (N_0).

inactivation, because the rate of inactivation for spores exposed to both agents simultaneously is greater than the rate of spore inactivation due to heating at a given temperature in the absence of aqueous formaldehyde plus the inactivation rate due to 1% formaldehyde at 24 C. Those spores suspended in 1% formaldehyde at a concentration of 10^8 spores per ml were completely inactivated on heating to 55 or 60 C for 4 hr. Sterility was determined by direct colony count and incubation in broth for 10 days, as described previously. Dilution bottles containing either 0.5% sodium sulfite or ammonium chloride were used to serially dilute spore suspensions in two different experiments, in an effort to neutralize the formaldehyde and, perhaps, reverse the formaldehyde-induced inactivation. No difference in survival level was observed between these experiment and those in which formaldehyde-treated spores were not exposed to these chemicals.

DISCUSSION

A previous study established that the germi-

nation of bacterial spores can be inhibited reversibly by low concentrations of either aliphatic or aromatic alcohols (5). The present investigation indicates that aqueous formaldehyde can also reversibly inhibit the spore-germination process. Structurally and chemically an aldehyde is different from an alcohol, yet both appear to affect spore germination in a similar manner. Insight into why formaldehyde should act as an alcohol in its effect on spore germination may be obtained from basic chemical considerations. Gaseous formaldehyde rapidly reacts with water to form a monohydrate, methylene glycol, $\text{CH}_2(\text{OH})_2$, which subsequently forms a series of low-molecular-weight, polymeric hydrates or poly-methylene glycols, having the type formula, $\text{HO}(\text{CH}_2\text{O})_n\text{H}$. The reaction of formaldehyde with water is shown in Fig. 5.

It has been determined (6) that at 30 C a 2%-formaldehyde solution contains 0.001% formaldehyde monomer and 99.999% methylene glycol and polymers of methylene glycol. In other words, formaldehyde in water is 99.999% in the form of a di-alcohol (methylene glycol) or polymeric di-alcohols. Therefore, formaldehyde acts like an alcohol in reversibly inhibiting spore germination because, in aqueous solution, formaldehyde exists essentially as a di-alcohol. That di-alcohols per se can inhibit spore germination was shown in a study in which ethylene glycol (10% v/v) reversibly inhibited the germination of *B. subtilis* var. *niger* spores.

The contention that aqueous formaldehyde reversibly inhibits spore germination (Fig. 4), because in solution formaldehyde essentially exists as a di-alcohol and alcohols are known to reversibly inhibit spore germination, invites interesting comparisons. For example, Table 1 compares the physical and sporostatic properties of ethanol and aqueous formaldehyde (methylene glycol). Calculations based on the data in Table 1 reveal that nearly identical amounts of ethanol and methylene glycol are required to inhibit the germination of a single *B. subtilis* var. *niger* spore (column A). This result suggests that these additives, of comparable molecular weight, steric volume, and chemical substituents, are interacting with

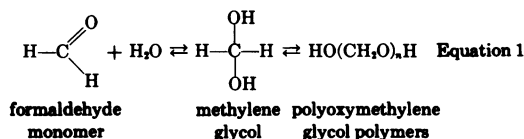


FIG. 5. Reaction of formaldehyde with water.

TABLE 1. Comparison of the physical and sporostatic properties of ethanol and methylene glycol

Additive	Structure	Mol wt	Germination inhibiting concn	Column A ^a
Ethanol	$\text{CH}_3-\text{CH}_2-\text{OH}$	46.07	1.4% ^b	1.46×10^{12}
Methylene glycol	$\text{HO}-\text{CH}_2-\text{OH}$	48.03	0.8%	1.0×10^{12}

^a Figures in this column refer to the calculated number of atoms of additive required to inhibit one *B. subtilis* var. *niger* spore. Calculated from the germination-inhibiting concentration, inhibited spore concentration (1×10^8 spores per ml), and Avogadro's number.

^b Data obtained from reference 5.

the spore in the same manner to inhibit spore germination, perhaps by combining with spore enzyme(s) required for spore germination. The observation that the rate of reversal of spore inhibition by aqueous formaldehyde is approximately five times slower (Fig. 3) than that observed for ethanol suggests that, whereas both additives may be interacting with the same spore component and with the same stoichiometry, they do so in different manners.

A further difference between alcohol and aqueous formaldehyde is that exposure of spores to formaldehyde results in decreased spore survival levels, whereas exposure of spores to alcohol is not sporocidal. For example, spore suspensions A and D in Fig. 4 both achieved essentially the same extent of germination, and yet the viable spore concentration in solution A was 9.4×10^7 spores per ml and that of solution D was 4.5×10^5 spores per ml. Both of these spore suspensions had the same initial optical density of 0.61. These two observations, (i) that the inhibition of germination occasioned by a formaldehyde solution is fully reversible on removal of the aqueous formaldehyde from the spore environment, and (ii) that the survival level of the formaldehyde-treated, resuspended spores (suspension D) was more than 100 times lower than the control levels, strongly imply that the sporostatic and sporocidal properties of aqueous formaldehyde are not related.

The equilibrium between formaldehyde and its hydrate forms (see Fig. 5) may also serve as the basis for understanding the synergistic inactivation of spores by heat and aqueous formaldehyde (Fig. 4). It is suggested that, whereas the inhibition of spore germination is

due to methylene glycol, the inactivation of spores is a result of an irreversible reaction between the free formaldehyde monomer present in a formaldehyde solution and the spores suspended in such a solution. The equilibrium between formaldehyde monomer and its diol form in water (methylene glycol) is not greatly affected by changes in temperature up to 60 C (6). Therefore, the extreme temperature dependence observed for the inactivation of spores in aqueous formaldehyde may be a result of the increased rate of reaction between free formaldehyde monomer and the spores with increasing temperature. Consistent with such an interpretation is the observation that spores suspended in 1% formaldehyde solution and heated to 50, 55, or 60 C all exhibited approximately the same survival level after treatment for 30 min, and thereafter had different rates of inactivation (Fig. 4). This result can be understood if one assumes that sufficient thermal energy was present at 50 to 60 C to allow the equilibrium concentration of formaldehyde monomer to be completely utilized in combining with and inactivating a certain fraction of the total spore population. The subsequent differences in spore inactivation rate from 50 to 60 C could then reflect the influ-

ence of temperature on the reestablishment of the equilibrium between formaldehyde monomer and methylene glycol in an aqueous formaldehyde solution.

ACKNOWLEDGMENTS

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