

Temperature-Induced Changes in the Sporicidal Activity and Chemical Properties of Glutaraldehyde

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Freshly prepared 2% acid and alkaline glutaraldehyde solutions were stored at 4, 20, and 37 C. At intervals, samples were removed and changes in pH, ultraviolet spectrum, and sporicidal activity (against *Bacillus pumilus* spores) were recorded. Alkaline solutions stored at 4 C showed little changes in these properties, whereas such solutions stored at 37 C became turbid and showed a decrease in pH, marked changes in ultraviolet spectrum, and an almost complete loss of sporicidal activity. Intermediate results were obtained with alkaline solutions stored at 20 C. In contrast, acid 2% glutaraldehyde solutions (initial pH 3.5) showed comparatively few changes in their properties. Treatment of spores with freshly prepared glutaraldehyde solutions (0.5%) at temperatures above 40 C reduced the effect of pH on sporicidal activity.

Glutaraldehyde is an important microbicidal agent which is lethal at alkaline pH to bacteria, bacterial spores, fungi, and viruses (1, 4, 5-7, 9-12; S. Thomas and A. D. Russell, *J. Appl. Bacteriol.*, in press), although specific tests conducted question tuberculocidal activity (9, 10). Solutions of the dialdehyde are stable at acid pH, but when "activated" for use at alkaline pH their shelf life is short (1, 12). We have been interested in the effects of temperature on the chemistry, physical properties, and sporicidal activity of glutaraldehyde, and results of these findings are presented in this paper.

MATERIALS AND METHODS

Glutaraldehyde solutions. Cidex, a 2% solution of glutaraldehyde, was kindly provided by Arbrook, Inc., Arlington, Tex. It was stored at 4 C. When required, 2 × 300-ml volumes were removed, one 300-ml portion being made alkaline by the addition of 0.3% (wt/vol) sodium bicarbonate. The 300-ml volumes of acid and alkaline solutions were subsequently stored at 4, 20, and 37 C, and at intervals samples were removed and monitored for pH, spectrophotometric analysis, and sporicidal activity against *Bacillus pumilus* spores, as described below.

Spore preparation. Nutrient agar (Oxoid, Ltd., London, England) in Roux flasks was seeded with *B. pumilus* ATCC 27142 (E.601) and incubated for 5 days at 37 C. The growth was washed off the surface with sterile glass-distilled water and centrifuged, and the pellet was washed three times with sterile water and finally resuspended in sterile water to give 10¹⁰ to 10¹¹ spores/ml. No preheating of the spore suspensions

was made prior to sporicidal tests, since this may result in increased spore sensitivity to antibacterial agents (10).

Sporicidal tests. A suitably diluted spore suspension (1 ml) was added to 2% alkaline glutaraldehyde (previously stored for the desired period at 4, 20, or 37 C) to give an initial spore level of approximately 10¹⁰/ml. Samples were removed at intervals and, after dilution of glutaraldehyde to a sub-inhibitory level, viable survivors were determined by means of a plating method.

The same procedure was adopted with the 2% acid glutaraldehyde solutions (previously stored for the desired period at 4, 20, or 37 C). Additional sporicidal tests were also made with these solutions, which were made alkaline by addition of 0.3% sodium bicarbonate immediately prior to inoculation with spores.

pH changes. Changes in pH during storage at 4, 20, and 37 C of the 2% acid and alkaline glutaraldehyde solutions were recorded with the Cambridge pH meter.

Spectral changes. Changes in the ultraviolet (UV) spectrum of the 2% acid and alkaline glutaraldehyde solutions during storage were recorded in the Unicam SP 800 spectrophotometer, using 1-cm cells and distilled water as the blank. The glutaraldehyde solution was diluted four times before examination. In some experiments, 0.5% solutions of acid and alkaline glutaraldehyde were allowed to equilibrate at elevated temperatures (40 to 95 C), and the absorbance was measured at 235 and 280 nm in a Hilger and Watts spectrophotometer fitted with a water-jacketed cell carrier.

Sporicidal activity of glutaraldehyde at elevated temperatures. Acid or alkaline glutaraldehyde solutions (0.5%) were held at the desired temperature until equilibrium had occurred. They were then

inoculated with bacterial spores, and samples were removed at intervals for viable counts as previously described.

RESULTS AND DISCUSSION

pH changes. Changes in pH of 2% alkaline glutaraldehyde solutions during storage at 4, 20, and 37 C are shown in Table 1. At 4 C, there was no change in pH over the 21-day period, a slight decrease with the solution stored at 20 C, and a more marked pH decrease with the solution stored at 37 C. In contrast, 2% acid solutions (initial pH 3.5) stored for 3 months at 4, 20, and 37 C had pH values of 3.6, 3.6, and 3.3, respectively, at the end of this period.

Spectral changes. Ultraviolet spectrum changes of 0.5% alkaline glutaraldehyde solutions during storage at 4, 20, and 37 C are indicated in Fig. 1, which demonstrates that the solution stored at 4 C (Fig. 1a) shows virtually the same spectrum after 4 weeks. In contrast, there are some changes in the spectrum of the solution stored at 20 C (Fig. 1b) and very marked alterations in the solution stored at 37 C (Fig. 1c), with a shift in the peak from 280 nm to one of slightly higher wavelength.

Acid 0.5% glutaraldehyde solutions stored at 4, 20, and 37 C showed a different response pattern (Fig. 2), in that negligible changes occurred at 280 nm even after a prolonged storage for several months at any one of these temperatures. There were, however, increases in the absorbance at 235 nm with increases in the storage period, especially at 37 C (Fig. 2c) and, to a lesser extent, at 20 C (Fig. 2b). In these cases, therefore, there was a significant increase in the 235 nm/280 nm ratio, which is believed to be an indication of increasing polymerization (8).

Sporicidal activity. Two-percent alkaline glutaraldehyde, as a freshly prepared solution, is rapidly lethal to *B. pumilus* spores at 37 C (Fig. 3). In contrast, alkaline solutions stored at 37 C became cloudy and within a short storage period were no longer very actively sporicidal,

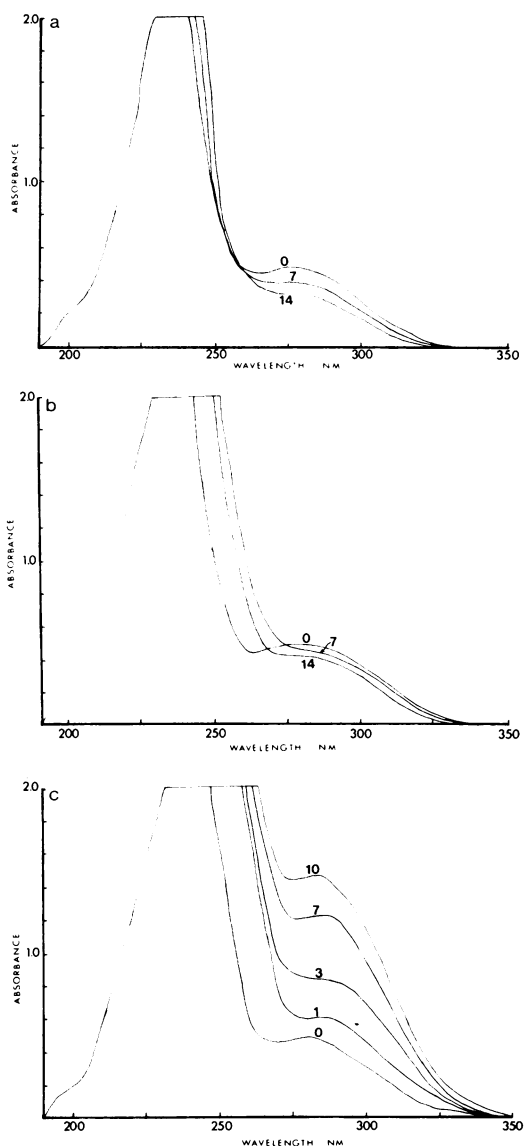


FIG. 1. Ultraviolet spectrum changes of alkaline 0.5% glutaraldehyde during storage at different temperatures. (a) 4 C, (b) 20 C, (c) 37 C. Numbers represent weeks at a particular temperature.

TABLE 1. pH changes in 2% alkaline glutaraldehyde solutions stored at different temperatures

Storage temp	Day					
	0	1	8	10	14	21
4 C	7.9	7.8	7.9	7.8	7.8	7.8
20 C	7.9	7.7	7.65	7.65	7.5	7.5
37 C	7.9	7.3	7.17	7.1	7.0	6.9

whereas alkaline solutions stored at 4 C maintained their stability and anti-spore activity for considerably longer periods (Table 2). Intermediate results were obtained with alkaline solutions held at 20 C.

Glutaraldehyde solutions are considerably less sporicidal and bactericidal at acid pH than at alkaline pH (1, 5, 8, 11, 12), and this point is again illustrated in Fig. 3, which shows the sporicidal activity of acid and alkaline 2%

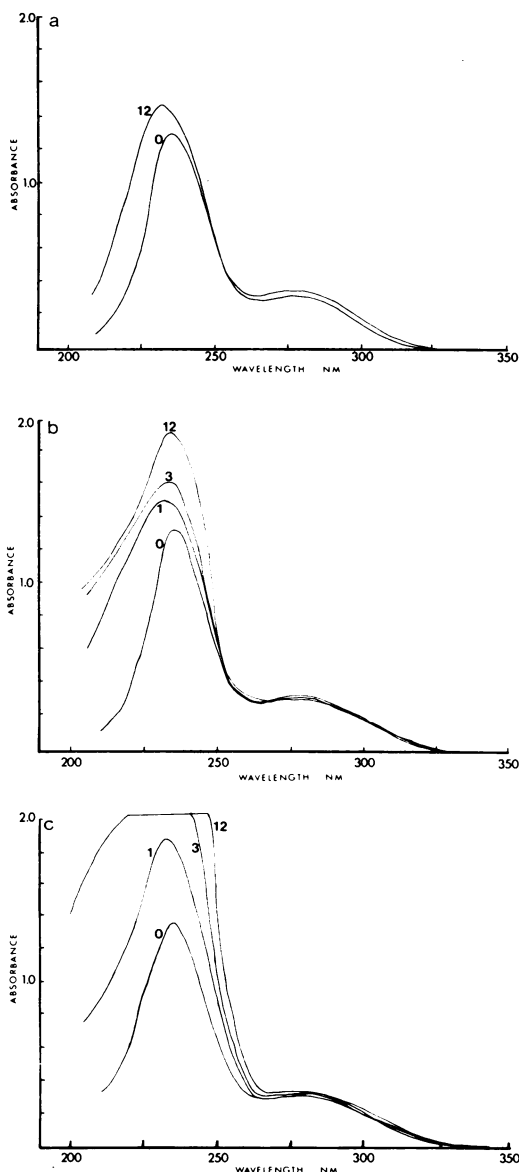


FIG. 2. Ultraviolet spectrum changes of acid 0.5% glutaraldehyde during storage at different temperatures. (a) 4 C, (b) 20 C, (c) 37 C. Numbers represent weeks at a particular temperature.

solutions at 37 C. Thomas and Russell (J. Appl. Bacteriol., in press) have previously shown that, at 20 C, 2% alkaline glutaraldehyde is considerably more lethal than a 2% acid solution, which exerts little sporicidal activity against these spores over a 60-min period at this lower temperature. If, as seems possible, glutaraldehyde acts as a protein cross-linking agent on the surface layers of microbial cells (6, 7, 12), then it

would be expected that this would occur more rapidly as the pH increases (2, 3). Storage of acid glutaraldehyde at 4, 20, or 37 C neither increased nor decreased the comparatively low level of sporicidal activity. In contrast, these stored acid solutions made alkaline immediately prior to spore inoculation were highly sporicidal, the viable spores per milliliter after 10 min of contact at 37 C being 6×10^3 , 3×10^2 , and 1×10^8 for solutions stored at 4, 20, and 37 C, respectively.

One of the objections to using glutaraldehyde as a disinfectant has been its comparatively short shelf life after "activation." Storage of activated solutions at 4 C overcomes this criticism, and alkaline solutions are still highly active sporicidally even after storage for 18 months at this low temperature (Table 2).

Sporicidal activity and spectral changes at elevated temperatures. Figure 4 shows the effects of 0.5% acid or alkaline glutaraldehyde on bacterial spores at elevated temperatures. It is significant at temperatures above 40 C that the discrepancy in sporicidal activity between alkaline and acid glutaraldehyde is reduced, although it does not entirely disappear. There

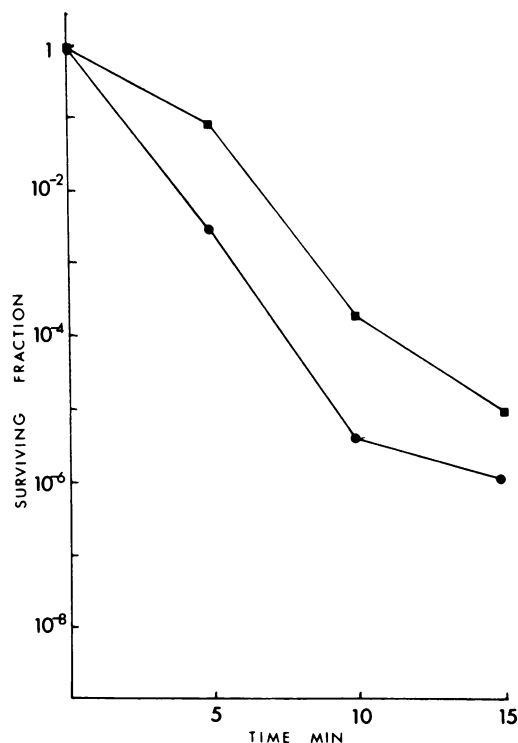


FIG. 3. Sporicidal activity of 2% alkaline (●) and acid (■) solutions of glutaraldehyde against *B. pumilus* spores at 37 C.

TABLE 2. Sporicidal activity of 2% alkaline glutaraldehyde^a after storage at different temperatures

Storage temp	Period of storage	No. of <i>B. pumilus</i> spores per ml ^b after treatment at 37 C for		
		10 min	20 min	30 min
Control ^c				3×10^7
4 C	1 week	3.5×10^6	7×10^3	< 10
	3 weeks			
	7 weeks			
	14 weeks			
	18 months			
20 C	1 week	1.4×10^7	3.5×10^5	< 10
	3 weeks			
	7 weeks			
	14 weeks			
	18 months			
37 C	1 week	1.4×10^7	2.2×10^7	5×10^5
	3 weeks			
	7 weeks			
	14 weeks			
	18 months			

^a Solutions of glutaraldehyde (2% alkaline) were stored at various temperatures; samples were removed, allowed to equilibrate at 37 C, and then tested for their sporicidal activity at 37 C.

^b Number at 0 min in each case was 3×10^7 /ml.

^c Control in absence of glutaraldehyde.

are two possibilities to account for this: (i) the higher temperatures cause some changes in the spores enabling greater penetration by the aldehyde, or (ii) changes in the dialdehyde molecule at alkaline pH occur on exposure to higher temperatures. Although the former may be a contributory factor, we have found a marked difference in the spectrophotometric properties of the acid and alkaline forms of glutaraldehyde with respect to temperature. Under acid conditions, the absorbance peak at 280 nm for glutaraldehyde increases markedly with increasing temperature (range tested, 40 to 95 C), whereas that at 235 nm remains relatively constant. Under alkaline conditions, however, the absorbance peaks at both 235 and 280 nm are greatly increased, the latter to the same extent as for the acid form. It has also been noted that the effect of temperature is reversible in the case of the 280-nm peak, but irreversible with the 235-nm peak when the alkaline glutaraldehyde is cooled to room temperature and reexamined. These results may appear to disagree with those of Rasmussen and Albrechtsen (8); however, the latter authors used long storage periods (up to 20 weeks).

Thus, the differences in the properties of the two solutions could form a possible basis for

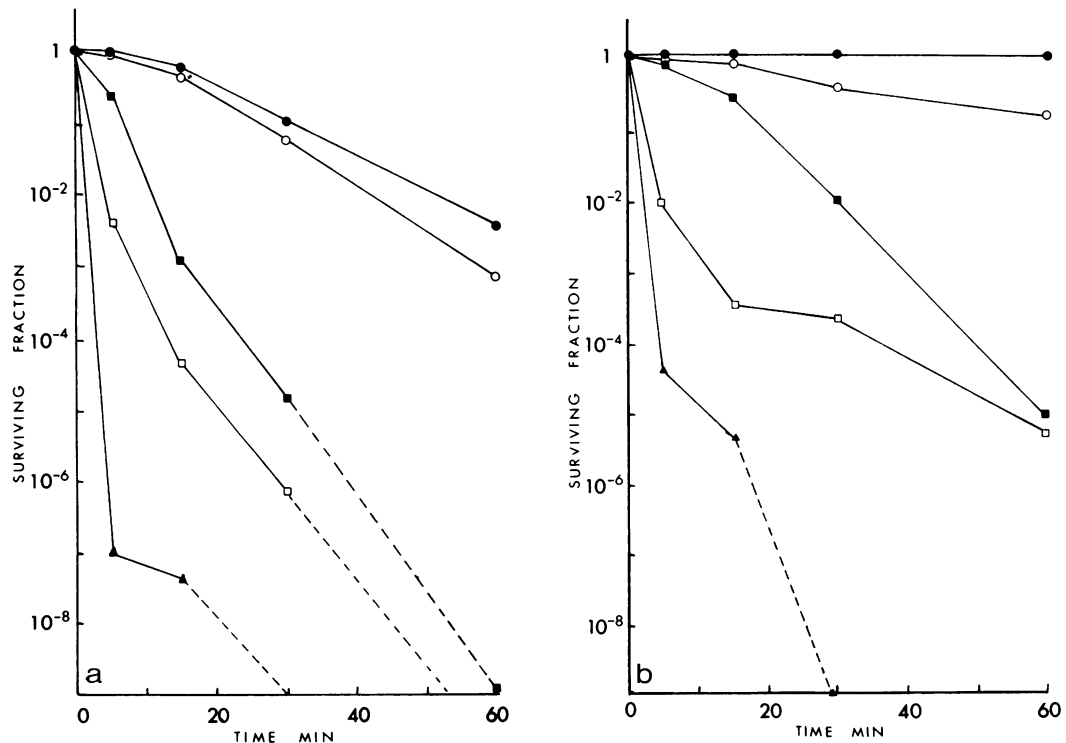


FIG. 4. Effect of elevated temperatures on the sporicidal activity of 0.5% solutions of (a) alkaline and (b) acid, glutaraldehyde against *B. pumilus* spores. Temperatures: ●, 20 C; ○, 30 C; ■, 40 C; □, 50 C; ▲, 60 C.

explaining the differences in the sporicidal and bactericidal activity of glutaraldehyde at acid and alkaline pH.

Overall comments. Two-percent alkaline glutaraldehyde is considerably more potent than acid glutaraldehyde as a sporicidal agent at 20 C (Thomas and Russell, *J. Appl. Bacteriol.*, in press), but increasing temperature reduces the difference; above approximately 40 C there is little difference between 0.5% acid and alkaline glutaraldehyde solutions acting as sporicidal agents over a 60-min period. This conclusion supports that reached by Sierra and Boucher (11). There are, however, marked chemical changes which occur in alkaline glutaraldehyde solutions stored for periods of 3 weeks or more at 37 C, and these changes are reflected in a complete loss of sporicidal activity.

The results also demonstrate the need for further research to be carried out on the chemical nature of the glutaraldehyde molecule, especially in relation to the nature and level of its sporicidal activity.

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