Inactivation of Enterovirus by Glutaraldehyde

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A study on the rate of inactivation by glutaraldehyde of coxsackievirus was conducted using different concentrations, temperatures, and pH values. It was found, that 2% glutaraldehyde at pH 7.4 and 25°C, as recommended for a sporicide, reduced the titer of infectious virus by 2 log₁₀ U in 1 min or less. The reduction was not negatively affected by high concentrations of organic matter (serum and animal spillings) in the reaction mixtures.

Glutaraldehyde has been studied (3, 4) as a disinfectant against a number of bacteria and bacterial spores, but the literature on the effect on viruses seems very limited (5–7). Prompted by the need for disinfectants for use where chlorine compounds and formalin seems less desirable, the present study was carried out using an apparently very stable strain of coxsackievirus B3.

The effect of glutaraldehyde was studied at different pH values, at different temperatures, in the presence or absence of serum, and using different concentrations. Each of these parameters was followed keeping the others at constant value. Thus a rather intensive practical study was carried out, but no attempt to elucidate the nature of the inactivating process was made.

MATERIALS AND METHODS

Glutaraldehyde. Glutaraldehyde (Merck no. 820603) was used in the form of a 25% solution in water.

Virus. A strain of coxsackievirus B3, which was isolated from Copenhagen sewage on several occasions, was used. The stock suspension had a titer of around 10⁸ mean infectious doses (ID₅₀)/ml, and the mixtures of glutaraldehyde and virus employed had initial virus titers of around 10⁻⁴ ID₅₀/ml.

Virus titrations. Virus titrations were carried out in HeLa cell tube cultures using 2 ml of medium per tube. The medium consisted of Hanks solution to which was added 0.5% lactalbumin hydrolysate, 2% calf serum, and antibiotics (100 IU of penicillin and 100 μg of streptomycin per ml). The calf serum employed did not contain neutralizing antibodies or other inhibitory effect against coxsackievirus. The titrations were carried out using 10-fold dilutions and inoculating 0.1 ml of each dilution in three cell cultures. Final readings of cytopathic effect were made on day 7 after the inoculations. Titration accuracy was estimated to around 10⁻³ ID₅₀.

Experimental procedure. The effect of glutaraldehyde on the virus was studied by following the rate of inactivation at pH 5, 7.4, and 9. For each pH value the inactivation was followed at a temperature of 5, 25, 37, and 50°C. For each pH temperature combination, series of experiments were established using three, or sometimes only two, different concentrations of glutaraldehyde in the range 0.1 to 2.0%. For each series a control without glutaraldehyde was used. Each concentration was employed in a mixture using tap water or 10% calf serum in medium 199 as a diluent for the stock suspension of virus. This was diluted 1:100 in the experimental mixtures. For all mixtures the rate of virus inactivation was determined by sampling for virus infectivity at different times after exposure to glutaraldehyde. The residual amounts of infective virus were measured at five or more time points after the initial exposure to glutaraldehyde.

The rates of virus inactivation. Virus inactivation rates were obtained by plotting the titration results against time for all individual experiments and reading the slope of the curve.

RESULTS

With a few exceptions, the rate of virus inactivation and its dependence on temperature, pH, the presence of high concentrations of organic matter (10% serum), and concentration of glutaraldehyde were determined. When the results of the individual experiments were plotted to determine the rate of inactivation, a reasonable approximation of a first order reaction was found during the first 2.0 to 2.5 log₁₀ U of decrease in virus infectivity.

When 0.1% glutaraldehyde was employed, the results were not reproducible and the rate of virus inactivation deviated so much from a first order reaction that it seems that the glutaraldehyde was not present in a constant concentration for more than the beginning of the inactivation period. Therefore the results obtained employing 0.1% were disregarded and not included in Fig. 1.
A general limitation of the tests is that the virus titers could not be followed below a value of approximately $10^4.5$ ID$_{50}$ because the glutaraldehyde proved to be toxic to the cells when the concentration inoculated was around 0.005%. On the other hand, it was desired to obtain virus suspensions which were truly different in composition. Therefore 1:100 dilutions of virus suspensions were employed as reaction mixtures. In this way the range of virus titers available for study in the experiments was limited to $10^4.5$ ID$_{50}$ and 3 log$_{10}$ units below this value. On several occasions cultures which were registered as toxically destroyed were assayed for virus, but in no such case was virus demonstrated. This confirmed that the cellular changes observed were caused by glutaraldehyde and not by virus. The results were expressed as the time in minutes for a 2 log$_{10}$ U reduction from the initial virus titer. The time estimates thus obtained are compiled in Fig. 1A through C. From the figures the following may be observed.

(i) Effect of pH. Except at 37°C and in the presence of high amounts of organic matter, where glutaraldehyde apparently functioned slightly faster at pH 9 than at pH 7.4 (i.e., if 10% calf serum was added to the virus-glutaraldehyde mixture), the rate of virus inactivation was somewhat higher at pH 7.4 than at pH 9. On the acid side a very pronounced decrease in the rate of virus inactivation was observed at all temperatures and concentrations studied. The rate of inactivation was about 10 times faster at pH 7.4 than at pH 5.

(ii) Effect of temperature. For all experiments presented in Fig. 1, the corresponding control virus suspension without glutaraldehyde added remained unchanged in titer during the experiment. The rate of inactivation was in general around three times as fast at 25°C than at 5°C, whereas the change in rate seems somewhat faster between 25°C and 37°C.

(iii) Effect of glutaraldehyde concentration. The rate of inactivation seems roughly proportional to the concentration employed when 0.5, 1.0, or 2.0% glutaraldehyde were compared under otherwise identical conditions.

(iv) Effect of serum presence. The efficiency of glutaraldehyde was not negatively affected by the presence of 10% serum and amino acids in the reaction mixture (Fig. 1). The opposite effect was observed; in all cases the rate of inactivation was faster in the presence of 10% serum than in the corresponding reaction mixture without serum.

Because of the importance of the observation regarding the positive effect of organic matter on the inactivating capacity of glutaraldehyde, an additional experiment was carried out employing the following reaction mixtures. The stock virus suspension was diluted 1:100 in (i) distilled water, (ii) tap water, (iii) 10% serum in Hanks solution, and (iv) 20% mouse spillings in Hanks solution. In all four cases 0.5% glutaraldehyde was added, and the pH was adjusted to

![Fig. 1](image-url)
Fig. 2. The rate of inactivation of coxsackievirus B3 using 0.5% glutaraldehyde at pH 7.4 and 25 C. Symbols: x, control virus titrations; O, distilled water as diluent; ®, tap water; A, 10% calf serum in Hanks’ solution; ▲, 20% mouse spillings in Hanks’ solution; A shows that A and ▲ coincide. The stipled line indicates the level of cell toxicity of the glutaraldehyde, thus the last points of each group do not indicate virus activity and are not included in the curves.

7.4. The result of this experiment are shown in Fig. 2. The virus suspended in distilled water was not inactivated in 60 min, whereas the rates for groups (ii) and (iii) corresponded to that previously found (Fig. 1), and the virus suspended in mouse spillings was even faster inactivated than the other groups.

DISCUSSION

Klein and Deforest (5) studied the inactivation of seven different viruses using, among other substances, bicarbonate-buffered glutaraldehyde. Their description is very brief, but it may be concluded that at room temperature and presumably at pH 7.4 all viruses examined were inactivated by 2% glutaraldehyde. This result is in agreement with our results.

We demonstrated that the pH affects the virus inactivation in much the same way as indicated for bacteria (4), i.e., at pH 5 the rate of inactivation is much lower than at pH 7.4, whereas the rate was little affected when the pH was raised from 7.4 to 9.

Although it thus seems possible to disinfect in an alkaline milieu, the instability reported (2, 4) for glutaraldehyde at a high pH value should be kept in mind.

The temperature dependence for the rate of inactivation, which was found in the range of 5 to 37 C, seems quite ordinary for a chemical process. The apparently faster increase per degree between 25 and 37 C than between 5 and 25 C is also a common occurrence for virus inactivation.

None of the publications cited contain information on the kinetics of the inactivation, but the results of the present report indicate that the reaction, with some approximation, follows a first order reaction pattern with a linear relationship between time and reduction in log units of active virus. The deviations become, however, very pronounced when the reactions have run over more than 2 log units of virus, and sometimes before. This is a common occurrence for rates of inactivation by heat, formalin, oxidation, etc. The conclusion should, however, be that minimal time periods for disinfection with a certain concentration of the chemical in question should be indicated with due regard to these deviations, and a suitable number of minutes should be added for safety.

In the present work a limitation in the kinetic studies was imposed by the cell toxicity of the glutaraldehyde, so that the rate of inactivation of the virus could not be followed beyond what corresponds to a glutaraldehyde concentration estimated to be approximately 0.005%. Graham and Jaeger (4) apparently did not encounter the same problem by inoculation of glutaraldehyde-treated material in mice, but in their work the treated tissue was washed three times before inoculation. In the work of Klein and Deforest (5) the problem was mentioned briefly: “germicide carried over in the early dilutions was frequently toxic for tissue culture cells, and thus we could not determine complete viral inactivation.” Therefore, caution must be recommended because the deviations from straight line relationship is further motivated by the cell toxicity problem. In the work of Stonehill et al. (7), the toxicity for mice and rats was studied and also the effect on skin, etc. The conclusion was that the compound is “slightly to moderately toxic and should be handled with the same precautions as for the safe use of formaldehyde.”

The practical use of a number of potentially potent disinfectants is limited or made completely impossible by the adverse effect of organic material. This is especially a problem to varying degrees with the halogens or halogenated substances. We consistently found that
the rate of inactivation in the presence of serum was faster than when the diluent was plain tap water. Not only serum but also animal spillings seem to accelerate the inactivation caused by glutaraldehyde. For its use as a disinfectant this is a very important advantage. The cautious conclusion seems to be that glutaraldehyde works as a disinfectant even in presence of high amounts of organic matter. From this it would be dangerous to conclude that it could be relied upon to disinfect soiled utensils without previous cleaning because the function as a fixative for proteins may make penetration of glutaraldehyde less efficient. The results of Graham and Jaeger (4) indicate, however, that an exposure for 2 h at 20°C was sufficient to allow penetration of glutaraldehyde into diced tissue (1 to 2 mm) of brain and liver.

The enteroviruses belong to the more stable viruses difficult to inactivate by chemical means. It is thus not surprising that Klein and Deforest (5) found a higher efficiency of glutaraldehyde toward adenovirus, herpesvirus, influenza virus, and even poxvirus, than against polio-, echo-, and coxsackievirus. The virus used in the present work was selected among the enteroviruses as a very stable strain. The coxsackievirus in question has been found repeatedly in domestic sewage, where it has resisted biological treatment and chlorination.

Although an enterovirus must be considered a reasonable indicator virus for chemical disinfection, it seems likely that papovaviruses (1) and parvoviruses (1) are even more stable, but they are more difficult to handle. If a study of a parvovirus were included, there is no guarantee that the human hepatitis viruses might not be more resistant. Consequently, it seems for the time being reasonable to carry out studies using enteroviruses and interpret the results with caution. It seems advisable to increase the time allowed in disinfection rather than the concentration used, since glutaraldehyde seems stable under the conditions used, rather expensive, and somewhat toxic (2).

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