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Activity of Glutaraldehyde at Low Concentrations (<2%) against Poliovirus and Its Relevance to Gastrointestinal Endoscope Disinfection Procedures

JEAN-LUC BAILLY,[1]* MARTINE CHAMBON,[1] HELENE PEIGUE-LAFEUILLE,[1] HENRI LAVERAN,[2] CHRISTOPHE DE CHAMPS,[2] AND DANIEL BEYTOUT[2]

Laboratoire de Bactériologie-Virologie[1] and Laboratoire d'Hygiène Hospitalière,[2] Faculté de Médecine et Pharmacie, 28 Place Henri-Dunant, 63001 Clermont-Ferrand Cedex, France

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The activity of glutaraldehyde (GTA) at low concentrations (<2%) against poliovirus was assessed by a suspension procedure. The inactivation kinetics showed that concentrations of ≤0.10% were effective against purified poliovirus at pH 7.2; a 1 $\log_{10}$ reduction was obtained in 70 min with 0.02% GTA, and a 3 $\log_{10}$ reduction was obtained in 30 min with 0.10% GTA. GTA activity at low concentrations was greatly enhanced at alkaline pH, but was completely abolished at acid pH. In contrast, the inactivation assays on poliovirus RNA showed that it was highly resistant to GTA at concentrations up to 1.0% at pH 7.2. At pH 8.3 a low inactivation was noticed with 1.0% GTA. Our results are of relevance to hospital practice in digestive endoscopy investigations because there has been an increasing tendency to use low concentrations of GTA and very short contact times in disinfection procedures.

Glutaraldehyde (GTA) is a saturated 5-carbon dialdehyde [CHO-$(CH_2)_3$-CHO], used since the early 1960s for disinfection and sterilization (for reviews, see references 10 and 29). The powerful activity of aqueous 2% alkaline GTA solutions (pH 7.5 to 8.5) against vegetative bacteria, fungi, and bacterial and fungal spores is now widely documented (for a review, see reference 10). Alkaline 2% GTA solutions have been shown to be good inactivators of viruses including enteroviruses (poliovirus, echovirus, and coxsackievirus) (22) and hepatitis A virus (26). These overall findings have resulted in the wide use of 2% GTA solutions for the cold sterilization of medical, surgical, and dental equipment which may be damaged by alternative chemicals or physical processes (29). In particular, they are preferred for the disinfection of gastrointestinal fiber-optic endoscopes (2, 25).

In recent years there has been a considerable increase in the number of digestive endoscopic examinations performed in patients infected with the human immunodeficiency virus. Hence, it has become even more essential that liquid disinfectants used for the disinfection of fiber-optic endoscopes have sufficient biocidal activity.

Two factors are of interest concerning this problem: the type of disinfectant to be used and the duration of contact of endoscopes with the disinfectant. There is a general agreement regarding the type of disinfectant; both the British and American Societies of Gastroenterology recommend the use of 2% alkaline GTA solutions in their endoscope disinfection protocols (1, 5). The contact time, however, is still under discussion, and recent international surveys on the disinfection procedures currently applied report an increasing tendency to reduce it to a few minutes (13).

When GTA was first used in disinfecting solutions, the main advantages claimed for its use were its broad spectrum of activity, its rapid microbicidal action, and its noncorrosive effect on metals, rubber, lenses, and other materials. In addition, it has been shown to be nonirritating to patients (29). Unfortunately, several recent studies have reported occupational skin and airway symptoms in endoscopy staff repeatedly exposed to 2% GTA solutions (15, 24). As a result, novel commercial formulations containing less than 2% GTA have recently been developed.

Both reduction of the contact times and reduction of the concentrations of GTA in the disinfectants are disturbing, because although low concentrations (<2%) of GTA have biocidal action against bacteria (10, 23) and spores (11, 28, 32), their effects on viruses have been studied to a far lesser extent. Hence, we have studied the virucidal action of GTA at low concentrations on poliovirus (Sabin strain), which is known to be very resistant to GTA-containing disinfectants (33). In this work, the ability of low concentrations of GTA to inactivate the infectivity of purified poliovirus was investigated. Some parameters of the inactivation process were determined at pH 7.2. The ability of GTA to inactivate poliovirus RNA infectivity was also examined.

MATERIALS AND METHODS

Virus and cells. Poliovirus type 1 (Sabin strain; World Health Organization Reference, Center for Enterovirus, Lyon, France) was used throughout this study. Vero cells, a continuous line of African green monkey kidney cells (Flow Lab. Inc., McLean, Va.), were grown in Eagle minimum essential medium (EMEM) (Flow Lab.) supplemented with penicillin (200 IU/ml), streptomycin (50 μg/ml), nonessential amino acids (1%), glutamine (1%), and 10% fetal calf serum (Boehringer, Meylan, France).

GTA preparation, storage, and control. Stock solutions of 10% GTA were prepared in phosphate-buffered saline (PBS) (Bio-Mérieux Lab.) from a 25% GTA solution (reagent grade I; SIGMA OSI, Paris, France). The stock solutions were divided into aliquots and stored at −20°C. The changes in the UV spectrum (220 to 300 nm) in these solutions were monitored during storage and before their use for inactivation assays.

* Corresponding author.

Virus growth and purification. Vero cell monolayers were inoculated with poliovirus at a multiplicity of infection of 10 to 20 MPNCU (most probable number of cytopathic units; see below) per cell. The inoculum was removed after 2 h of incubation at 37°C, and the monolayers were incubated again for 18 h with 15 ml of EMEM containing 2% fetal calf serum. The culture plates were frozen (−80°C) and thawed (+37°C), and the suspensions were pooled and centrifuged at 20,000 × *g* for 30 min at 4°C in an SW 28 rotor (Beckman). The pellets were resuspended in PBS, frozen, and thawed, and cellular material was removed by centrifugation. The supernatants were pooled, and the virus was pelleted by centrifugation in an SW 28 rotor at 125,000 × *g* for 4 h. The virus pellets were suspended in PBS and then purified by isopycnic centrifugation in CsCl in an SW 65 rotor (Beckman) at 165,000 × *g* for 40 h at 4°C. The virus-containing fraction was dialyzed against PBS for 24 h at 4°C. The virus titer was determined as described above, and the protein concentration was determined by the procedure of Lowry et al. (20). The purified virus suspension was diluted to 10^9 MPNCU/ml in PBS, divided into aliquots (20 μl), and stored at −80°C.

Virus titration. The endpoint dilution method was used for the virus titration assays. The concentration of cytopathic units was determined from the poliovirus cytopathic effect, observed on Vero cells cultured in 96-well tissue culture plates (Falcon 3072; Becton Dickinson). The plates were inoculated with a cell suspension containing 7×10^4 cells per ml (200 μl per well) and incubated under 5% CO_2 at 37°C for 24 h. The appropriate number of 10-fold virus dilutions was prepared in EMEM without fetal calf serum. The last 10-fold dilution was used to prepare seven 4-fold dilutions. Aliquots (50 μl) of these eight dilutions were dispensed into the microtiter plate wells at the rate of 11 replicates per dilution. EMEM plus 2% fetal calf serum was added (200 μl per well), and the plates were incubated at 37°C. The wells were scored microscopically as positive or negative for virus growth on days 4 and 5. The most-probable-number method was used to evaluate the number of cytopathic units (6). The MPNCU was calculated from a group of positive results obtained with four successive dilutions, using a computer program to solve the equation of "maximum likelihood" (17). The standard deviation (*S*) of the MPNCU was a constant and was calculated from the following formula: $S = 0.55 (\log d/N)^{1/2}$, where *d* is the dilution rate and *N* is the number of repetitions for each dilution (7). Confidence limits of the MPNCU ($P > 0.95$) were $\pm 1.96 \times S = \pm 0.25 \log_{10}$.

Poliovirus RNA transfections. Poliovirus RNA was phenol-chloroform extracted by a previously described method (19). The poliovirus RNA infectivity was then determined by transfection (34). Six 10-fold dilutions of RNA were performed in a volume of 100 μl, in HBSS buffer (50 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] per liter, 80 g of NaCl per liter, 0.37 g of KCl per liter, 0.125 g of $Na_2 HPO_4 \cdot 2H_2O$ per liter, 1.0 g of glucose per liter [pH 7.05]). An equal volume of a 1.0-mg/ml DEAE-dextran solution (Pharmacia Fine Chemicals, Uppsala, Sweden) was added to each dilution. The mixtures were stored at 0°C for 30 min. The recipient cells were cultured in six-well tissue culture plates (Falcon 3046; Becton Dickinson) for 24 h before the assays, to produce confluent monolayers. Before the transfection assays, the cell monolayers were washed twice with EMEM. Each mixture of RNA and DEAE-dextran was introduced onto the washed cell monolayers, maintained for 30 min at room temperature on the cells, and removed. The cells were then washed once and incubated with 4 ml of EMEM containing 2% fetal calf serum. The state of the cell monolayers and the degree of viral growth were monitored daily for 4 days and compared with those in cell controls treated and not treated with DEAE-dextran.

Inactivation assays. (i) Poliovirus inactivation. Purified poliovirus preparations were mixed with appropriate GTA dilutions to obtain final GTA concentrations of 0.02, 0.05, and 0.10% (wt/vol) and incubated in a water bath at 25°C. The treated preparations were then diluted 100-fold in cold EMEM (0°C) to stop the action of GTA on the virus. The viral infectivity was then determined from the diluted preparations.

(ii) RNA inactivation. RNA-containing solutions were prepared with phosphate buffers (0.01 M KH_2PO_4, 0.01 M NaH_2PO_4) at pH 7.2 and 8.3 and with HBSS buffer. RNA concentrations were determined by spectrophotometric analysis at 260 nm. The RNA was incubated with appropriate GTA dilutions to obtain final GTA concentrations of 0.2, 0.4, and 1.0% (wt/vol) at 25°C. After a 30-min incubation period, RNA was precipitated with 2.5 volumes of ethanol at −80°C. RNA was collected by centrifugation (20,000 × *g*) at 2°C for 30 min, washed once in 70% ethanol, dried, and resuspended in HBSS (100 μl). The inactivation rate was estimated by transfection and comparison with results for RNA preparations without GTA, treated under the same conditions.

RESULTS

Spectrophotometric control of GTA solutions. The UV spectrum (220 to 300 nm) was recorded to verify the chemical stability of the GTA solutions used. Temperature is known to enhance the formation of GTA polymers, which absorb at 235 nm, whereas monomers absorb more specifically at 280 nm (31). The UV absorbance ratio A_{235}/A_{280} was calculated and used as an index of the homogeneity of the GTA solutions. All the solutions used in the inactivation experiments had an index between 0.3 and 0.4. The variation in the A_{235}/A_{280} ratio was also studied by incubating GTA solutions at 25°C (Fig. 1). At pH 7.2, there was no variation in this ratio for a solution of GTA at 10.0%; for a solution of GTA at 1.0%, the variations were +0.06 and +0.12 after 2 and 4 h of incubation, respectively. The variation in the A_{235}/A_{280} ratio of a solution of GTA at 0.2% was almost the same as that of a 1.0% GTA solution. The 0.2% GTA solution at pH 8.3 was characterized by two differences from the solutions at neutral pH. First, the initial A_{235}/A_{280} ratio at pH 8.3 was nearly twice as high as for the solutions at pH 7.2. Second, the variations in this ratio were +0.32 and +0.63 after 2 and 4 h of incubation, respectively. Therefore, the variations observed in the GTA solutions at pH 7.2 can be considered negligible. However, it was not possible to ascertain whether these variations are identical when GTA is incubated with the virus. In contrast, the variations observed in the alkaline GTA solution were greater, indicating that changes in the chemical composition of GTA solutions take place during inactivation experiments.

Poliovirus inactivation kinetics with low concentrations of GTA. During preliminary assays we noticed slight differences in sensitivities to GTA among the various preparations of the poliovirus strain. Therefore, all the results presented in this study were obtained with a single stock of poliovirus. Aliquots of this stock used for inactivation experiments contained $10^{7.7}$ MPNCU in a volume of 20 μl, and the protein concentration was 200 μg/ml. Three kinetics experiments were performed with GTA at 0.02, 0.05, and 0.10% to

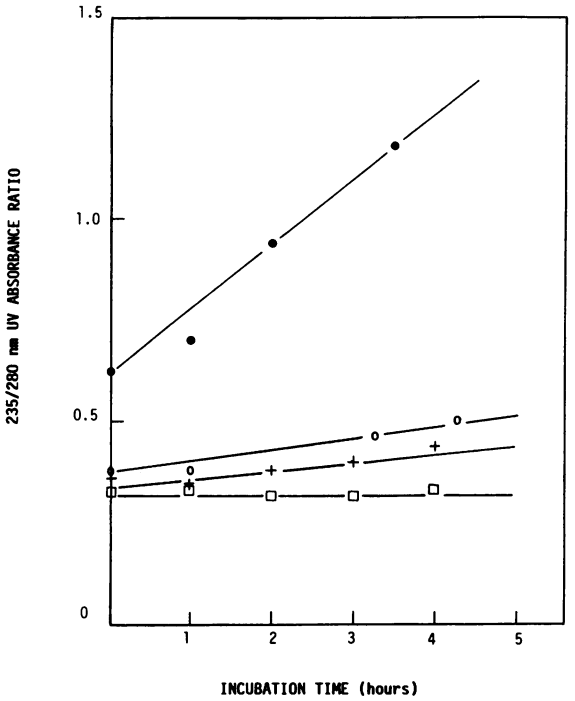

FIG. 1. Variation of absorbance of GTA solutions during incubation at 25°C. GTA solutions (pH 7.2) at 10.0% (□), 1.0% (+), 0.02% (○), and 0.02% (pH 8.3) (●) were incubated at 25°C. A_{235}/A_{280} ratios were calculated from UV spectra recorded between 220 and 300 nm.

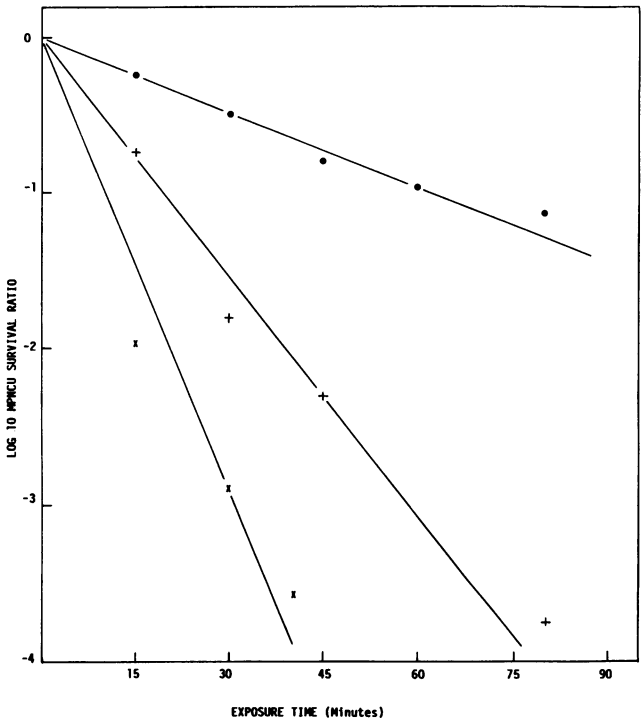

FIG. 2. Kinetics of inactivation of poliovirus type 1 with glutaraldehyde in PBS (pH 7.2). The virus-containing samples were incubated at 25°C with GTA at 0.02% (●), 0.05% (+), and 0.10% (x).

determine the poliovirus inactivation process (Fig. 2). Each point of the graph is the mean of 3 log reduction rates. The $\log_{10}$ reduction in the viral concentration was a linear function of the exposure time. The inactivation rates determined from the slopes of the three straight lines of Fig. 2 were plotted as a function of GTA concentration (Fig. 3). The inactivation rates increased linearly with the GTA concentration. Poliovirus inactivation by GTA is therefore a first-order reaction for the period studied and under the conditions described. Since no satisfactory inactivator of GTA is available, it was not possible to test concentrations beyond 0.10% because of the cellular toxicity of GTA at such concentrations. These results show that each cytopathic unit is equally likely to be inactivated per unit of time and that the inactivation process does not require cumulated effects (14). They also indicate that all the virus preparations were homogeneous.

Influence of pH on poliovirus inactivation by 0.05% GTA. Inactivation experiments were performed in phosphate buffers at pH 5.4, 7.2, and 8.3 (Table 1). At acid pH, no significant reduction in the viral titer was observed; in contrast, a 3.3 $\log_{10}$ decrease was observed at alkaline pH in 30 min. The inactivation rate of poliovirus at alkaline pH was twice as high as at neutral pH.

RNA transfection method. The RNA transfection procedure developed by Van der Werf et al. (34) with HeLa cells was applied to Vero cells. The reduction in RNA infectivity was estimated by the same dilution method as that used in virus titration. We first determined the effectiveness of RNA transfection. Serial dilutions of poliovirus RNA were incubated with recipient cell monolayers. As the initial concentration of RNA was known, it was established that the samples containing less than 10 μg of RNA (dilutions of $\geq 10^{-3}$) did not result systematically in a positive transfection under the conditions described (results not shown). It can be assumed that the transfection of such RNA concentrations is highly dependent on the experimental conditions and particularly on the state of the cells.

GTA effect on poliovirus RNA. The effect of GTA on poliovirus RNA in HBSS buffer or phosphate buffer (pH 7.2)

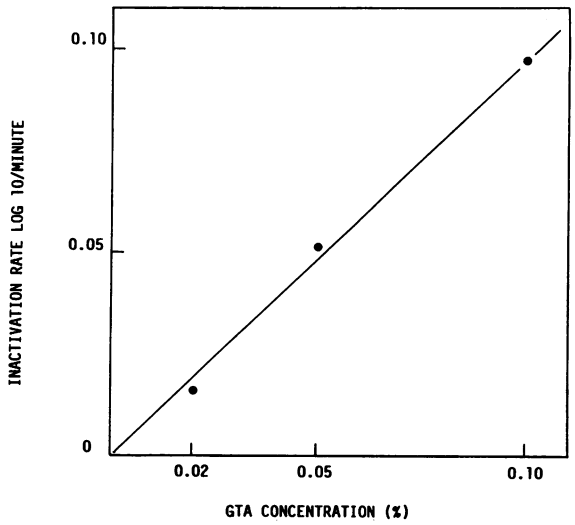

FIG. 3. Poliovirus inactivation rate with different GTA concentrations.

TABLE 1. Influence of pH on poliovirus inactivation by 0.05% GTA at 25°C[a]

Exposure time (min)	Change in viral titer (log_{10} MPNCU survival ratio) at pH:		
	5.4	7.2	8.3
15	−0.30	ND[b]	−2.54
30	−0.31	−1.56	−3.30

[a] Virus samples and GTA were diluted in phosphate buffers prepared at different pHs.
[b] ND, Not done.

was tested by incubating RNA for 30 min at 25°C with GTA at different concentrations (Table 2). The results indicated that there was no significant decrease in poliovirus RNA infectivity with 0.2, 0.4, and 1.0% GTA. Since it is known that pH is a critical parameter for GTA activity, 1.0% GTA was tested with poliovirus RNA at pH 8.3 (Table 3). The results of RNA transfections indicated a difference of two dilutions between the GTA-treated RNA and the RNA controls. This assay was performed in duplicate with two different RNA concentrations (21.6 and 12.8 μg/100 μl). Therefore, it was concluded that a 100-fold decrease in poliovirus RNA infectivity is obtained when RNA is treated with 1.0% GTA at pH 8.3.

DISCUSSION

It is well known that after spores, viruses have the greatest resistance to 2% GTA solutions (4) and that enteroviruses are less easily inactivated than other virus groups. It is therefore important to determine the action of low concentrations of GTA on highly resistant viruses.

In our study, the temperature, pH, and purity of GTA solutions, the parameters that govern GTA activity, were controlled and the most-probable-number method was used to assess the number of cytopathic units. Temperature and pH are known to greatly influence the chemical characteristics of neutral and alkaline GTA solutions (16, 21). The biocidal activity of these solutions can be modified by this instability (16). It was decided to use the A_{235}/A_{280} ratio as a spectrophotometric index of the chemical stability of GTA solutions. According to this criterion, all the neutral solutions used in this work were homogeneous in their composition: their spectrophotometric indices were between 0.3 and 0.4. The indices of alkaline solutions were about 0.6.

The results obtained by the most-probable-number method are more accurate when 96-well culture plates are used instead of tissue culture tubes. This technique has three

TABLE 2. Effect of GTA on poliovirus RNA at pH 7.2

GTA concn (%)	Reaction buffer	RNA concn (μg/100 μl)[a]	Cytopathic effect on tissue culture at RNA dilution[b] of:						
			1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
0	HBSS	4.4	+	+	+	+	−	ND[c]	ND
0	Phosphate	14.0	+	+	+	+	+	−	−
0.2	HBSS	4.4	+	+	+	−	−	ND	ND
0.4	HBSS	4.4	+	+	+	+	−	ND	ND
1.0	Phosphate	14.0	+	+	+	+	+	−	−

[a] The RNA concentrations indicated are the initial concentrations in the samples before GTA treatment.
[b] The RNA dilutions were prepared in HBSS buffer.
[c] ND, Not done.

TABLE 3. Effect of GTA on poliovirus RNA at pH 8.3[a]

GTA concn (%)	pH	RNA concn (μg/100 μl)	Cytopathic effect on tissue culture at RNA dilution[b] of:						
			1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
0	7.2	21.6	+	+	+	−	−	−	−
1.0	7.2	21.6	+	+	+	−	−	−	−
0	8.3	21.6	+	+	+	−	+	−	−
1.0	8.3	21.6	+	−	−	−	−	−	−
0	8.3	12.8	+	+	+	+	−	−	−
1.0	8.3	12.8	+	+	−	−	−	−	−

[a] The RNA treatments with GTA were performed in phosphate buffers at pH 7.2 and 8.3.
[b] The RNA dilutions were performed in HBSS buffer.

advantages: it can be used to measure the activity of antiseptics and disinfectants, cell cultures are easily reproducible, and assays can be performed with a large number of replicates. This method showed that the virucidal activity of GTA at low concentrations follows a first-order reaction, with a linear relationship between the inactivation rate and the GTA concentration (Fig. 3). This had been suggested in a previous study on the inactivation of coxsackievirus B3 (30). The kinetics of poliovirus inactivation indicate that this virus is sensitive to low GTA concentrations. A 3 log_{10} reduction in the infectious titer is obtained with 0.05% GTA in 60 min and with 0.10% GTA in 30 min. The inactivation of hepatitis A virus was recently studied under controlled conditions (26). The results showed that in 30 min at 23°C, the reductions were about 1 and 3 log_{10} with 0.02% and 0.10% GTA, respectively. Poliovirus and hepatitis A virus therefore have similar GTA sensitivities. The highest inactivation rate obtained for poliovirus was about 4 log_{10} in 40 min at pH 7.2 with 0.10% GTA. At pH 8.3 the highest inactivation rate was 3.3 log_{10} for a 30-min incubation with 0.05% GTA. Therefore, using purified viral suspensions, we established that the incubation time necessary to obtain inactivation rates ≥ 3 log_{10} is at least 30 min for the concentrations studied.

The conditions under which the poliovirus was inactivated in our study were different from those encountered in disinfection procedures of medical equipment. However, our results raise questions about how long the equipment should be in contact with the disinfectant during these procedures. The disinfection of endoscopes is interesting in this respect because the contact times with the disinfectant are sometimes lowered to a few minutes when alkaline 2% GTA solutions are used (5). These contact times (4 or even 2 min) seem to be sufficient to inactivate viruses such as hepatitis B virus and human immunodeficiency virus (13, 25). These times would become critical thresholds if disinfectants containing low concentrations of GTA were substituted for disinfectants with 2% GTA. This problem warrants consideration, since, although it is known that enteroviruses circulate abundantly in the population (18, 27) and that their hospital transmission occurs mainly by direct contact, via hands and equipment (9), the risk of transmission by endoscopes has not been specifically investigated. When considering endoscopy of immunocompromised patients, for whom enteroviruses represent a cause of serious disease (3, 8, 9), the use of disinfectants with low concentrations of GTA should be viewed with great caution.

To understand more fully the action of GTA on poliovirus particles, we attempted to determine the effectiveness of GTA against viral RNA. The results indicated that the

nucleic acid of poliovirus is much more resistant than the complete particles. At pH 7.2, RNA is not sensitive to GTA up to a concentration of 1.0% (Table 2). At pH 8.3, there is low inactivation (corresponding to a 100-fold decrease in the initial concentration of infectious RNA) after 30 min of incubation with 1.0% GTA (Table 3). This is small compared with the loss of viral infectivity under the same conditions, but may not be insignificant.

Hence, the poliovirus RNA is a bad reactive site for GTA. The other targets in the viral particles are the capsid proteins. The chemical resistance of the poliovirus RNA suggests, therefore, that the inactivation of the infectivity of viral particles is due to changes in the capsid rather than in the RNA. This interpretation is in agreement with results of studies of the interaction of GTA with proteins (12, 21).

Further studies should be made to determine the precise mode of action of low concentrations of GTA on viral capsids: is it a sealing effect or a denaturation that causes the alteration of their structural integrity? It would also be interesting to evaluate the consequences of the aggregation of viral particles on the effectiveness of GTA.

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