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Evidence for Conformational States of Poliovirions: Effects of Cations on Reactivity of Poliovirions to Guanidine (38690)

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The capsid of poliovirion is comprised of morphologically identical capsomeres which are geometrically arranged and maintained by noncovalent bonds in an energetically stable state (1–3). Treatment of these virions with heat, urea or guanidine, agents which are capable of breaking noncovalent bonds, was shown to inactivate virions but under similar conditions to have little effect on the infectivity of the RNA (4–6). Thus, these three agents inactivate the virion by their specific action on the capsid structure of the virion. Heat (45°–60°) and urea (4–7.2 *M*) have been reported to degrade poliovirions into empty capsids, a soluble polypeptide designated VP-4 and free RNA (7–9) whereas 6.5 *M* guanidine hydrochloride completely reduced the capsid structure into soluble polypeptides (5). Furthermore, cations and methionine were shown to stabilize poliovirus to heat and urea inactivation whereas cations sensitized the virion to inactivation by guanidine while methionine partially stabilized it to this reagent (10). This paper examines the effects of cations and methionine on the kinetics of poliovirus inactivation by guanidine and suggests how these agents affect the stability of poliovirus. In addition, the effects of Mg^{2+} on the stability of poliovirus to heat, urea and guanidine are summarized and the results interpreted in terms of possible conformational and functional states of the virion.

Materials and Methods. Virus. Mahoney strain type 1 poliovirus was grown in HeLa cells and purified as previously described (10). The purified virus was stored in phosphate-buffered saline (PBS), pH 7.2, at 4° and diluted one-hundred fold in distilled water before it was used, to reduce the amounts of salts carried over with the virus.

Sources. Ultrapure guanidine hydrochloride (Mann Research Laboratories, New York, NY); Baker analyzed $MgCl_2$, $CaCl_2$, NaCl (J. T. Baker Chem. Co., Phillipsburgh, NJ); methionine (General Biochemical, Chagrin Falls, OH); and CsCl (Varlacoid Chemical Co., Elizabeth, NJ) were used.

Experimental methods. The preparation of distilled water and glassware was previously described (11). From 1.8 to 5.4 ml of the prescribed reagent were preheated to 34°, in a constantly stirring water bath, equipped with a thermoregulator resulting in a sensitivity of ±0.1°. To the reagent was added one-tenth its final volume of virus (0.2–0.6 ml), followed by immediate mixing with a vortex mixer and reincubation. At the prescribed time, a 0.4 ml sample was removed and immediately diluted into cold 3.6 ml of Eagle's medium to stop the reaction and subsequently assayed for infectivity on monolayers of HeLa cells in 2 oz prescription bottles. Infectivity titer is expressed as plaque-forming units per ml. The degree of inactivation is expressed as the log V/V_o where V is the infectivity titer of the treated virus sample. The control titer V_o represents an untreated sample. Virus inactivation in water after 1 hr at 34° was negligible.

Results. The kinetics of poliovirus inactivation by guanidine hydrochloride (HCl) and the modulatory effects of cations and methionine. At 34°, a minimum concentration of 5 *M* guanidine HCl was required before appreciable virus inactivation was observed and this initial linear rate of inactivation increased as the concentration of guanidine was increased (Fig. 1). In 5 *M* guanidine, virus inactivation proceeded at a slow, constant rate for 60 min. However, in 6 *M* guanidine the initial rate of virus inactivation was maintained for 45 min after which the rate of inactivation increased, while in 7 *M* guanidine the initial rate of inactivation

was maintained for only 4 min followed by a decrease in the rate of inactivation. Thus, increasing the concentration of guanidine HCl resulted in a change in the shape as well as the slopes of the inactivation curves of poliovirus.

The effect of cations on poliovirus structure was examined by determining the kinetics of poliovirus inactivation at 34° in 5 *M* guanidine HCl plus various concentrations of cations. Figure 2 shows that increasing the $MgCl_2$ concentration from 0.5 to 1.0 *M* progressively enhanced the rate of virus inactivation as well as changed the shape of the 5 *M* guanidine inactivation curve to a two-slope curve similar to that observed with 6 and 7 *M* guanidine (Fig. 1). The effect of 1 *M* $CaCl_2$ on inactivation by 5 *M* guanidine was very similar to that of 1 *M* $MgCl_2$ while the effect of 1 *M* NaCl was less than that of 0.5 *M* $MgCl_2$. These results confirm our earlier finding (10) that divalent cations are more effective than monovalent cations in sensitizing poliovirus to guanidine inactivation.

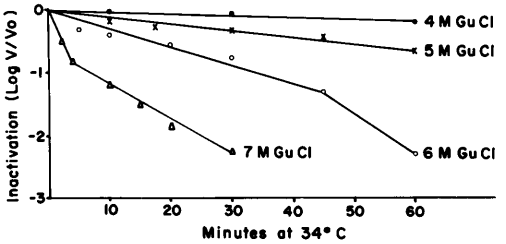

FIG. 1. The kinetics of poliovirus inactivation at 34° in various concentrations of guanidine hydrochloride: 4.0 *M* (○——○), 5.0 *M* (×——×), 6.0 *M* (○——○) and 7.0 *M* (△——△). Inactivation is expressed as log reduction in virus titer (PFU/ml).

Stabilization of poliovirus by methionine (10) was further examined by comparing the kinetics of poliovirus inactivation at 34° in 6 *M* guanidine HCl alone and with added 0.2 *M* methionine or 0.5 *M* $MgCl_2$. Figure 3 shows that 0.2 *M* methionine effectively reduced the rate of inactivation of virus by 6 *M* guanidine to that observed in 5 *M* guanidine (Fig. 1), while 0.5 *M* $MgCl_2$ enhanced the rate of inactivation in 6 *M* guanidine to a degree similar to that observed in 5 *M* guanidine plus 1.0 *M* $MgCl_2$ (Fig. 2). Thus, as the concentration of guanidine was increased, the minimum concentration of Mg^{2+} required to maintain an enhanced rate of poliovirus inactivation was decreased.

The inactivating effects of inorganic salts and guanidine HCl on poliovirus. Since poliovirions became increasingly more sensitive to guanidine as the concentration of cations was increased, the effect of extremely high concentrations of inorganic salts on poliovirus was determined. Figure 4 shows that after 30 min at 34°, poliovirus infectivity was essentially unaffected in water, 2.0–3.0 *M* $MgCl_2$, 3.0 *M* $CaCl_2$, 4.0 or 5.0 *M* NaCl, 5.0 or 6.0 *M* CsCl as well as 2.0 *M* $MgCl_2$ + 1.0 or 2.0 *M* guanidine HCl. On the other hand, the virus titer was

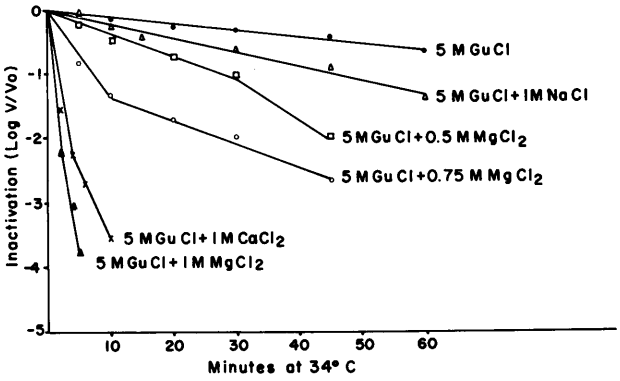

FIG. 2. The effects of cations on the kinetics of poliovirus inactivation at 34° by 5.0 *M* guanidine HCl. At zero time poliovirus was added to 5.0 *M* guanidine HCl containing the following cations: none (○——○), 1.0 *M* NaCl (△——△), 0.5 *M* $MgCl_2$ (□——□), 0.75 *M* $MgCl_2$ (○——○), 1.0 *M* $MgCl_2$ (△——△) and 1.0 *M* $CaCl_2$ (×——×). Inactivation is expressed as log reduction in virus titer (PFU/ml).

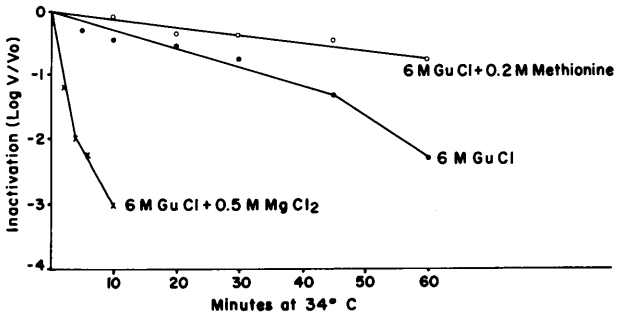

FIG. 3. The effects of 0.5 *M* $MgCl_2$ and 0.2 *M* methionine on the kinetics of poliovirus inactivation by 6.0 *M* guanidine HCl at 34°. At zero time poliovirus was added to 6.0 *M* guanidine HCl containing: 0.2 *M* methionine (O——O) or 0.5 *M* $MgCl_2$ (×——×) or no additives (O——O). Inactivation is expressed as log reduction in virus titer (PFU/ml).

reduced by greater than four logs in 2.0 *M* $MgCl_2$ + 3.0 *M* guanidine HCl and greater than five logs in 3.5–4.0 *M* $MgCl_2$ as well as 4.0 *M* $CaCl_2$. These results show that high concentrations of divalent but not monovalent cations can inactivate poliovirus and indicate again that divalent cations are more effective than monovalent cations in affecting the structure of the virion. Furthermore, in the presence of 2.0 *M* $MgCl_2$ even noninactivating concentrations of guanidine HCl (3.0 *M*) resulted in extensive virus inactivation.

To examine the possibility that the mechanisms of inactivation of poliovirus by high concentrations of divalent cations and guanidine HCl were the same, the effects of temperature on the inactivating reactions and the nature of the viral degradation products resulting with each were determined. At 34°, the virus titer was reduced by more than two logs after 30 min in 7 *M* guanidine HCl, three logs after 10 min in 6 *M* guanidine HCl + 0.5 *M* $MgCl_2$ and five logs after 30 min in 4 *M* $MgCl_2$. However, when the incubation temperature was reduced to 23°, the titer of poliovirus after 30 min incubation in 7 *M* guanidine HCl or 6 *M* guanidine HCl + 0.5 *M* $MgCl_2$ was virtually unaffected. On the other hand, the greater than five logs reduction in virus infectivity observed after incubation in 4 *M* $MgCl_2$ for 30 min at 34° was also observed at 23 and 3°; however, no inactivation was observed when the temperature was reduced to −20°.

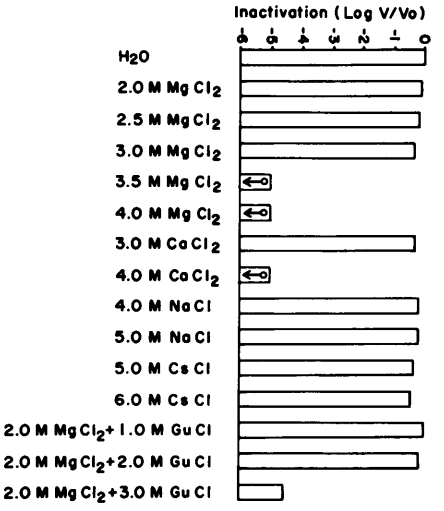

FIG. 4. The effect of high concentrations of salts on the reactivity and infectivity of poliovirus. Poliovirus was incubated for 30 min at 34° in all the solutions listed, where GuCl represents guanidine HCl. Symbols attached to descending arrows indicate that the final titer is something less than the level recorded. Inactivation is expressed as log reduction in virus titer (PFU/ml).

The products of poliovirus inactivation were examined by first incubating concentrated virus suspensions for 30 min at 34° in 4 *M* $MgCl_2$ or 8 M guanidine HCl. Both treatments reduced the virus titer by at least five logs. These samples were then dialyzed against PBS to remove the magnesium or guanidine salts and centrifuged in a CsCl density gradient for 26 hr at 4°. The infectious virus which bands at a density

of 1.34 was absent in both samples. Instead, the sample treated with 4 *M* $MgCl_2$ revealed a broad visible band with an average density of 1.28 while the sample treated with 8 *M* guanidine revealed a narrow band with a density of about 1.53. Thus, the differential effect of temperature as well as differences in the degradative products indicate that the mechanisms of inactivation by guanidine and $MgCl_2$ of poliovirus are different.

Discussion. Operationally, cations increase the effective concentration of guanidine in the inactivation of poliovirus. The complex family of inactivation curves observed with increasing concentrations of guanidine (Fig. 1) was reproduced in 5 *M* guanidine when supplemented with increasing concentrations of $MgCl_2$ (Fig. 2). Since guanidine HCl is itself a cation and at least 5 *M* guanidine is required for appreciable virus inactivation, cationic sensitization of the virus is an inherent event in the mechanism of guanidine inactivation, which appears to be the result of a series of complex reactions. Despite the ability of 3.5 and 4.0 *M* $MgCl_2$ alone to inactivate virus, the temperature requirements and the nature of the viral degradation products of the inactivation reaction indicate it is not the same as that observed with guanidine. The fact that the effects of $MgCl_2$ and guanidine are cooperative but not the same are in agreement with previous reports that guanidine effectively disrupts all noncovalent bonds of most proteins whereas high concentrations of inorganic salts primarily disrupt the subunit interactions of quarternary macromolecules such as icosahedral viruses, enzymes, ribosomes and hemoglobins and only in rare instances partially disrupt some of the internal structure of proteins (12–14).

Since ions preferentially affect the ionic bonds of proteins (13, 14), it is proposed that the capsid structure of poliovirions is held together by noncovalent bonds and stabilized by ionic bonds. The critical area of the virion is conceived as two interacting subunits which may represent two polypeptides of a single capsomere or two different capsomeres. The interaction of the subunits are between hydrogen or hydrophobic bonds and stabilized by ionic bonds. The primary effect of the cations is on the ionic bonds of the capsid protein which in turn affect the other intermolecular interactions of the capsid proteins in such a way as to strengthen or occlude the bonds preferentially attacked by heat or urea while weakening or exposing those bonds preferentially attacked by guanidine. Based on data in this paper and in the one which accompanies it (11), four environmentally induced conformational states of the virion (which are reversible, and do not destroy the virion's infectivity potential) can be detected by differences in their stability to inactivation by heat, urea and guanidine.

In the presence of little or no Mg^{2+}, State I, the virion is rapidly inactivated by heat (45°) or urea (2 *M* at 34°) but is resistant to guanidine HCl (4 *M* at 34°). In 0.5 *M* Mg^{2+}, State II, the virion is stable to heat (45°), urea (2 *M* at 34°) and guanidine HCl (3 *M* at 34°). In the range from 1 to 3 *M* Mg^{2+}, State III, the virion is still stable to heat (45°) and urea (3 *M* at 34°) but becomes increasingly sensitive to inactivation by guanidine (3 *M* at 34°). In concentrations of 3.5 *M* Mg^{2+} and greater, State IV, the virus is rapidly inactivated at 3° and 34° but is completely stable at −20°. Presumably, the thermal vibrations at 3° and 34° but not at −20° are enough to destroy the virion's weakened structural integrity brought about by the effect of $\frac{3}{5}$ *M* $MgCl_2$. It has been suggested that there are one or more specific sites on the virion through which the RNA is released in the normal infection of a cell and that heat and urea can artificially trigger this reaction (4, 15), with release of essentially all the VP-4 polypeptides that are structured in the pentameric arrangement of the capsomeres at the vertices of the icosahedral capsid. If this is correct, the conformational states of the virion described in this paper may play a role not only in the environmental stability of the virion but also in activation of the functional sites on the virion during the infectious process. These conformational changes of the virion and their proposed

role in the normal infectious process of poliovirus are visualized as follows:

Free	Cell Membrane	Cell Cytoplasm
State II →	State I →	B form → Free RNA + Empty Capsid

In the natural environment of poliovirus the total effect of all modulators probably simulates 0.5 *M* Mg^{2+} and the free virus is normally in State II. This is the most stable conformation against various environment conditions it may encounter. Upon attachment and interaction with the membranes of cell, the virion undergoes a change in conformation to State I, thus destabilizing the site (s) on the virion through which the RNA is to be released.

If the analogy with urea is accurate, the release of RNA is preceeded by a preliminary priming reaction since it was previously shown that urea inactivation is a two step process with prior conversion of the native virion to a sensitized B form.

In the final step, release of the viral RNA is triggered, resulting in an empty capsid and the free viral RNA in the cell cytoplasm. Evidence for the involvement of at least two distinct steps in the uncoating or penetration of poliovirus was previously reported by Chan and Black (17) as well as Holland and Hoyer (18) who concluded as we do, that unless a proper cell receptor induced conformational change of the virion occurs, the ensuing penetration and uncoating are not completed.

Summary. The kinetics of inactivation of poliovirions were determined in the presence of various concentrations of monovalent and divalent ions, methionine, guanidine and combinations of these at various temperatures. Four states of the virion, under control of the ionic environment, could be recognized which were reversibly interchangeable and yet retained infectious potential. These states were detected by differential sensitivity of the virion to inactivation of infectivity by heat, urea and guanidine. The data are interpreted in terms of conformational structural changes under control of the ionic environment. It is proposed that these various structural conformations are meaningful to the various biologic functional states of the virus.

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