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The Inhibitory Effects of $MgCl_2$ on the Inactivation Kinetics of Poliovirus by Urea (38689)

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The capsid of poliovirions is comprised of capsomeres which are held together by noncovalent bonds (1, 2). Treatment of these virions with agents known to break noncovalent bonds such as heat (45–60°) or urea (4–7.2 *M*) resulted in the following detectable changes: (a) inactivation (b) conversion to H or C type antigenicity and (c) the release of infectious RNA and VP-4 polypeptides from a morphologically intact capsid structure (3–9), indicating that heat and urea inactivate the virions by altering their capsid structure rather than their RNA. Furthermore, environmental agents or modulators such as cations and some organic compounds (methionine, choline, betaine) which stabilized poliovirus against heat inactivation were also shown to stabilize the virus against urea inactivation (10, 11). These results indicate that the mode of inactivation of poliovirus by heat or urea is similar. In order to gain insights into the mechanism of urea inactivation of poliovirus, this paper examines the effects of $MgCl_2$ on the kinetics of inactivation of poliovirus by urea.

Materials and Methods. Water and glassware. Since ordinary distilled water and soft glass vessels resulted in some inhibition of the inactivation of purified poliovirus by urea, only water which had been demineralized and distilled once in a metal distiller followed by two successive distillations in pyrex glass distillers was used. All glassware which were of hardglass (pyrex) were cleaned by conventional methods followed by soaking for 30 min in aqua regia (part HNO_3 + 3 parts HCl) and rinsed successively with tap water and distilled water. Falcon plastic pipettes were used.

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

Sources. Ultrapure urea (Mann Research Laboratories, New York, NY) and Baker analyzed $MgCl_2$ (J. T. Baker Chem. Co., Phillipsburgh, NJ) were used.

Experimental methods. 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 and V_o that of the untreated sample. Virus inactivation in water after 1 hr at 34° was negligible.

Results. The effects of urea and Mg^{2+} on the kinetics of poliovirus inactivation. The inactivation kinetics of poliovirus in various concentrations of urea were determined at 34°. Figure 1 shows that the rate and degree of inactivation of poliovirus is dependent on the concentration of urea and that the inactivation curve is characterized by three distinct slopes: an initial shoulder, followed by a rapid exponential decrease in the virus infectivity and a final slow exponential decrease representing a resistant or persistent fraction of virus. Increasing the concentration of urea resulted in increasing the rate of virus inacti-

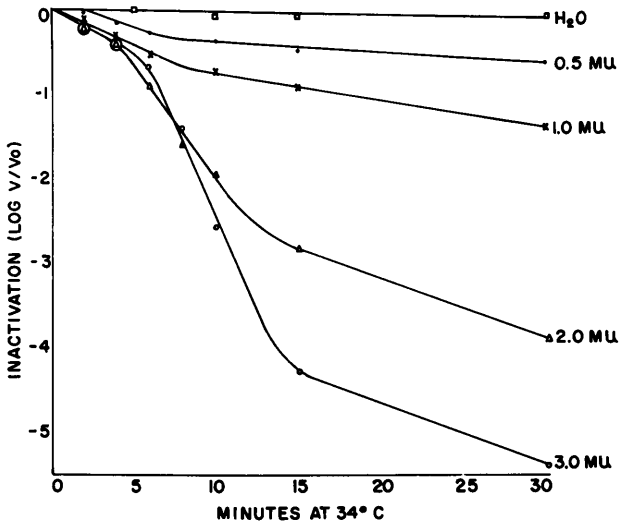

FIG. 1. The inactivation kinetics of poliovirus at 34° in water (□——□) and in various concentrations of urea: 0.5 *M* (○——○), 1.0 *M* (×——×), 2.0 *M* (△——△), and 3.0 *M* (○——○). Inactivation is expressed as log reduction in virus titer (PFU/ml).

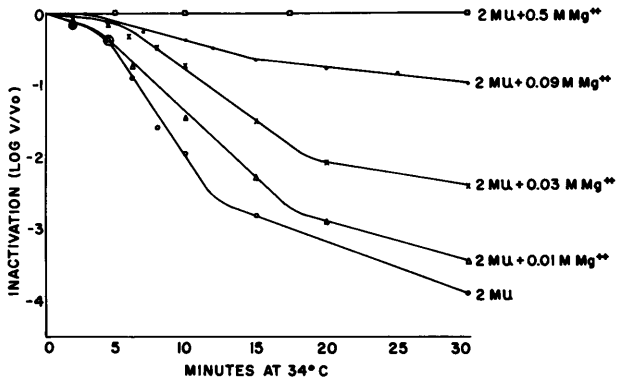

FIG. 2. The stabilizing effect of 0.01 *M* (△——△), 0.03 *M* (×——×), 0.09 *M* (○——○) and 0.5 *M* (□——□) $MgCl_2$ on inactivation of poliovirus by 2.0 *M* urea (○——○) at 34°. Inactivation is expressed as log reduction in virus titer (PFU/ml).

vation (steeper second slope) as well as reducing the fraction of persistent virus. However, its effect on the duration of the initial shoulder was negligible.

In preliminary experiments, 1–3 *M* $MgCl_2$ completely stabilized poliovirus against inactivation by up to 3 *M* urea. The effects of various concentrations of $MgCl_2$ on the kinetics of poliovirus inactivation in 2 *M* urea at 34° were determined. Figure 2 shows that the stabilizing effect of $MgCl_2$ ranged from minimal at 0.01 *M* to complete at 0.5 *M*. However, even in the presence of partially inhibitory concentrations of $MgCl_2$, inactivation proceeded in a similar three stage manner. Increasing the concentration of $MgCl_2$ resulted in decreasing the rate of virus inactivation as well as increasing the fraction of persistent virus. However, the presence of $MgCl_2$ had a negligible effect on the duration of the initial shoulder, indicating that Mg^{2+} did not affect the events responsible for the expression of the shoulder in the inactivation curve.

The mechanism of urea inactivation of poliovirus and the effects of Mg^{2+}. The multihit inactivation curves (Figs. 1, 2) suggest that urea inactivates poliovirus by a two-

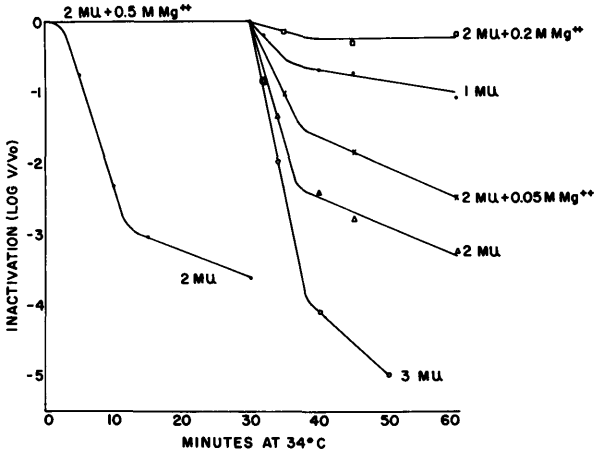

FIG. 3. The specific action of $MgCl_2$ on the inactivation kinetics of poliovirus by 2 *M* urea at 34°. At zero time, poliovirus was added to 34° solutions of 2 *M* urea and 2 *M* urea + 0.5 *M* $MgCl_2$. After 30 min of incubation in 2 *M* urea + 0.5 *M* $MgCl_2$, the sample was diluted 1:100 in cold 2 *M* urea and an aliquot of this sample added to 34° solutions of 1.0 *M* urea (○——○), 2.0 *M* urea (△——△), 3.0 *M* urea (○——○), 2.0 *M* urea + 0.05 *M* $MgCl_2$ (×——×) and 2.0 *M* urea + 0.2 *M* $MgCl_2$ (□——□). Inactivation is expressed as log reduction in virus titer (PFU/ml).

step mechanism. The initial shoulder or lag can be most easily explained by a conversion of the native virus into an intermediate or sensitized form before it is inactivated. To determine whether Mg^{2+} inhibits this initial sensitizing reaction as well as the subsequent inactivation step, poliovirus was incubated at 34° in 2 *M* urea + 0.5 *M* $MgCl_2$ for 30 min. Figure 3 shows that this pretreatment did not affect the infectivity of the virus. This sample was diluted one-hundred fold in cold 2 *M* urea to selectively dilute out the Mg^{2+} and aliquots were then added to 34° solutions of urea and urea + $MgCl_2$. The rates of inactivation in the various solutions are shown in Fig. 3. In all cases, inactivation proceeded exponentially, without a shoulder, the rate being dependent on the concentration of urea and the amount of Mg^{2+} present. Thus, the higher the concentration of urea, the faster the rate of virus inactivation and the smaller the fraction of persistent virus. Mg^{2+} was again able to reduce the rate of virus inactivation and to increase the fraction of the persistent virus. In effect, the same family of curves was obtained as shown in Figs. 1 and 2, but significantly, without the initial shoulder. Thus, Mg^{2+} does not appear to affect the sensitizing reaction. These results support the hypothesis that urea inactivation of poliovirus involves at least two successive reactions and that Mg^{2+} stabilized poliovirus by selectively inhibiting the second reaction. As further test of this hypothesis, virus was first incubated in 2 *M* urea at 34°. After 2, 10 and 15 min of incubation, an aliquot was removed and added to a 34° solution with a final concentration of 2 *M* urea + 0.5 *M* $MgCl_2$. After 30 min of incubation, the titers of all the samples were compared. Figure 4 shows that the titer in the original 2 *M* urea solution was reduced by over 3.5 logs in 30 min. However, the titers of samples which were transferred at 2, 10 and 15 min to a solution of 2 *M* urea + 0.5 *M* $MgCl_2$ were not further inactivated. Thus, addition of 0.5 *M* $MgCl_2$ at any time during exponential inactivation stopped further inactivation indicating again that Mg^{2+} stabilized the virus against the inactivating reaction.

The reversibility of the initial shoulder in the urea inactivation curve of poliovirus was suggested by Cooper (6). In order to show more definitively that the first step in the two-step urea inactivation mechanism is reversible, poliovirus was first incubated at 34° for 30 min in 2 *M* urea +

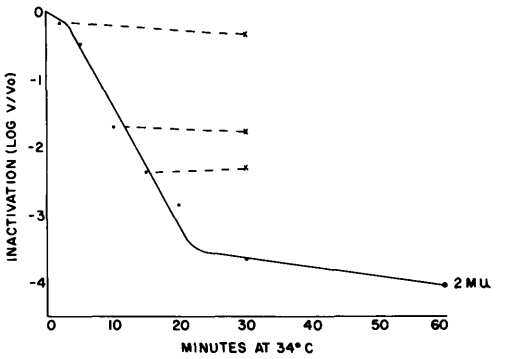

FIG. 4. Inhibition of the inactivation of poliovirus in 2.0 *M* urea at 34° by the addition of 0.5 *M* $MgCl_2$. At zero time, poliovirus was added to a 34° solution of 2.0 *M* urea (O——O). After 2, 10, and 15 min of incubation in 2 *M* urea, an aliquot sample was removed, added to a 34° solution of 2.0 *M* urea + 0.5 *M* $MgCl_2$ (O——X), and incubated for a total of 30 min. Inactivation is expressed as log reduction in virus titer (PFU/ml).

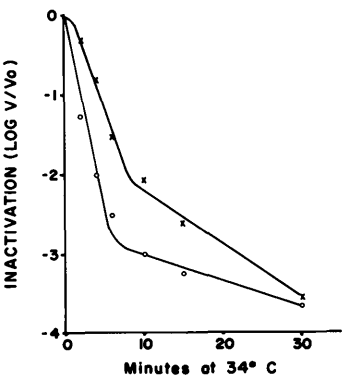

FIG. 5. The effect of diluent on poliovirus incubated for 30 min at 34° in 2.0 *M* urea + 0.5 *M* $MgCl_2$. Poliovirus preincubated for 30 min at 34° in 2.0 *M* urea + 0.5 *M* $MgCl_2$ was initially diluted 1:10 in cold water (X——X) or cold 2.0 *M* urea (O——O). Aliquots from both samples were then added to 34° solutions of 2.0 *M* urea and their rate of inactivation measured. Inactivation is expressed as log reduction in virus titer (PFU/ml).

0.5 *M* $MgCl_2$. As was shown in Fig. 3, this treatment did not affect the infectivity of the virus but did sensitize the virus population to urea. An aliquot of this sample was then diluted one-hundred fold in cold distilled water to dilute out both the Mg^{2+} and the urea, while another aliquot was similarly diluted one-hundred fold in cold 2 *M* urea to dilute out the Mg^{2+} while keeping the urea concentration constant. Both samples were then added to a 34° solution of 2 *M* urea and their rate of inactivation measured. Figure 5 shows that the inactivation curve of the sample diluted in water displayed an initial shoulder, while no shoulder was observed in the sample diluted in 2 *M* urea. These results indicate that when the virus was diluted in cold urea, the virus population remained sensitized and when exposed to urea at 34° it was immediately inactivated. Thus, no initial lag or shoulder was observed under these conditions. On the other hand, when the virus was diluted in water to reduce the concentration of urea and Mg^{2+}, some of the sensitized virus in the sample reverted to the native desensitized state. Thus, when the virus was re-exposed to urea at 34°, the native form of the virus had to be resensitized before it was inactivated, resulting in the observed initial shoulder. However, the shortness of the shoulder indicates that not all the sensitized virus reverted to the native state.

The third slope in the three stage urea inactivation curve describes an abrupt reduction in the rate of virus inactivation, indicating that a certain fraction of the virus population (the persistent fraction) is more resistant to urea inactivation. However, the size and stability of that fraction is determined by the concentration of urea and Mg^{2+} present (Figs. 1–3). To determine how environmental factors effect the initiation of the persistent fraction, the inactivation curves of poliovirus in 2 *M* urea and in 2 *M* urea + 0.02 *M* $MgCl_2$ at 34° were first determined (Fig. 6). After 30 min of incubation, both inactivation curves were already in their third slopes and as expected, the presence of Mg^{2+} induced a larger fraction of persistent virus. An aliquot of the 30-min sample from both curves was then diluted one-hundred fold in fresh 2 *M* urea to effectively change the environment of both persistent virus fractions to that of fresh 2 *M* urea. The inactivation rates of both these samples were then determined at 34° and compared with the slope of the persistent virus kept in its original environ-

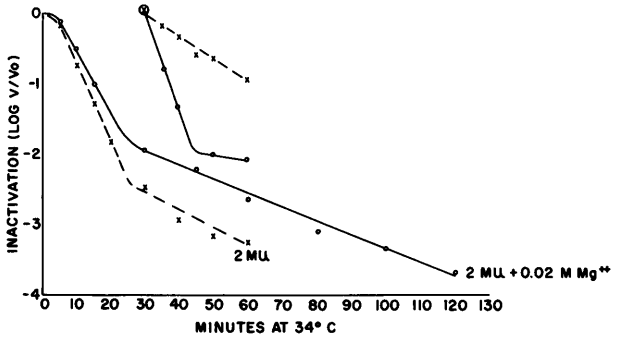

FIG. 6. Factors controlling the third slope of the urea inactivation curve. At zero time, poliovirus was added to 34° solutions of 2 *M* urea (×——×) and 2.0 *M* urea + 0.02 *M* $MgCl_2$ (○——○). After 30 min of incubation, an aliquot from both samples was diluted 1:10 in cold 2 *M* urea and then added to 34° solutions of 2.0 *M* urea. The inactivation rates of the 30-min sample from the 2 *M* urea (×——×) and the 2 *M* urea + 0.02 *M* $MgCl_2$ (○——○) inactivation curve are plotted at the top of the graph. Inactivation is expressed as log reduction in virus titer (PFU/ml).

ment. Figure 6 shows that the inactivation rate of the persistent virus produced by 2 *M* urea did not change when it was diluted into fresh 2 *M* urea. However, the inactivation rate of the persistent virus produced by 2 *M* urea + 0.02 *M* $MgCl_2$ was immediately accelerated when it was diluted in fresh 2 *M* urea, until another, smaller fraction of persistent virus was established. Thus, the persistent fraction established in the presence of urea and Mg^{2+}, unlike that produced in urea alone, is due to a reversible fixation of a portion of virus population into a stable state by Mg^{2+}. Operationally, Mg^{2+} reduces the effective concentration of urea (Figs. 2, 3) but is operative only in its continuous presence (Fig. 6). To gain insight as to factors responsible for the production of the persistent virus by urea, fresh virus or fresh urea was added to the persistent fraction established in urea and the inactivation rates determined at 34°. The freshly added virus was inactivated to the same degree as virus which had been inactivated to produce the persistent fraction, indicating that urea activity in the persistent fraction had not been reduced and that the stabilizing factor was not present in excess amount in the environment. Furthermore, when fresh additional urea was added to the persistent fraction, rapid virus inactivation occurred until another, smaller persistent fraction related to the total urea concentration was obtained, indicating that the persistent fraction was comprised of virus which was resistant only to that concentration of urea from which it was obtained.

Discussion. As previously reported (6, 9) the urea inactivation curve of poliovirus is characterized by three distinct slopes: an initial delay or shoulder, followed by a rapid exponential decrease in virus infectivity and a final slow exponential decrease. The initial shoulder of this inactivation curve indicates that the mechanism of urea inactivation of poliovirus is due to a process of cumulative damage or in its simplest form, a two-step reaction (12, 13). Evidence for a two-step reaction mechanism was demonstrated by the selective action of Mg^{2+} which at optimum concentrations completely stabilized the virus to urea inactivation but at sub-optimal concentrations inhibited the second slope (inactivation) but did not affect the duration of the initial shoulder. The following model is proposed to schematically outline the events of urea inactivation of poliovirus in the absence and presence of Mg^{2+}.

$$\begin{array}{ccccc} & & \text{Bmg} & & \\ & & \updownarrow\ Mg^{2+} & & \\ A & U & B & U & I \\ & \rightleftarrows & & \rightarrow & \end{array}$$

There are specific sites on the native form of poliovirions (A) which are sensitive to urea. These sites are involved in maintaining

the VP-4 polypeptide in the virion and are stabilized by noncovalent bonds such as hydrogen or ionic bonds. When polio virions (A) are exposed to urea (U), the more susceptible hydrogen bonds are readily broken, converting the native virion (A) into an intermediate form (B) which is still infectious. This initial reaction (A → B), is dependent on the presence of urea and corresponds to the observed initial lag or shoulder in the inactivation curve. It is the intermediate form (B) which is then inactivated by further reaction with urea to a product (I) at a rate corresponding to the observed second slope. The inactivation reaction (B → I) results from the breaking of the ionic bonds which stabilizes the VP-4 polypeptides onto the virion. However, Mg^{2+} binds to and strengthens these ionic bonds, forming a stable Bmg complex ($B + Mg^{2+} \rightarrow Bmg$) and thereby inhibits the inactivation reaction. The B and Bmg forms are in dynamic equilibrium, the proportion of B to Bmg being a function of the concentration of Mg^{2+}. At optimal concentrations of Mg^{2+}, essentially only Bmg forms are available and the virus population is completely stable to urea. As the optimal concentration of Mg^{2+} is progressively decreased, the proportion of B forms progressively increases and the rate of inactivation of virus proportionately increases in any given concentration of urea.

Besides stabilizing poliovirus against urea inactivation, Mg^{2+} has also been shown to stabilize poliovirus against heat inactivation (10, 14, 15). Furthermore, both heat and urea degrade poliovirions into empty capsids VP-4 polypeptides and RNA (4–9). These results imply that there are common and specific sites on poliovirions which are susceptible to the action of both heat and urea and that the mechanisms of heat and urea inactivation are similar. However, the kinetics of poliovirus inactivation by urea is characterized by a multi-hit curve whereas heat inactivation of the virus is characterized by a single-hit phenomenon (5, 12, 15). One explanation for this apparent discrepancy is that the two sets of bonds stabilizing the VP-4 polypeptides in the virions are unequally available to urea but are equally available to heat. Thus, the kinetics of heat inactivation of poliovirus may in actuality be a pseudo-single-hit reaction.

The third slope or the final tailing effect of the urea inactivation curve indicates that a certain fraction of the virus population was or became more resistant to urea inactivation. The level at which this third slope was initiated could be controlled by varying the concentration of urea or by the addition of Mg^{2+} to the urea solution. This raises the question as to the origin of the virus population which is stable to urea. Two possibilities exist. First, that the virus population is initially heterogenous with respect to its resistance to urea and by varying the concentration of urea, one merely selects for an existing stable virus population. However, this possibility is unlikely as it implies that an infinite variation in the packaging of the virions must occur to account for the heterogenous reactivity of the virus population to urea. The second more likely possibility is that the virus population is initially homogenous with respect to its reactivity to urea and is made heterogenous by the action of urea and Mg^{2+}. The persistent fraction observed in the urea plus Mg^{2+} inactivation curve was clearly induced by the stabilizing effect of Mg^{2+}. The size of the fraction as well as the maintenance of the persistent virus population was shown to be dependent on the concentration and continued presence of Mg^{2+}. However, the present data do not allow one to select between these possibilities regarding the establishment of the persistent virus fraction in urea alone.

Summary. By analyzing the inhibitory effects of Mg^{2+} on the three-stage urea inactivation curve of poliovirus, it was concluded that urea inactivates poliovirus via a two-step reaction as follows: urea initially converts the native virus into an intermediate state which is still infectious but is now highly sensitive to inactivation. This reaction is unaffected by Mg^{2+} and is reversible. In the subsequent reaction, the sensitized virus is either irreversibly inactivated by urea or reversibly stabilized by Mg^{2+}. In addition, the inactivation curve revealed that a fraction of relatively stable virus population was established in the presence of urea and that the size of this

persistent virus population depended on the concentration of urea. It was not determined whether urea induced the formation of this stable fraction of virus or merely selected for a preexisting stable population of virus. However, evidence was presented that (1) the persistent virus population was not due to the depletion or inactivation of urea in the sample, (2) whatever stabilized the virus, it could not be removed or reversed by simple dilution as in the case of Mg^{2+}, (3) no excess stabilizing material was made to stabilize the addition of untreated virus and finally (4) the persistent virus population was resistant to that concentration of urea in which it was observed but could be further inactivated by a higher concentration of urea, only to result in a smaller fraction of persistent virus population.

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