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Measurement of the Inactivation Kinetics of Poliovirus by Ozone in a Fast-Flow Mixer

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Inactivation kinetics of poliovirus type 1 in ozone demand-free water was investigated by utilizing a fast-flow mixing apparatus. Ozonated water and a solution of ozone demand-free water containing a known quantity of poliovirus type 1 were introduced simultaneously into a mixing chamber, both at a constant rate. This mixture was then passed through a narrow tube of known length and diameter into a neutralizing solution. By altering the rate of introduction and/or tube length, different contact periods between ozone and virus could be determined with an accuracy of 0.01 s. Inactivation of the poliovirus occurred in two steps. During the first step, which lasted for 0.2 to 1.0 s, 95 to 99% of the virus was inactivated, depending on the ozone concentration (which ranged from 0.1 to 2.0 mg/liter). The second step apparently continued for several minutes; in this period the remainder of the virus was inactivated. An obvious dose-response relationship was demonstrated during the first step of the inactivation curve. The pH of the water slightly affected the viral inactivation rate, but these small differences seem to have no practical value.

In an earlier publication (5) we reported on a two-stage ozone (O_3) inactivation curve of poliovirus type 1. During the first stage, which lasted for less than 10 s, approximately 99% of the viruses were inactivated, whereas the remainder of the viruses were killed during the second stage, which continued for several minutes. However, a dose-response relationship between O_3 concentrations and viral inactivation rate could not be demonstrated. This is not in accordance with the assumption that disinfection is a first-order reaction (3). It should be pointed out that, because of technical reasons, 8 s lapsed before the first sample could be withdrawn, during which time the majority of the viruses underwent inactivation. It was hypothesized that during these 8 s a dose-response relationship indeed exists, an occurrence which could, however, not be demonstrated because of the rapid inactivation rate.

The present study was undertaken to elucidate the kinetics of O_3 action on poliovirus type 1 during very short periods of time, e.g. 0.2 to 1.0 s, and to verify a possible dose-response relationship. The effect of pH on the viral inactivation rate was also investigated.

MATERIALS AND METHODS

Virus. Poliovirus type 1 (Brunhilde) was grown in BGM cells (2) and concentrated by phase separation (8). The concentrate was centrifuged for 30 min at 12,000 × *g* to remove cell debris. The supernatant was centrifuged for 2 h at 100,000 × *g*, and the pellet was resuspended in 0.005 M phosphate buffer, pH 7.2. The partially purified virus concentrate (2×10^9 plaque-forming units per ml) was stored in glass vials (0.5 ml each) at −70°C. The virus stock was diluted 1,000-fold in 0.02 M phosphate buffer before each experiment; the pH of the buffer was adjusted according to the requirements of the experiment. The same virus stock was used throughout.

Ozone. O_3 was generated from oxygen with an ozonizer (Ozone Generator model 501; Fisher Laboratories) and bubbled through 2×10^{-4} M phosphate buffer, pH 5.0, for several minutes. The concentrated O_3 solution was diluted to the desired O_3 concentration for each experiment. Determination of O_3 concentration was performed by the method of Schechter (7).

Ozone demand-free water. All buffers were prepared from triple-distilled water. Ozone demand-free water was obtained by adding alkaline potassium permanganate to the water during the third distillation.

Viral inactivation kinetics experiments. A fast-flow mixer (D. P. Ballou, Ph.D. thesis, University of Michigan, Ann Arbor, 1971) was used, with the following modifications. The contents of two syringes, one containing O_3 and the other containing diluted virus suspension, were injected simultaneously into a mixing chamber; from there the mixture passed through a narrow tube (diameter, 1.07 mm) into a quenching solution (0.002 M tetrasodium pyrophosphate, 0.0004 M sodium sulfite). The movement of the plungers was electronically controlled. Contact time of ozone and virus could be varied by changing the injection rate and the length or diameter of the narrow tube.

Before the start of an inactivation kinetics experiment, O_3 and virus solutions were prepared and kept

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at 4°C; the same solutions were used throughout a given experiment. Samples were taken at contact times ranging from 0.2 to 1.0 s. O_3 concentration was determined before and after each experiment. Only the experiments in which O_3 reduction was 5% or less were incorporated into the results.

Calibration of the fast-flow mixer. Calibration of the fast-flow mixer was performed by the method of Barman and Gutfreund (1), using alkaline hydrolysis of *p*-nitrophenol acetate to *p*-nitrophenol. The mixer was calibrated for contact times between 0.2 and 1.0 s, with an accuracy of 0.01 s.

RESULTS

Inactivation of poliovirus type 1 by ozone. O_3 concentrations ranged between 0.06 and 2.5 mg/liter. Figure 1 shows the viral inactivation kinetics with 0.06, 0.19, 0.4, and 1.24 mg of O_3 per liter at pH 7.2. Here too, the characteristic two-stage inactivation curve is apparent (5). However, in the current experiments it was possible to measure the duration of the inactivation period of the first stage, and a direct correlation between this period and the O_3 concentration could be shown: the higher the O_3 concentration, the shorter the duration of the first stage. In fact, at O_3 concentrations of 0.06 and 0.19 mg/liter, the two-stage inactivation curve does not even appear in Fig. 1. This is probably due to the very short sampling period (1 s or less).

Dose-response relationship between poliovirus type 1 inactivation rate and O_3 concentration. The results shown in Fig. 1 suggest a probable dose-response relationship between poliovirus type 1 inactivation rate and O_3 concentration. To verify this relationship, additional inactivation experiments with various O_3 concentrations at pH 7.2 were carried out. The time required for the inactivation of 95% of the virus was calculated for each experiment, and the end results were plotted on a log-log scale as a function of time versus concentration (Fig. 2). A dose-response relationship is obvious; the coefficient of correlation is −0.804.

Effect of pH on poliovirus type 1 inactivation. In addition to pH 7.2, the following pH's were tested with regard to poliovirus type 1 inactivation: 3.0, 5.0, 9.0, and 10.0. The characteristic two-stage inactivation curve appeared at all of the above pH's. The results of 95% viral inactivation were calculated and plotted as described above (Fig. 3). An obvious dose-response relationship existed for all of the pH's tested, as well as slight differences in the rates of viral inactivation for the different pH's. The coefficients of correlation for the curves of the pH's were as follows: pH 3.0, −0.9504; pH 5.0, −0.9067; pH 9.0, −0.7392; and pH 10.0, −0.9442.

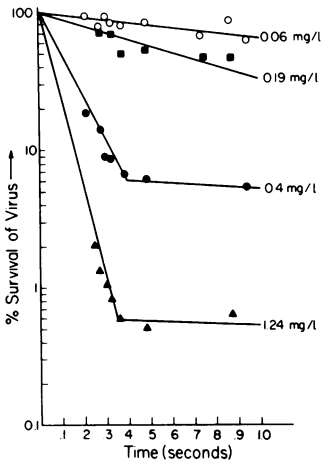

FIG. 1. *Inactivation kinetics of poliovirus type 1 with various concentrations of ozone in a fast-flow mixer.*

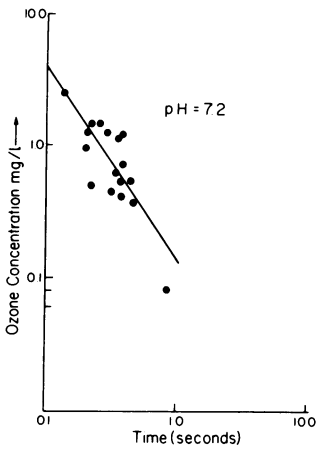

FIG. 2. *Time-concentration relationship for 95% inactivation of poliovirus type 1 by ozone (log-log scale).*

DISCUSSION

The main objective of the present study was to determine whether there exists a dose-response relationship between O_3 concentrations and their ability to inactivate poliovirus type 1. During a previous study there were indications that such a relationship would be demonstrable, provided that very short reaction times were applied. A fast-flow mixer, enabling reaction times of 0.2 to 1.0 s with an accuracy of 0.01 s, was used in the current experiments.

The kinetics curve obtained in the present work is similar to that shown in our previous study (5). Unlike the previous work, the first stage could actually be measured in the current

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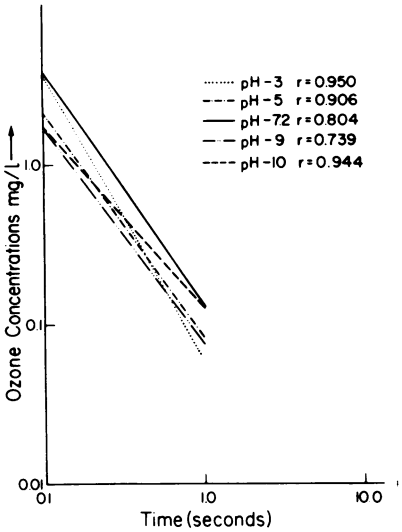

FIG. 3. *Effect of pH on time-concentration relationship for 95% inactivation of poliovirus type 1 by ozone (log-log scale). r, Correlation coefficient.*

experiments, and an O_3 concentration-viral inactivation rate (dose-response) relationship is apparent (Fig. 1 and 2).

From the first part of the slope of the inactivation kinetics curve (Fig. 1), it can be seen that the reaction is of a first order with respect to the virus, since the O_3 concentration is in excess and constant throughout the reaction. The first-order equation is, therefore: $\ln [(V/V_0) \times 100] = kt$ (equation 1), where V is the virus concentration (in plaque-forming units per milliliter) at time t (in seconds), V_0 is the virus concentration (in plaque-forming units per milliliter) at time zero, and k is the pseudo first-order specific rate constant (in seconds^{-1}) and depends on the O_3 concentration in any given experiment. If k is defined in terms of the O_3 concentration, then equation 1 becomes: $\ln [(V/V_0) \times 100] = K[O_3]^n t$ (equation 2), where K is the overall chemical reaction rate constant (in [microliters per liter]$^{-n}$·seconds^{-1}), $[O_3]$ is the ozone concentration (in microliters per liter), and n is the exponent of the ozone concentration ($n = 1$ if the reaction is of the first order with respect to the ozone, etc.). For 95% inactivation, equation 2 becomes: $\ln 5 = K[O_3]^n t_{95}$ or $[O_3]^n t_{95} = 1.61/K$ (equation 3), which is a variation of the equation known as Watson's law (9).

Evaluation of n and K is usually accomplished by the logarithmic form of equation 3: $n \log[O_3] + \log t_{95} = \log 1.61/K$ (equation 4). Plots of $\log [O_3]$ versus $\log t_{95}$ are shown in Fig. 2, and the values for n and K at different pH's as shown in Fig. 3 are compiled in Table 1.

TABLE 1. *Reaction rate constant (K) and O_3 concentration exponent (n) at various pH values*

pH	Concn exponent	Reaction rate constant ([μl/liter]$^{-n}$·s^{-1})
3.0	0.6	8.4
5.0	0.7	9.5
7.2	0.68	6.5
9.0	0.73	10.5
10.0	0.88	9.7

The units of K depend on the value of n and are therefore (microliters per liter)$^{-n}$·seconds^{-1}. A value of n equal to unity seems natural since this would make the overall reaction of the second order (first order with respect to the virus and first order with respect to ozone). The deviation from unity may be due to experimental aberration. Deviations of a similar magnitude for aqueous disinfectants have been observed previously. However, for ozone it may well be that more than one species is responsible for the disinfection process (i.e., O_3, OH, etc.) (6), and this will give rise to a complicated kinetics curve and cannot simply be described as in equation 2. Ozone species may vary with pH and other chemical factors in the water.

The pH of the water may have an effect on the virus by causing viral clumping. It has been demonstrated (4) that viruses have a tendency to clump at pH's below 6.0. At pH 7.0 and above, the virus clumps disaggregate very rapidly. It is obvious that such aggregation of viruses in the water would affect the efficiency of any disinfection agent. It is reasonable to assume that our results are a combination of the effects exerted by the pH on both the O_3 and the virus. However, the differences in the inactivation kinetics at the various pH values are so small as to be of no practical significance.

In conclusion, it can be stated that these experiments carried out in a fast-flow mixer with contact times varying from 2.0 to 1.0 indicate that: (i) a clear dose-response relationship between O_3 concentration and virus inactivation rate can be demonstrated; (ii) pH variations between 3 to 10 do not, apparently, lead to meaningful differences in virus inactivation rates by ozone; (iii) the kinetic reaction is of the first order with respect to the virus; and (iv) certain anomalies in relation to virus inactivation by ozone may be associated with varying ozone species that may develop under varying substrate conditions and for virus clumping and disaggregation under varying conditions.

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