

Comparison of Ozone Inactivation, in Flowing Water, of Hepatitis A Virus, Poliovirus 1, and Indicator Organisms

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In steadily flowing water at 20°C and pH 7, five organisms had the following order of resistance to ozone (at constant levels of ozone): poliovirus 1 (PV1) < *Escherichia coli* < hepatitis A virus (HAV) < *Legionella pneumophila* serogroup 6 < *Bacillus subtilis* spores. The tests were repeated at 10°C with HAV, PV1, and *E. coli*. Ozone inactivation of HAV and *E. coli* was faster at 10°C than at 20°C. At 20°C, 0.25 to 0.38 mg of O₃ per liter was required for complete inactivation of HAV but only 0.13 mg of O₃ per liter was required for complete inactivation of PV1.

Drinking water, a natural transmission route for hepatitis A virus (HAV), bacteria, and other viruses (9), requires precautionary inactivation of such waterborne organisms. Disinfection with ozone is steadily gaining importance as an alternative and supplement to chlorine disinfection.

Earlier studies have shown that HAV is more resistant to chlorine under certain conditions than some types of bacteria (8, 14). However, the literature gives quite disparate reports on the resistance of enteroviruses to ozone (1, 3, 11). Differences in laboratory conditions play a significant role here. Fortunately, new methods of growing and propagating HAV in cell cultures have made it possible to use precise numbers of HAV for inactivation experiments (6).

We compared ozone inactivation of HAV and poliovirus 1 (PV1) under conditions of constant flow (2). This made it possible to maintain a steady concentration and effectiveness of ozone despite ozone loss due to reaction with viruses and other substances. This arrangement corresponds best to the conditions in a water treatment plant.

Unless otherwise specified, all ozone levels in this report refer to constantly maintained concentrations of ozone in flowing water.

MATERIALS AND METHODS

Viruses. The virus strains tested were the HAV/HFS/GBM strain of HAV and the vaccine strain of Behring Werke for PV1. Human fibroblast cells (HFS) were used for culture. The growth medium was Earle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) plus 10% fetal calf serum, glutamine, and antibiotics.

Samples (0.2 ml) of HAV and PV1 were diluted (up to 10⁻⁸) in 1.8 ml of Earle minimal essential medium with 5% fetal calf serum by 1-in-10-dilution steps. A sample (0.2 ml) of each dilution was applied to each well of microdilution plates, one set of plates for HAV and one for PV1 with HFS monolayers cultured and incubated with 5% CO₂. Antigenicity to newly produced HAV was demonstrated after 3 weeks as described elsewhere (5), whereas each well of the PV1 microdilution plates was examined microscopically after 1 week and evaluated for cytopathogenic effects. The infectious titer, 50% tissue culture infective dose (TCID₅₀), was determined for HAV and PV1 by using Kärber's method (12).

Bacteria. Three species of bacteria were used for compar-

ison in the inactivation experiments: *E. coli* (ATCC 11229), *Bacillus subtilis* (DSM 2277), and *Legionella pneumophila* serogroup 6 (our own isolate from drinking water). CFU were counted after plating onto Mueller-Hinton agar plates or buffered charcoal yeast extract plates.

Ozone measurement. Ozone concentrations were measured with diethyl-*p*-phenylendiamine and a potassium iodide-phosphate buffer (7). The resulting dye was measured spectrophotometrically at 510 nm.

Ozonation method. The experimental apparatus was structured as shown in Fig. 1. Phosphate-buffered saline solution (pH 7.2) was pumped from a supply container into a thermostatically controlled glass column whose temperature was maintained at 20 or 10°C during the experiments. Ozone entered the same column from a 0.5/50A-type ozoniser (Prominent Dosiertechnik, Heidelberg, Federal Republic of Germany) at a concentration of 10 to 30 g of O₃/m³ of air and was distributed in the form of small bubbles. The ozonated water moved continuously (10 ml/min) out of the glass column through a valve into a 100-ml reaction vessel in which the maximum possible ozone concentration was 1.4 to 1.6 mg of O₃ per liter. The bacterial or viral suspension was

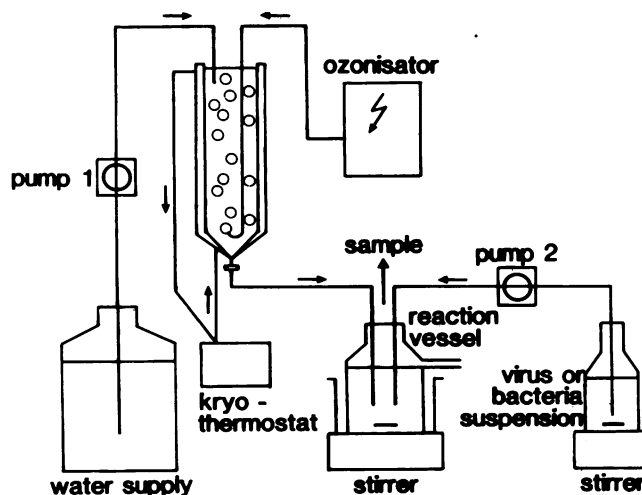


FIG. 1. Structure of experimental apparatus. There was a constant flow of ozone and viral or bacterial suspensions into the reaction vessel.

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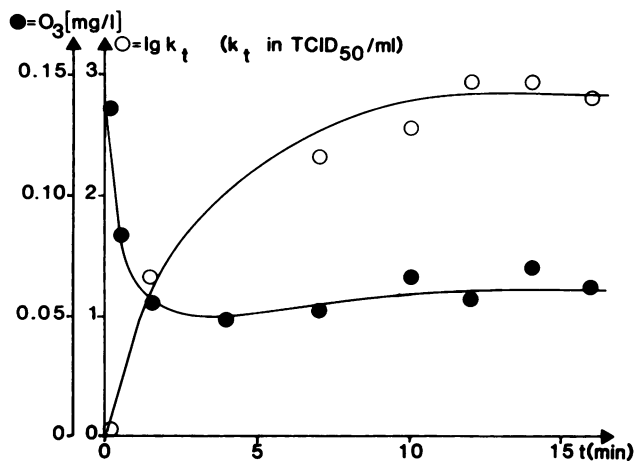


FIG. 2. HAV inflow rate E of $10^{3.5}$ TCID₅₀/ml per minute at 20°C. The ozone and virus concentrations are shown.

pumped continuously (1 ml/min) into this reaction vessel from a reserve container. Adequate mixing was ensured by magnetic stirrers in both the reserve container and the reaction vessel.

Samples were taken directly from the reaction vessel at fixed intervals: 1 ml for the virological and bacteriological examination and 3 ml for the determination of ozone concentration. The 1-ml samples were put into tubes containing 30 μ l of a 0.1% Na₂S₂O₃ solution.

Reaction kinetics. Constant flow conditions were used to ensure constant ozone concentration, which is prerequisite for a first-order reaction. Equation 1 describes the modified inactivation process (11).

$$K_t = K_o e^{-\lambda t} \quad (1)$$

where K_t is the concentration of viruses or bacteria at time t (TCID₅₀/milliliter or CFU/milliliter), K_o is the initial concentration of viruses or bacteria, and λ is the inactivation constant (minute⁻¹).

For maintenance of steady flow, the inflow rate (2), E , was calculated as follows:

$$E = Mp/V \quad (2)$$

where E is the virus and bacteria inflow rate (TCID₅₀/milliliter per minute or CFU/milliliter per minute), M is the initial concentration (TCID₅₀/milliliter or CFU/milliliter) of

TABLE 1. HAV inactivation by ozone at 20°C ($E = 10^{3.5}$ TCID₅₀/ml per minute)

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K_{∞} ^a (TCID ₅₀ /ml)	λ (min ⁻¹)	$t(10^4)$ (min)	Reduction ^b (%)
0.10	0.03	$10^{3.5}$	1.0	9.20	0.0
0.14	0.06	$10^{2.9}$	4.4	2.10	77.3
0.16	0.08	$10^{2.7}$	6.9	1.30	85.5
0.29	0.10	$10^{2.4}$	11.7	0.80	91.4
0.50	0.18	$10^{2.3}$	14.8	0.60	93.2
0.85	0.22	$10^{1.6}$	87.3	0.10	98.8
0.94	0.24	$10^{1.0}$	353.0	0.03	99.7
1.04	0.25	$10^{0.7}$	599.0	0.02	99.8
1.22	0.38	$<10^{0.7}$	ND ^c	ND	100.0

^a K_{∞} , Constant concentration of HAV.

^b Reduction from inflow to outflow.

^c ND, No virus activity detected in 0.8 ml.

TABLE 2. HAV inactivation by ozone at 10°C

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K_{∞} ^a (TCID ₅₀ /ml)	λ (min ⁻¹)	$t(10^4)$ (min)	Reduction ^b (%)
0.32	0.06	$10^{2.4}$	12.2	0.75	91.8
0.48	0.10	$10^{1.7}$	68.9	0.13	98.5
0.79	0.27	$<10^{0.7}$	- ^c	-	100.0

^a K_{∞} , Constant concentration of HAV.

^b Reduction from inflow to outflow.

^c -, Negative.

viruses and bacteria, p is the pumping rate of viruses and bacteria (in milliliters per minute), and V is the volume (in milliliters) of the reaction vessel.

Equation 1, taken with the inflow rate E , gives

$$K_t = (E/\lambda) (1 - e^{-\lambda t}) \quad (3)$$

The inactivation constant, λ , was calculated for $t \rightarrow \infty$ with the constant concentration (K_{∞})

$$K_{\infty} = E/\lambda \quad (4)$$

The effectiveness of ozone in inactivating the various organisms was quantified by calculating the time required for reduction by a factor of 10^4 in each instance.

$$t(10^4) = \ln(10^4)/\lambda \quad (5)$$

Figure 2 shows the constant flow conditions of the HAV experiment. Constant concentrations of ozone and HAV were established after about 10 min, with an initial ozone concentration of 0.14 mg of O₃ per liter and an HAV inflow rate E of $10^{3.5}$ TCID₅₀/ml per minute.

RESULTS

The results of the inactivation experiments are shown in Tables 1 to 8.

Table 1 shows the inactivation of HAV at 20°C.

Although there was no measurable inactivation of HAV at 0.03 mg of O₃ per liter, 77.3% of HAV was inactivated when the ozone concentration was doubled. The die-off constant was 11.7 min⁻¹ at 0.1 mg of O₃ per liter, and reduction by a factor of 10^4 was calculated as complete after 0.8 min. HAV was almost completely inactivated at 0.25 mg of O₃ per liter, and no surviving viruses were found at 0.38 mg of O₃ per liter.

HAV die-off was considerably more rapid when the temperature was reduced from 20 to 10°C (Table 2).

Reduction by a factor of 10^4 required only about 1/4 as much time at 10°C as at 20°C, and inactivation was 91.8%

TABLE 3. PV1 inactivation by ozone at 20°C

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K_{∞} ^a (TCID ₅₀ /ml)	λ (min ⁻¹)	$t(10^4)$ (s)	Reduction ^b (%)
0.03 ^c	0.02	$10^{2.7}$	12.0	45.0	91.87
0.09 ^c	0.04	$10^{2.2}$	43.0	12.7	97.71
0.17 ^d	0.10	$10^{1.0}$	6,000.0	0.1	99.98
0.19 ^c	0.13	$<10^{0.7}$	- ^e	-	100.00

^a K_{∞} , Constant concentration of PV1.

^b Reduction from inflow to outflow.

^c $E = 10^{3.8}$ TCID₅₀/ml per min.

^d $E = 10^{4.8}$ TCID₅₀/ml per min.

^e -, Negative.

TABLE 4. PV inactivation by ozone at 10°C
($E = 10^{3.6}$ TCID₅₀/ml per min)

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K _∞ ^a (TCID ₅₀ /ml)	λ (min ⁻¹)	t(10 ⁴) (s)	Reduction ^b (%)
0.04	0.02	10 ^{1.6}	95.5	5.8	98.95
0.05	0.03	10 ^{1.4}	153.0	3.6	99.34
0.13	0.08	<10 ^{0.7}	- ^c	-	100.00

^a K_∞, Constant concentration of PV1.^b Reduction from inflow to outflow.^c -, Negative.

complete at 0.06 mg of O₃ per liter. No further viruses were detectable at 0.27 mg of O₃ per liter.

PV1 was considerably more sensitive to ozone than HAV was. Table 3 shows the inactivation of PV1 at 20°C.

The time required for reduction by a factor of ca. 10⁴ at a steady concentration of 0.1 mg of O₃ per liter was calculated at 1/10 s for PV1 but 47 s for HAV. PV1 activity was already undetectable at 0.13 mg of O₃ per liter.

PV1 inactivation was also tested at 10°C. Table 4 shows the clear difference in resistance at the two temperatures. The inactivation constant for PV1 at 0.02 mg of O₃ per liter was 95.5 min⁻¹ at 10°C but only 12 min⁻¹ at 20°C. Viruses were no longer demonstrable at 0.08 mg of O₃ per liter.

E. coli was tested as an indicator bacterium for fecal contamination of water. Table 5 shows the results of its inactivation at 20°C.

E. coli was only slightly more sensitive to ozone than HAV was. The time required to reduce it by a factor of ca. 10⁴ at 20°C was 0.6 min at 0.1 mg of O₃ per liter compared with 0.8 min for HAV. PV1 was reduced some 300 times faster than *E. coli* was at this ozone concentration. No further surviving *E. coli* cells were detectable at 0.31 mg of O₃ per liter. Inactivation of *E. coli*, like that of HAV and PV1, was much faster at 10°C and was already complete at 0.1 mg of O₃ per liter (Table 6).

L. pneumophila serogroup 6 was used as further test bacterium for ozone resistance at 20°C and proved considerably more ozone resistant than did *E. coli* (Table 7).

At 20°C, λ was 4 min⁻¹ for *L. pneumophila* and 95.5 min⁻¹ for *E. coli* at ca. 0.17 mg of O₃/liter, but 14.8 min⁻¹ for HAV, that is, *L. pneumophila* was also more resistant than HAV. *L. pneumophila* was 99.5% inactivated at 0.32 mg of O₃ per liter, whereas HAV was already 99.7% inactivated at 0.24 mg of O₃ per liter.

Two series of experiments were undertaken with *B. subtilis*. However, there was no significant reduction of the number of spores with the ability to propagate at 0.18 or 1.2 mg of O₃ per liter.

TABLE 5. *E. coli* inactivation by ozone at 20°C

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K _∞ ^a (CFU/ml)	λ (min ⁻¹)	t(10 ⁴) (min)	Reduction ^b (%)
0.10 ^c	0.07	3.4 × 10 ⁴	2.9	3.1	66.0
0.15 ^c	0.10	6.8 × 10 ³	14.7	0.6	93.2
0.36 ^d	0.16	3.0 × 10 ⁴	95.5	0.1	99.0
0.43 ^c	0.31		CI ^e	CI	100.0

^a K_∞, Constant concentration of *E. coli*.^b Reduction from inflow to outflow.^c E = 10⁵ CFU/ml per min.^d E = 3 × 10⁶ CFU/ml per min.^e CI, Complete inactivation.TABLE 6. *E. coli* inactivation by ozone at 10°C
($E = 10^6$ CFU/ml per min)

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K _∞ ^a (CFU/ml)	λ (min ⁻¹)	t(10 ⁴) (min)	Reduction ^b (%)
0.10	0.06	2.4 × 10 ⁴	42.7	0.220	97.65
0.25	0.10	7.5 × 10 ³	134.0	0.070	99.25
0.27	0.17	1.0 × 10 ²	10,000.0	0.001	99.99
0.36	0.31		CI ^c	CI	100.00

^a K_∞, Constant concentration of *E. coli*.^b Reduction from inflow to outflow.^c CI, Complete inactivation.

DISCUSSION

The inactivation experiments showed clear-cut differences in resistance to ozone among the viruses and bacteria tested. Figure 3 gives an overview of their inactivation behavior. Die-off constants rose nearly exponentially as ozone concentration increased. A dose-dependent response is recognizable.

A temperature dependency of resistance to ozone by HAV and *E. coli* is clearly recognizable; ozone effectiveness diminished as temperature rose. That HAV was more resistant to ozone than PV1 coincides with the results of Flehmig et al. (4), in which HAV showed greater stability under heat than PV1. It is difficult to explain why this is so, since the two viruses are very similar in structure (17). Comparative experiments have indicated that ozone inactivates viruses and bacteria faster than chlorine (8, 14, 16), but caution is necessary because of differences in laboratory conditions and particularly because these experiments were not performed with constant concentrations under constantly flowing water.

Even though ozone concentrations corresponding to our calculated values for λ and t(10⁴) may vary in other methods of measurement, the dependence on temperature and the order of resistance to ozone of the microorganisms remain constant. Our experiments showed the following order of resistance to ozone at 20°C: PV1 < *E. coli* < HAV < *L. pneumophila* serogroup 6 < *B. subtilis* spores. Supplementary experiments with spores will be conducted in the future.

The inactivation time was defined as the time required for reduction by a factor of 10⁴. Inactivation times, except for those of the spores, amounted to seconds or a few minutes.

In river water, up to about 300 PFU enteroviruses per liter

TABLE 7. *L. pneumophila* serogroup 6 inactivation by ozone at 20°C

Initial O ₃ concn (mg/liter)	Constant O ₃ concn (mg/liter)	K _∞ ^a (CFU/ml)	λ (min ⁻¹)	t(10 ⁴) (min)	Reduction ^b (%)
0.20 ^c	0.14	2.4 × 10 ⁴	2.6	3.50	63.1
0.26 ^c	0.17	1.6 × 10 ⁴	4.0	2.30	75.4
0.50 ^d	0.20	1.0 × 10 ⁴	8.0	1.20	87.5
0.64 ^d	0.25	6.0 × 10 ³	13.2	0.70	92.5
0.85 ^d	0.28	1.8 × 10 ³	43.7	0.20	97.8
1.00 ^d	0.32	4.3 × 10 ²	185.0	0.05	99.5
1.40 ^e	1.20		CI ^f	CI	100.0

^a K_∞, Constant concentration of *L. pneumophila* serogroup 6.^b Reduction from inflow to outflow.^c E = 6.5 × 10⁴ CFU/ml per min.^d E = 8 × 10⁴ CFU/ml per min.^e E = 2 × 10⁷ CFU/ml per min.^f CI, Complete inactivation.

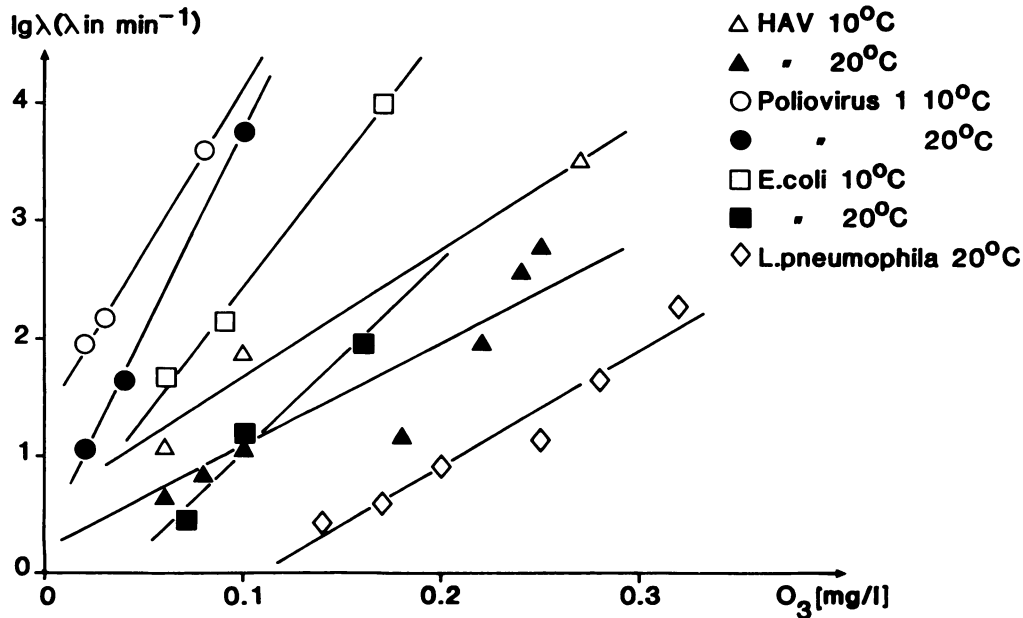


FIG. 3. Inactivation rates of HAV, PV1, *E. coli*, and *L. pneumophila* serogroup 6 by ozone at 10 and 20°C.

may be present (18). In raw water, 3 to 100 most probable number of cytopathogenic units (MPNCU)/liter were found, and 0.02 to 0.0006 MPNCU/liter were still present after chlorination, sedimentation, filtration, and ozonation (15). In some samples, enteroviruses or rotaviruses were still isolatable, even at residual chlorine levels of more than 0.2 mg/liter and a total coliform rate of <1 CFU/100 ml (13).

Considering these conditions, our rates of inflow during the experiments were relatively high: ca. 10^4 TCID₅₀/ml per minute for the viruses and ca. 10^6 CFU/ml per minute for the bacteria.

The concentrations of ozone vary in actual drinking water disinfection. Some researchers consider a residual ozone concentration of 0.4 mg/liter for 4 min adequate (1).

Generally, our results confirm this: 10^4 reduction time, $t(10^4)$, at this concentration was calculated to be only a few seconds. On the other hand, 1.2% of the initial HAV concentration remained present at every point in our reaction container (including the outflow) under continuous flow, ideal mixing, and ozone concentrations causing a reduction time, $t(10^4)$, of 0.1 min.

Since the equations used here also apply for large vessels, e.g., drinking water treatment basins, ideal mixing is not optimal for drinking water disinfection. Rather, water treatment containers should be designed in such a way as to ensure a specified retention time for every water molecule or microorganism.

Viruses and bacteria in certain population areas often remain detectable in drinking water even after disinfection. This is chiefly due to aggregate formation and protection by colloids. In addition to an adequate reaction time and a sufficiently high concentration of the antimicrobial substance, prefiltration for the elimination of aggregates should therefore not be omitted from drinking water preparation.

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