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Liquid-Phase Study of Ozone Inactivation of Venezuelan Equine Encephalomyelitis Virus

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Ozone, in a liquid-phase application, was evaluated as a residue-free viral inactivant that may be suitable for use in an arboviral research laboratory. Commonly used sterilizing agents may leave trace residues, be flammable or explosive, and require lengthy periods for gases or residues to dissipate after decontamination of equipment such as biological safety cabinets. Complete liquid-phase inactivation of Venezuelan equine encephalomyelitis virus was attained at 0.025 mg of ozone per liter within 45 min of exposure. The inactivation of $10^{6.5}$ median cell culture infective doses ($CCID_{50}$ of Venezuelan equine encephalomyelitis virus per milliliter represented a reduction of 99.99997% of the viral particles from the control levels of $10^{7.25-7.5}$ $CCID_{50}$/ml. A dose-response relationship was demonstrated. Analysis by polynomial regression of the logarithmic values for both ozone concentrations and percent reduction of viral titers had a highly significant r^2 of 0.8 ($F = 63.6$; df = 1, 16). These results, together with those of Akey (J. Econ. Entomol. 75:387–392, 1982) on the use of ozone to kill a winged arboviral vector, indicate that ozone is a promising candidate as a sterilizing agent in some applications for biological safety cabinets and other equipment used in vector studies with arboviruses.

Viral inactivation by physical, chemical, and biological means has been studied for many years for application in a wide variety of public and veterinary health fields, biomedical research laboratories, and medical care facilities. Of these applications, the most common is probably the production of noninfective viral antigens for vaccines or viral serotyping (14). Other uses include production of potable water, treatment of wastewater, sterilization of hospital or laboratory equipment, laboratories, and bioclean rooms, and disinfection or sterilization of surfaces and spaces potentially contaminated by viruses. Laboratories that deal daily with viruses as a part of research, diagnostic, or production activities often are concerned with the above applications.

Arboviral research presents additional needs, as both the virus and the arthropod vectors must be considered in sterilization procedures. In such research, biological safety cabinets are often modified by sealing openings and attaching gloves or sleeves so that research can be safely conducted with a virus and a vector without escape of the arthropod vector or release of the virus. The vapor-phase (gaseous) sterilizing agents now used with such cabinets may leave trace residues, e.g., formaldehyde, or are flammable or explosive, e.g., ethylene oxide, and may require lengthy aeration periods to dissipate gases or residues after sterilization.

In an effort to find more suitable sterilizing agents for arboviral research, we have considered the use of ozone, the most powerful oxidizer known (25). Ozone has a virucidal activity against poliomyelitis virus far surpassing that of chlorine (8). Ozone for viral inactivation has had common use as a liquid-phase application for potable water production and wastewater treatment (4, 10, 20, 27). Ozone was used in a (vapor-phase) test as early as 1942 by Elford and van den Ende in an effort to reduce airborne bacterial infections (12).

Recently, ozone has been tested as a vapor-phase sterilizing agent to inactivate microorganisms in bioclean and hospital rooms. Yamayoshi (28) compared ozone (0.120 mg/liter) with formaldehyde for sterilizing a bioclean room. This concentration of ozone required 72 h of exposure to ensure sterilization of *Bacillus subtilis*, although *Penicillium* sp. and *Escherichia coli* were much more susceptible to sterilization by ozone. In a similar study, Masaoka et al. (19) repeatedly used 0.80 mg of ozone per liter to treat three bioclean rooms. They compared ozone with formaldehyde and concluded that, although formaldehyde was effective as a bactericide when penetration was necessary, ozone was superior because it was easy to control, left no residues, and could be rapidly expelled by ventilation. Dyas et al. (11) used relatively low concentrations of ozone to kill bacterial and fungal species in (i) a cupboard (0.087 m^3) and (ii) a hospital room (66 m^3). They succeeded at ozone concentrations of 0.0006 to 0.0018 mg/liter with a 4-h exposure period in the cupboard but were not able to detect ozone in the much larger hospital room (ca. 758-fold larger than the cupboard).

Viral inactivation studies with candidate agents can be divided into a two-step sequence of investigation: (i) liquid phase and (ii) vapor phase. Here we report on the use of ozone for liquid-phase inactivation of Venezuelan equine encephalomyelitis (VEE) virus, an arboviral pathogen that is commonly used in biological safety cabinets as recommended for safe laboratory practice.

MATERIALS AND METHODS

Ozone. Ozone was produced with a model 10 generator (Ampliformer International, Littleton, Colo.) as previously described (1). An iodometric method (21), spectrophotometrically confirmed (24), was used to determine the concentrations of ozone bubbled into the test medium. Draeger gas detector tubes (Bullard Safety, Denver, Colo.) that could indicate ozone at 0.0001 to 0.0028 and 0.02 to 0.60 mg/liter were used to determine ozone concentrations in the

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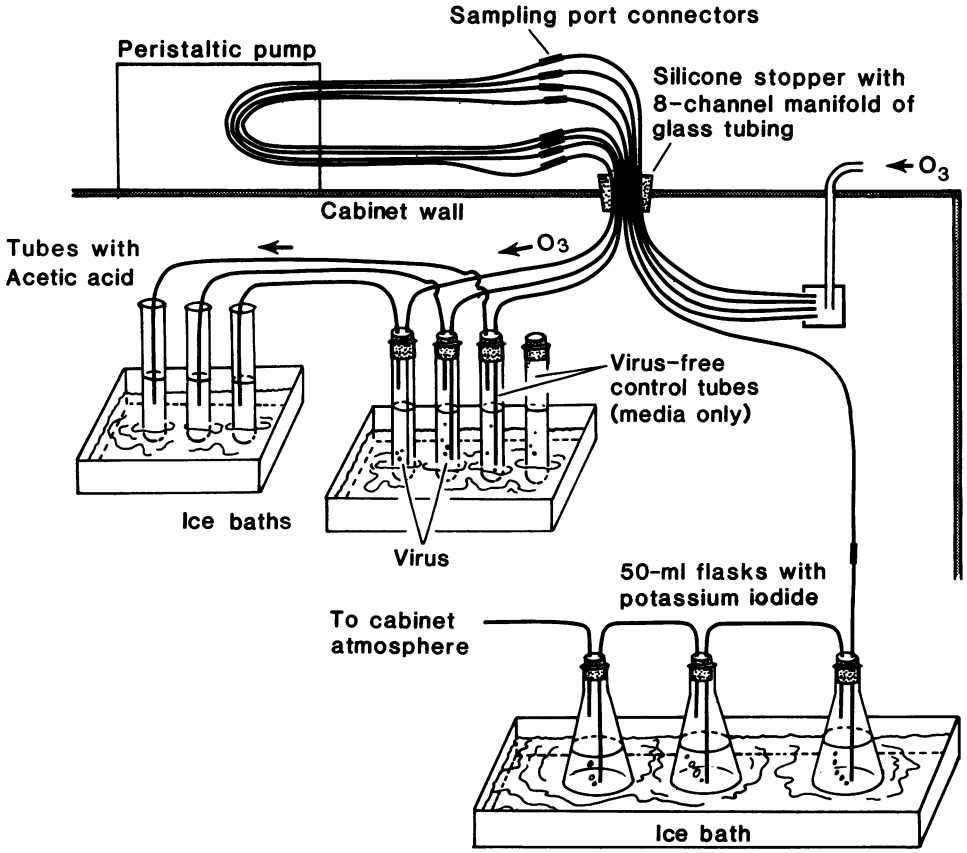

FIG. 1. Schematic drawing of pumping system used to deliver ozone to test tubes containing virus and medium or medium only and to a series of trap flasks. Ozone was also sampled in line from the delivery lines by disconnecting a line connector at the pump.

test cabinet (1). Tubes for 0.0001 to 0.0028 mg/liter were also used to monitor ozone concentrations of ambient air for personnel safety in the laboratory. A Draeger Multi-Gas Detector pump (model 21/31) was used to draw sample gases through the tubes.

Test apparatus. Tests were conducted in a closed cabinet (0.3 m^3) previously described (1). Ozone was introduced into the test cabinet through a R3603 Tygon entry line. Four smaller Tygon lines were connected to the ozone entry line inside the cabinet (Fig. 1). The other ends of these four lines were each connected to one channel of an eight-channel manifold of glass tubing that also passed through the cabinet. Outside the cabinet, via the eight-channel manifold, these four lines were connected to a four-channel peristaltic pump, and the four output lines from the pump were connected to the other four channels of the eight-channel manifold and passed back into the cabinet. By this arrangement, the inlet and outlet ports of each pump channel were inside the cabinet, but the pump remained outside. A sampling port connector on each channel line on the outside of the cabinet allowed ozone to be sampled in each channel line. By this means, ozone production and variations in level were monitored during the tests by sampling the ozone delivered in the channel lines. Inside the cabinet, the ozone in one of the four lines was bubbled into a series of three 50-ml Erlenmeyer flasks that contained 50 ml of cold potassium iodide (0 to 4°C ice bath). These three flasks were used to trap the incoming ozone for determination of the total ozone delivered by the system. In practice, the ozone was trapped by the first two flasks, and the third flask never received ozone. The other three lines were passed through three stoppered test tubes (7.53 by 1.96 cm diameter; 6 ml) held in a 0 to 4°C ice bath. The stoppers were custom-made of silicone rubber (1). The tubes contained 2 ml of solution. An output line from each of the three test tubes that received ozone passed excess ozone and any aerosol, possibly virus contaminated, into an inactivant of 4% acetic acid. This prevented the cabinet interior from being contaminated with virus in the event that ozone was an ineffecive inactivant.

Virus source and assay. The virus used in this study was an attenuated vaccine strain of VEE virus, TC-83 (3). Viral stocks were prepared in Vero (African green monkey kidney) cell cultures and stored until used in a stabilizing medium of buffered lactose-peptone (2). The viral concentration was $10^{7.25-7.50}$ median cell culture infectious doses ($CCID_{50}$)/ml. After being treated with ozone, serial 10-fold dilutions of the virus were made in Pucks saline solution A (23). Each of four rolling-tube cultures of Vero cells was inoculated with 0.1 ml per dilution of virus. Tubes were examined daily for cytopathic effect and incubated for 5 days. Earlier work had shown that this was a more sensitive assay than the plaque assay method. Viral titers were determined by the Spearman-Karber method (13).

Procedures. Ozone was bubbled into two tubes that contained both virus and cell culture medium and one tube of cell culture medium only (control). A fourth tube, which contained cell culture medium only, was sealed as a control free of ozone and virus. A fifth tube, also a control, that contained virus and cell culture medium was held outside the cabinet in a 4°C incubator and was not treated with ozone.

Exposure times ranged between 7.5 and 75 min, with two test replicates for each exposure time. After ozone testing, tubes for viral assay were held at 4°C for 24 h to allow residual ozone to dissipate; in preliminary tests, residual ozone killed the cells of the assay system if treated samples were assayed within 4 h after exposure to ozone. Residual ozone was not quantified in the tubes of cell culture medium (control) with respect to time but was detected by assay as late as 12 h after treatment. This effect lasted less than 24 h. Residual ozone concentrations in the trap flasks were determined immediately after the completion of each test and were used for the data analyses. Spot checks of the pH of treated and untreated preparations were made before and immediately after treatment. The pH of all tubes of cell culture medium (with and without virus) was 6.9 before treatment and was unchanged by ozonization.

Viral inactivation data were analyzed by first-, second-, and third-degree polynomial regression equation analysis together with analysis of variance (programs from Hewlett Packard Co., Denver, Colo.) and by probit analysis (9). Transformed (logarithmic, square, and square root) and untransformed values of several parameters were analyzed, e.g., time, ozone concentration, and reduction and percent reduction of viral titers (expressed as numbers of viral particles).

RESULTS

Reduction of VEE viral titers increased proportionally with increased ozone concentrations and exposure times (Table 1). Exposure times and the amount of ozone produced were highly correlated ($F = 139$; df = 1, 7; $r^2 = 0.95$), as determined by first-degree polynomial regression equation analysis (Fig. 2). This correlation was observed despite two apparent anomalies in the amount of ozone produced at 7.5 and 45 min of exposure time (Table 1). Examination of the regression plot showed that too little ozone was produced at 10 and 60 min and too much was produced at 45 min of exposure time. These differences reflected changes in the amount of ozone produced by the generator. Viral inactivation data were more highly correlated with ozone concentrations than with exposure times.

TABLE 1. Inactivation of VEE virus by ozone

Ozone concn (mg/liter)	Exposure (min)	Reduction of viral titer[a]	
		$CCID_{50}$/ml log_{10}	%
0.0007	10.0[b]	1.75	98.22172
		2.00	99.00000
0.0012	7.5	3.00	99.90000
		3.25	99.94377
0.0058	15.0	6.00	99.99990
		6.25	99.99994
0.0070	20.0	6.00	99.99990
		3.50	99.96838
0.0140	30.0	5.75	99.99982
		6.00	99.99990
0.0150	37.5	6.00	99.99990
		6.25	99.99994
0.0230	60.0[b]	6.50	99.99997
		6.25	99.99994
0.0250	45.0	6.25	99.99994
		6.50	99.99997
0.0350	75.0	6.50	99.99997
		6.50	99.99997

[a] Pretreatment titer was $10^{7.25}$ $CCID_{50}$/ml for 0.007to 0.0150 mg of ozone per liter and $10^{7.50}$ for 0.0230 to 0.0350 mg of ozone per liter. There were two replicates for each ozone concentration and exposure time.

[b] The amount of ozone produced was less than expected during these runs.

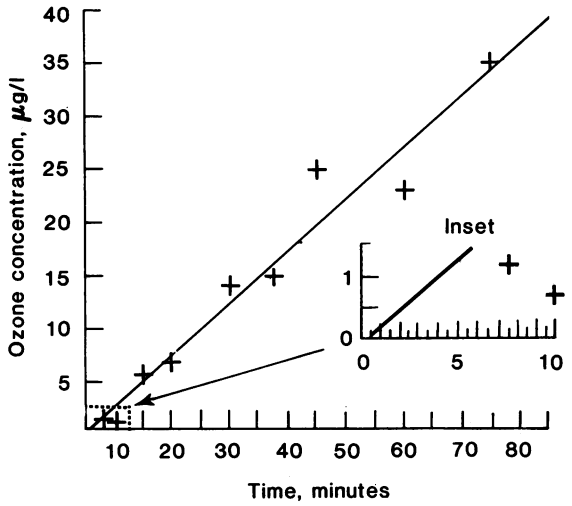

FIG. 2. Plot of exposure time against residual ozone concentration. Regression line was generated by a first-degree polynomial regression equation.

Complete inactivation of VEE virus by ozone was attained at ozone concentrations of 0.023 to 0.025 mg/liter and 45 to 60 min of exposure (Table 1). Treatment of VEE virus at 0.035 mg of ozone per liter, 75-min exposure time, did not increase viral inactivation. The inactivation measured was at the limit of viral detection of the assay method. The inactivation of $10^{6.5}$ $CCID_{50}$ of virus per ml represented a reduction of 99.99997% of the viral particles from the control levels of $10^{7.25}$ or $10^{7.5}$ $CCID_{50}$/ml. Viral inactivation data were analyzed most effectively by first-degree polynomial regression equation analysis of the logarithmic values both for ozone concentrations and for percent reduction of viral particles. The polynomial regression equation analysis of these parameters yielded a highly significant r^2 value of 0.8 with an F value of 63.6 (df = 1, 16) for the regression analysis (Fig. 3). Confidence intervals (26) were calculated also, and those for 95% are shown in Fig. 3. Treatment of data by second- and third-degree polynomial regression equation

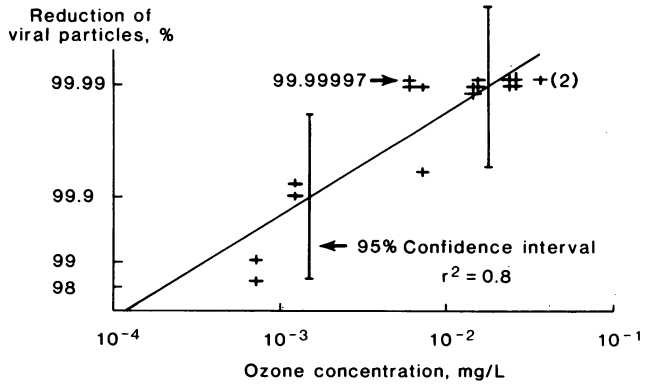

FIG. 3. Ozone inactivation of VEE virus. Inactivation is expressed as percent reduction of viral particles and was calculated as follows: $100 - 100V/V_0$, in which V was viral titer after treatment and V_0 was the initial (control) titer. The regression line was generated by a first-degree polynomial regression equation.

analysis increased the r^2 but did not reduce the confidence limits significantly. Because the upper threshold of viral reduction was reached at a level of 0.023 mg of ozone per liter, additional analyses were made on two data sets that consisted of the points up to or including this threshold (first 12 or 14 data points in Table 1). Polynomial regression equation analysis of these data sets produced r^2 values that were not significantly different from those of the complete data set; additionally, both the reduced and full data sets were untractable to probit analysis.

DISCUSSION

The results clearly indicated that VEE virus, an alphavirus, was effectively and completely inactivated by ozone at 0.023 to 0.025 mg of residual ozone per liter. The treatment reduced the initial viral titers by 6.25 to 6.5 $\log_{10}$. It is probable that this concentration of ozone would be effective against similar viruses.

Comparisons of the results reported here with those of other studies are difficult, because tests of viral inactivants are not generally standardized, unlike comparable tests with bacteria, e.g., use of *E. coli* and *B. subtilis* with formaldehyde or ethylene oxide. Nevertheless, some parameters that must be considered in comparisons of these results with other studies on ozone for viral inactivations include (i) the titer of the virus to be inactivated, (ii) the time used or needed for inactivation, (iii) the presence of materials that protect the virus from inactivation, (iv) ozone demand of the viral medium, and (v) the pH of the viral medium. Also, in comparing studies that have relatively large differences between the viral titers used, the percent reduction in viral particles is an appropriate parameter for reporting the efficiency of the treatment. In this study, the efficiency of the treatment as expressed by percent particle reduction was such that only 3 of every 10,000 viral particles remained. The reduction may have been even greater, because that value represented the limit of the viral assay system itself.

Recent liquid-phase studies (6, 17, 18) on the mechanisms and mechanics of virus inactivation by ozone have used higher viral titers than did many of the earlier studies on ozone inactivation of viruses for water treatment. We chose to study inactivation of $10^{7.25-7.5}$ $CCID_{50}$ of VEE virus per ml, because that is representative of the titers of our solutions of stock viruses and is the "worst spill case" that we would encounter.

The concentrations of ozone used in this study were lower than those used in a number of other studies on liquid-phase inactivation of enteric viruses. To inactivate poliovirus type 1, Katzenelson et al. (17) and Roy et al. (22) used concentrations of ozone between 0.06 and 2.50 mg/liter and 0.30 and 0.80 mg/liter, respectively. The several minutes needed to achieve inactivation in our study can probably be attributed to the lower ozone concentrations and possibly to the presence of cellular fragments or proteins in the medium that may have protected the virus from the ozone. The latter could have been verified had we quantified the ozone demand of the virus-containing samples. This was not done because of safety considerations with respect to the high titers of virus used in the study.

Katzenelson et al. (16) and Kim et al. (18) considered a dose-response relationship to ozone. Their studies indicated that a two-stepped reaction may be involved. Our study indicated a definite dose-response relationship, although the 95% confidence intervals were rather large (Fig. 3). This agrees with a second study by Katzenelson et al. (17) that showed a dose-response relationship for poliovirus with a plot of $\log_{10}$ ozone concentration $\times$ $\log_{10}$ time and an associated r value of 0.8. Inactivation plots shown by Burleson et al. (7) supported a dose-response relationship for ozone treatment of vesicular stomatitis virus and encephalomyocarditis virus. They did not observe an all-or-none effect. The difficulty of observing a dose-response relationship is a function of two related parameters: ozone concentration and sampling intervals during exposure time. If the ozone concentrations and efficiency of exposure of the agent to ozone are relatively high, then the sampling interval must be small enough to demonstrate the response. For many studies of potable and waste water, these sampling times are apparently on the order of seconds or fractions thereof. Inactivation studies may benefit by using ozone concentrations low enough to permit a sampling interval of minutes rather than seconds.

Although this study showed that VEE virus can be inactivated by ozone, it would be informative to understand the mechanism of alphavirus inactivation by ozone. VEE virus has a core of single-stranded RNA surrounded by a capsid and a lipid envelope (15). The inactivation mechanism may involve a reaction of ozone with the membrane constituents of the lipid envelope as discussed by Bolton et al. (5) for vesicular stomatitis virus. The possibility of damage to coat proteins and to RNA itself has been reviewed by Kim et al. (18) and by Roy et al. (22). The latter investigator reported that poliovirus was inactivated by damage to the RNA.

Additionally, a study on vapor-phase inactivation of VEE viral aerosols will be needed before ozone can be used as a sterilizing agent in biological safety cabinets. This is particularly true because it is generally accepted that aerosols of VEE virus are relatively stable (15). Also, it will be necessary to consider cost factors, delivery difficulties, availability of ozone-resistant materials, and safety factors. The latter two topics have been discussed by Akey (1). Nevertheless, based on the present study and on the earlier work reported by Akey (1) that ozone was an effective fumigant against a vector of bluetongue virus, the biting gnat *Culicoides variipenis* (Coquillett), ozone appears to be a promising candidate as a sterilizing agent in some applications for biological safety cabinets and other equipment used in vector studies with arboviruses.

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