APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1982, p. 603–608
0099-2240/82/030603-06$02.00/0

Vol. 43, No. 3

Ozone Inactivation of Cell-Associated Viruses

MAURA A. EMERSON,† OTIS J. SPROUL,‡ AND CHARLES E. BUCK*

Departments of Sanitary Engineering and Microbiology, University of Maine, Orono, Maine 04469*

Received 23 July 1981/Accepted 30 November 1981

The inactivation of HEp-2 cell-associated poliovirus (Sabin 1) and coxsackievirus A9 was investigated in three experimental systems, using ozone as a disinfectant. The cell-associated viral samples were adjusted to a turbidity of 5 nephelometric turbidity units. The cell-associated poliovirus and coxsackievirus samples demonstrated survival in a continuous-flow ozonation system at applied ozone dosages of 4.06 and 4.68 mg/liter, respectively, for 30 s. Unassociated viral controls were inactivated by the application of 0.081 mg of ozone per liter for 10 s. Ultrasonic treatment of cell-associated enteric viruses did not increase inactivation of the cell-associated viruses. The batch reactor with a declining ozone residual did not effect total inactivation of either cell-associated enteric virus. These cell-associated viruses were completely inactivated after exposure to ozone in a batch reactor using continuous ozonation. Inactivation of cell-associated poliovirus required a 2-min contact period with an applied ozone dosage of 6.82 mg/liter and a residual ozone concentration of 4.70 mg/liter, whereas the coxsackievirus was completely inactivated after a 5-min exposure to an applied ozone dosage of 4.81 mg/liter with an ozone residual of 2.18 mg/liter. These data indicate that viruses associated with cells or cell fragments are protected from inactivation by ozone concentrations that readily inactivate purified virus. The cell-associated viral samples used in this research contained particles that were 10 to 15 μm in size. Use of a filtration system before ozonation would remove these particles, thereby facilitating inactivation of any remaining viruses associated with cellular fragments.

The necessary reuse of large quantities of renovated wastewater for human consumption may pose a potential health hazard to large segments of the population. The documentation of numerous outbreaks of waterborne disease lends credence to this possibility (4, 5, 11). Wastewater treatment processes alone are inadequate for the elimination of viral pathogens (15). The transmission of viral disease by low-level contaminated water supplies must also be considered as a possible source of endemic disease. Therefore, it is necessary to rely on some method of terminal disinfection to combat this potential health hazard (1).

It has been shown that viruses in the natural environment may be adsorbed to or embedded within solid materials (3, 12). The viral complexes that result may exhibit a completely different response to disinfection in comparison with viruses in the free state, enabling the infectious agent to pass through the disinfection process in a viable state. As a result of this possible particulate protection of virus and bacteria, the national interim primary drinking water regulations allow a maximum contamination level of 1 nephelometric turbidity unit (NTU), with up to 5 NTU allowed if it can be demonstrated that this turbidity level does not interfere with disinfection, prevent maintenance of effective disinfection throughout the distribution system, or interfere with microbial determinations.

Hoff (8) found that turbidity interference, when chlorine is used as the disinfectant, depended more on the type than on the level of turbidity. Interference with disinfection was very limited or nonexistent when turbidity was caused by inorganic materials such as clay or inorganic precipitates. However, organic materials such as cells and solids which were associated with coliforms showed a definite protective effect. Further evidence is needed to determine exactly what conditions are necessary for the destruction of microorganisms during disinfection when organic materials causing turbidity are present.

Further evidence is needed to elucidate the parameters necessary for the inactivation of enteric viruses associated with organic materials. The objective of this research was to determine the disinfection potential of ozone on se-

† Present address: Chemistry Department, United States Naval Academy, Annapolis, MD 21402.

‡ Present address: Department of Civil Engineering, The Ohio State University, Columbus, OH 43210.

lected enteric viruses associated with HEp-2 cells, which would simulate a potential source of pollution in renovated water. We used human epithelial cells (HEp-2) infected with poliovirus (Sabin type 1) or coxsackievirus A9 to simulate cells which may be sloughed from the intestinal tract of infected individuals.

MATERIALS AND METHODS

Glassware and solutions. Glassware used in sample preparation or ozone concentration determination was treated to ensure that it did not exert a demand for competitive oxidation by the ozone. Glassware was cleaned with chromic acid and then rinsed eight times with tap water and five times with distilled water. After immersion in a strong ozone solution for 1 to 3 h, the glassware was dried overnight at 180°C and stored until used.

Ozone demand-free triple-distilled water was prepared by subjecting the water to high concentrations of ozone. Any excess ozone was removed by autoclaving or allowed to dissipate at room temperature. An ozone demand-free 0.0005 M mono- and dibasic sodium phosphate, pH 7, buffer solution was prepared in 0.01 M NaCl. This buffer solution was used in all of the experimental work with the exception of control samples of unassociated poliovirus and coxsackievirus. The control samples had the free viruses suspended in ozone demand-free 0.01 M NaCl buffered to pH 7 with $NaHCO_3$.

Cell cultures. The HEp-2 cell line was propagated in a growth medium consisting of Eagle minimal essential medium with Earle balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% mycoplasma-tested and virus-screened newborn calf serum (GIBCO). Penicillin sodium NF (The Upjohn Co., Kalamazoo, Mich.), 200 U/ml, streptomycin sulfate USP (Eli Lilly & Co., Indianapolis, Ind.), 200 μg/ml, and amphotericin B (E. R. Squibb & Sons, New York), 5 μg/ml, were routinely added to growth and maintenance media.

The LLC-MK2 cell line was maintained as described for HEp-2 cells. However, the antibiotic levels incorporated in the growth and maintenance media were 100 U of penicillin sodium NF, 100 μg of streptomycin sulfate USP, and 5 μg of amphotericin B per ml.

Virus stock. Concentration and purification of unassociated poliovirus (Sabin type 1) and coxsackievirus A9 were conducted according to the procedures described by Guskey and Wolff (7), Boardman and Sproul (2), and Foster et al. (6). Crude virus stocks of poliovirus and coxsackievirus which were used to infect HEp-2 cells to obtain cell-associated virus were prepared as described by Hoff (8).

Titration of viruses. The method of Wolf and Quimby (17), with minor modifications, was used for enumeration of poliovirus and coxsackievirus. If more than 10 plates were required, cell suspensions from several 32-oz (ca. 946-ml) prescription bottles were pooled to obtain a uniform cell suspension. A 5-ml portion of the pooled cell suspension was transferred to each tissue culture plate and incubated at 37°C in an atmosphere of 5% CO_2 until confluent monolayers were obtained.

An overlay, consisting of 2 parts of melted 2% purified agar at 50°C, 3 parts of 2× minimal essential medium containing 4% newborn calf serum, penicillin (300 U/ml), streptomycin sulfate (300 μg/ml), and amphotericin B (5 μg/ml) at 37°C, was prepared by inverting the mixture several times. A 5-ml portion of the overlay was transferred to each plate containing the adsorbed virus. After the overlay had solidified, the plates were incubated for 84 to 96 h at 37°C in an atmosphere of 5% CO_2 and 100% humidity.

HEp-2 cell-associated virus. The procedures of Hoff (8) were used to obtain cell-associated viruses. The initial sample for exposure to ozone was prepared by suspending the pellet in 15 ml of buffer solution. Turbidity was determined with a Hellige turbidimeter (Hellige Inc., Garden City, N.Y.) and expressed in nephelometric turbidity units. The turbidity of the initial sample was adjusted to a level to give a final turbidity of either 1 or 5 NTU after dilution in the reactor. A portion of the initial sample was reserved to determine the titer of the cell-associated virus before ozonation.

Determination of ozone concentration. The ozone concentration was determined by the spectrophotometric method of Shechter (14) with some modifications. The preparation of a standard curve for measurement of ozone concentrations ranging from 0.01 to 0.03 mg/liter required the addition of equal portions of neutral potassium iodide to the triple-distilled water reference blank as well as to the standard ozone solution. This served to nullify adsorbance due to the presence of the potassium iodide. This addition was not necessary when measuring ozone levels greater than 0.03 mg/liter.

Disinfection of cell-associated viruses. Samples of cell-associated viruses were exposed to ozone in the sharp continuous-flow apparatus (13), a batch reactor with a continually declining ozone residual, and a batch reactor with constant ozone residual. The Sharp continuous-flow apparatus as modified for this work is described by Foster et al. (6).

The declining ozone residual experiments were carried out in a 500-ml Erlenmeyer flask fitted with a stopcock sampling post set in the bottom of the flask. The flask was filled with 500 ml of buffer solution which had been preozonated to the desired ozone value. The contents were mixed throughout the experiment with a magnetic mixer. Triplicate portions were taken for the initial ozone determination. The cell-associated viruses were added with a syringe to give the desired turbidity levels in the solution. Three milliliter samples for virus determination were taken at specified intervals and immediately mixed with 1 ml of 0.025 N sodium thiosulfate to destroy the ozone.

A 2,000-ml reagent bottle was used for the constant ozone concentration reactor. Ozone was added continuously during the experiment through a glass sparger placed near the bottom of the bottle. The solution was preozonated to obtain the desired initial ozone concentration before addition of the cell-associated viruses. Sampling for ozone and virus determination was done as in the declining-rate reactor.

Ultrasonic treatment of viruses. A comparison of the ozone inactivation of HEp-2 cell-associated poliovirus was performed in the Sharp apparatus, using duplicate portions of a sample, one of which had been sonically treated. The initial sample was divided, and one por-

tion was treated for 30 s at 50 kc/s with a sonic dismembrator.

Light microscopy. Light micrographs were obtained by using a 35-mm camera attached to a Phase Star, Spencer microscope. Replicate samples of presonicated HEp-2 cell-associated poliovirus and HEp-2 cell-associated poliovirus which had not been sonically treated were withdrawn after 10, 20, and 30 s of exposure to ozone in the Sharp apparatus. The physical state of the remaining cellular material at each contact time was compared with that of cellular material of the initial samples which were not exposed to ozone. Initial samples were diluted with buffer solution to a turbidity of 5 NTU to facilitate comparison with samples from the Sharp apparatus.

RESULTS

Inactivation of HEp-2 cell-associated poliovirus (Sabin type 1) in the Sharp apparatus. The association of poliovirus with a mass of cellular material protected the virus from inactivation after exposure to various concentrations of ozone in the Sharp apparatus (Table 1). Although the unassociated poliovirus was completely inactivated at applied ozone concentrations as low as 0.28 mg/liter, applied ozone dosages as high as 4.06 mg/liter, with a 40-s residual ozone concentration of 2.56 mg/liter, did not totally inactivate the cell-associated poliovirus. However, the increase in ozone concentration resulted in an increase in the inactivation of the cell-associated poliovirus.

The protective effect of physical cohesion of cellular material was investigated by using replicate samples, one of which had been ultrasonically treated before ozonation in the Sharp apparatus. The ultrasonic treatment of the cell-associated virus complex before ozonation resulted in increased inactivation of poliovirus when 1 NTU was present (Table 2). However, when 5 NTU was present, the ultrasonic treatment had little or no effect on the inactivation of poliovirus even though the ozone concentrations ranged from 3.75 to 4.06 mg/liter. Concurrent with these experiments, light microscopy displayed distinct differences in the physical state of the replicate samples. The sample which was not sonically treated showed distinct aggregates of cellular material, whereas there were no aggregates in the sonically treated cells.

Ozonated replicates demonstrated a complete absence of cellular aggregates after a 10-s exposure to 0.76 mg of ozone per liter. The absence of cellular material was demonstrated in both the control and the replicate previously subjected to ultrasonic treatment.

Inactivation of HEp-2 cell-associated coxsackievirus A9 in the Sharp apparatus. Table 3 shows the inactivation data for HEp-2 cell-associated coxsackievirus A9 at turbidity levels of 1 and 5 NTU. Complete inactivation of the cell-associated coxsackievirus was not obtained at the highest ozone dosage possible with this apparatus in the 30-s contact time available. In general, 1-NTU preparations had greater inactivations than did the 5-NTU samples, but complete inactivation was not obtained at either turbidity level.

Inactivation of cell-associated poliovirus in a batch reactor. The results presented in Table 4 indicate a decline of the ozone residual in the batch reactor concurrent with survival of the HEp-2 cell-associated poliovirus. Total inactivation of the cell-associated virus was not obtained in this system with contact times as long as 75 min.

Inactivation of cell-associated virus with continuous ozonation. Continuous ozonation coupled with extended contact times in a batch reactor yielded complete inactivation of both HEp-2

TABLE 1. Effect of various concentrations of ozone on the inactivation of unassociated and HEp-2 cell-associated poliovirus at 5 NTU[a]

Virus type	Ozone concn (mg/liter)		Initial titer (PFU/ml of ozonated stream)	Inactivation (%)		
	Initial	Residual[b]		10 s	20 s	30 s
Unassociated[c]	0.50	—[d]	8.3×10^4	100	100	100
	0.28		4.5×10^4	100	100	100
	0.015	0.011	1.4×10^4	99.7	99.95	99.98
Cell associated	4.06	2.56	1.6×10^6	99.7	99.90	99.96
	3.91	3.15	2.0×10^6	99.96	99.997	99.990
	3.75	2.85	2.5×10^5	99.6	99.8	99.8
	2.84	1.79	8.0×10^5	99.6	99.8	99.7
	2.74	1.07	1.4×10^6	99.2	99.5	99.7
	2.30	2.30	2.6×10^5	99.92	99.996	99.990
	1.30	0.34	6.5×10^6	97	95	99.3
	0.65	0.02	5.1×10^5	98	98	99.3

[a] pH 7.0 ± 0.2; temperature, 20°C ± 1°C.
[b] At 40 s.
[c] $NaHCO_3$ buffer system.
[d] —, Not determined.

TABLE 2. Effect of ultrasonic treatment on the inactivation of HEp-2 cell-associated poliovirus at 1 and 5 NTU and at various ozone concentrations[a]

Turbidity (NTU)	Ozone (mg/liter)		Ultrasonic treatment	Initial titer (PFU/ml of ozonated stream)	Inactivation (%)		
	Initial	Residual[b]			10 s	20 s	30 s
1	0.76	0.31	−	2.2×10^4	81	93	90
	0.76	0.39	+	4.8×10^4	97	98	98
5	4.06	2.56	−	1.6×10^6	99.7	99.90	99.96
	4.06	3.09	+	1.2×10^6	99.9	99.95	99.99
	3.91	3.15	−	2.0×10^6	99.96	99.997	99.990
	3.91	2.78	+	6.6×10^5	99.8	99.97	99.99
	3.75	2.85	−	2.5×10^5	99.6	99.8	99.8
	3.75	3.34	+	1.3×10^5	99.9	99.92	99.93

[a] pH 7.0 ± 0.2; temperature, 20°C ± 1°C.
[b] At 40 s.

TABLE 3. Effect of various concentrations of ozone on the inactivation of unassociated and cell-associated coxsackievirus at 1 and 5 NTU[a]

Virus type	Ozone concn (mg/liter)		Initial titer (PFU/ml of ozonated stream)	Inactivation (%)		
	Initial	Residual[b]		10 s	20 s	30 s
Unassociated[c]	0.081	0.042	1.3×10^4	100	100	100
	0.032	0.027	9.1×10^3	99	99.7	99.90
	0.012	0.010	9.1×10^3	97	99.2	99.8
Cell associated						
1 NTU[d]	4.68	2.53	2.4×10^4	99.7	99.98	99.96
	3.69	2.93	1.3×10^5	99.92	99.98	99.98
	3.59	2.66	9.8×10^4	99.4	99.91	99.97
	2.99	1.88	3.7×10^5	99.7	99.93	99.98
5 NTU	4.68	2.34	3.7×10^5	99.8	99.98	99.98
	3.69	2.93	3.0×10^5	99.0	99.7	99.9
	3.59	2.41	1.1×10^6	99.4	99.8	99.7
	2.99	1.85	1.9×10^5	96	92	99.8

[a–c] See Table 1.
[d] Data from reference 6.

TABLE 4. Effect of ozone on the inactivation of HEp-2 cell-associated poliovirus at 5 NTU in a batch reactor[a]

Initial ozone (mg/liter)	Residual ozone (mg/liter)					Initial titer (PFU/ml)	Inactivation (%)				
	1 min	2 min	15 min	60 min	75 min		1 min	2 min	15 min	60 min	75 min
3.93	1.56	1.56	0.55	0.10	—[b]	1.2×10^5	99.990	99.95	99.98	99.997	—
2.51	0.86	0.76	0.34	0.11	0.17	1.5×10^5	99.6	99.0	99.7	99.3	99.0

[a] pH = 7.0 ± 0.2; temperature, 20°C ± 1°C.
[b] —, Not determined.

TABLE 5. Effect of various concentrations of ozone on the inactivation of HEp-2 cell-associated virus at 5 NTU in a batch reactor with continuous ozonation[a]

Virus	Initial ozone (mg/liter)	Residual ozone (mg/liter)					Initial titer (PFU/ml)	Inactivation (%)				
		1 min	2 min	5 min	10 min	15 min		1 min	2 min	5 min	10 min	15 min
Poliovirus	6.82	4.70	4.70	4.10	4.45	4.70	2.3×10^5	99.93	100	100	100	100
Poliovirus	6.50	4.85	5.45	4.65	4.75	5.05	3.6×10^4	99.90	100	100	100	100
Coxsackievirus	5.33	3.76	3.56	3.21	2.68	2.55	1.9×10^4	96	99.7	99.8	99.8	100
Coxsackievirus	5.19	3.78	3.39	2.64	2.90	2.95	7.1×10^4	99.6	99.97	100	100	100
Coxsackievirus	4.81	2.73	2.20	2.18	2.42	2.51	1.9×10^5	99.99	99.996	100	100	100

[a] pH 7.0 ± 0.2; temperature, 20°C ± 1°C.

cell-associated poliovirus and coxsackievirus (Table 5). The system provided higher ozone residuals as well as extended contact times. The cell-associated poliovirus was completely inactivated after a 2-min contact time with initial ozone concentrations of 6.82 and 6.50 mg/liter. Ozone residuals of 4.70 and 5.45 mg/liter, respectively, were measured at the 2-min point. The cell-associated coxsackievirus was inactivated after a 5-min exposure to an initial ozone of 5.19 mg/liter and a residual ozone of 2.64 mg/liter.

The enhanced survival of coxsackievirus compared with that of poliovirus was obtained at a lower initial ozone concentration and consequently lower ozone residual. Initial concentrations of ozone used in the poliovirus experiments were 6.82 and 6.50 mg/liter, whereas the greatest applied dosage of ozone used in the trials with coxsackievirus was 5.33 mg/liter.

DISCUSSION

This investigation sought to determine the disinfection potential of ozone for the inactivation of HEp-2 cell-associated poliovirus and coxsackievirus at the highest turbidity level, 5 NTU, accepted by the national interim primary drinking water regulations. Previous research (6) had established inactivation data for these HEp-2 cell-associated enteric viruses at a turbidity level of 1 NTU, using the Sharp continuous-flow disinfection apparatus. We used ozone as the disinfectant in the Sharp apparatus as well as in two batch reactor systems to determine the range of applied ozone and residual ozone concentrations required for complete inactivation of HEp-2 cell-associated enteric viruses.

Current industrial practices advocate the use of an applied dosage of 0.5 to 1.5 mg of ozone per liter to achieve a 5-min contact period with ozone residuals of 0.1 to 0.2 and 0.4 mg/liter for disinfection of viruses and bacteria in clean water (5). This investigation indicates that such levels would be inadequate for the inactivation of poliovirus and coxsackievirus associated with cellular material. Complete inactivation of these cell-associated viruses was achieved in a batch reactor using continuous ozonation. Poliovirus (Sabin type 1) was inactivated to nondetectable levels after a 2-min exposure to ozone with an initial residual concentration of 6.50 mg/liter and a final residual of 5.45 mg/liter. Coxsackievirus A9 was inactivated to nondetectable levels after a 5-min exposure to 5.19 mg/liter initially and a final residual ozone concentration of 2.64 mg/liter.

Interpretation of these data is subject to qualification by current practices at treatment facilities. Those facilities that use filtration before the application of a disinfectant effect a 99% removal of 2.5- to 150-μm particles, with all particles greater than 10 μm removed (16). The HEp-2 cell-associated virus complexes ranged from 10 to 15 μm, a size which would dictate their removal by filtration if it were used before ozonation. These data indicate the necessity of filtration in view of the enhanced survival of cell-associated viruses subjected to disinfection by ozone.

The role of aggregates or organic materials in the production of a two-stage inactivation phenomenon has been suggested as an explanation for the altered inactivation rate after initial exposure to ozone (9). This investigation noted the presence of cellular aggregates, which were dispersed to individual cells and cellular fragments by ultrasonic treatment. Although aggregate dispersal caused an increase in the percentage of inactivation, the two-step phenomenon in the inactivation kinetics using HEp-2 cell-associated poliovirus at turbidity levels of 1 and 5 NTU clearly remains. At the contact times available in the Sharp continuous-flow apparatus, at 5 NTU, a decrease of 2.5 to 3 log units of cell-associated poliovirus occurred in the initial 10 s, but a portion of the population survived the 30-s exposure to an ozone concentration of 3.09 mg/liter. The replicate samples at 1 NTU also demonstrate the biphasic inactivation in both the control and the replicate subjected to ultrasonic treatment. Kim et al. (10) also noted the presence of a two-step rate of inactivation during ozone inactivation of the bacteriophage f2, although their electron microscopy studies did not reveal the presence of phage aggregates. Therefore, the presence of aggregates of organic material may not always be responsible for the altered inactivation rates commonly observed.

In conclusion, cell-associated enteric viruses were protected from inactivation by exposure to ozone. Complete inactivation of the cell-associated enteric virus at a turbidity level of 5 NTU was not achieved with contact times and ozone concentrations presently used in water treatment facilities. With decreased initial titers of cell-associated virus, as would be present after filtration, satisfactory inactivation might be obtained. In addition, the 5-NTU standard represents a heterogeneous sample rather than a homogeneous system consisting solely of cell-associated enteric viruses. Further research may elucidate the specific parameters required for the disinfection of a heterogeneous sample consisting of organic and inorganic particulate matter. However, one must conclude from these data that viral survival after exposure to 0.4 mg of ozone per liter after 5 min is certainly possible. This possibility would be enhanced by the use of a malfunctioning filtration system or the

absence of such a filtration system in a water treatment facility.

ACKNOWLEDGMENT

This work was supported in part by research grant R804587 awarded by the Municipal Environmental Research Laboratory of the U.S. Environmental Protection Agency.

LITERATURE CITED

1. **Berg, G.** 1970. Integrated approach to the problem of viruses in water, p. 339–364. *In* Proceedings of the National Specialty Conference on Disinfection. American Society of Civil Engineers, New York.
2. **Boardman, G. D., and O. J. Sproul.** 1977. Adsorption as a protective mechanism for waterborne viruses. Completion report, project A-030-Me. Land and Water Resource Institute, University of Maine, Orono.
3. **Carlson, G. F., F. E. Woodward, D. F. Wentworth, and O. J. Sproul.** 1968. Virus inactivation on clay particles in natural waters. J. Water Pollut. Control Fed. **40**:R89–R106.
4. **Committee on Environmental Quality Management.** 1970. Engineering evaluation of virus hazard in water. J. Sant. Eng. Div. Am. Soc. Civ. Eng. **96**:111.
5. **Diaper, E. W. J.** 1975. Disinfection of water and wastewater using ozone, p. 211–231. *In* D. J. Johnson (ed.), Disinfection water and wastewater. Science Publishers, Inc., Ann Arbor, Mich.
6. **Foster, D. M., M. A. Emerson, C. E. Buck, D. S. Walsh, and O. J. Sproul.** 1980. Ozone inactivation of cell- and fecal-associated viruses and bacteria. J. Water Pollut. Control Fed. **52**:2174–2184.
7. **Guskey, L. E., and D. A. Wolff.** 1972. Concentration and purification of poliovirus by ultra filtration and isopycnic centrifugation. Appl. Environ. Microbiol. **24**:13–17.
8. **Hoff, J. C.** 1978. The relationship of turbidity to disinfection of potable water, p. 103–117. *In* C. W. Hendricks (ed.), Evaluation of the microbiology standards for drinking water. EPA-57019-78-006. U.S. Environmental Protection Agency, Washington, D.C.
9. **Katzenèlson, E., B. Kletter, and H. D. Shuval.** 1974. Inactivation kinetics of viruses and bacteria in water by use of ozone. J. Am. Water Works Assoc. **66**:725–729.
10. **Kim, C. H., D. M. Gentile, and O. J. Sproul.** 1980. Mechanism of ozone inactivation of bacteriophage f2. Appl. Environ. Microbiol. **39**:210–218.
11. **Long, W. N., and F. A. Bell, Jr.** 1972. Health factors and reused waters. J. Am. Water Works Assoc. **64**:220–225.
12. **Schaub, J. A., and B. P. Sagik.** 1975. Association of enterovirus with natural and artificially introduced colloidal solids in water and infectivity of solids-associated virions. Appl. Microbiol. **30**:212–222.
13. **Sharp, D. G., R. Floyd, and J. D. Johnson.** 1976. Initial fast reaction of bromine on reovirus in turbulent flowing water. Appl. Environ. Microbiol. **31**:173–181.
14. **Shechter, H.** 1973. Spectrophotometric method for determination of ozone in aqueous solutions. Water Res. **7**:729–739.
15. **Sproul, O. J.** 1976. Removal of viruses by treatment processes, p. 167–179. *In* G. Berg et al. (ed.), Viruses in water. American Public Health Association, Inc., Washington, D.C.
16. **Tate, C. H., J. S. Lang, and H. L. Hutchinson.** 1977. Pilot plant tests of direct filtration. J. Am. Water Works Assoc. **69**:379–384.
17. **Wolf, K., and M. C. Quimby.** 1973. Fish virology: procedures and preparation of materials for plaquing fish viruses in normal atmosphere. U.S. Department of the Interior, Washington, D.C.