APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1986, p. 450–459
0099-2240/86/090450-10$02.00/0

Vol. 52, No. 3

Mechanism of Inactivation of Enteric Viruses in Fresh Water

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Received 10 March 1986/Accepted 12 June 1986

Fresh water obtained from nine sources was shown to cause inactivation of poliovirus. Further testing with four of these water samples showed that enteric viruses from different genera were consistently inactivated in these freshwater samples. Studies on the cause of inactivation were conducted with echovirus type 12 as the model virus. The results revealed that the virucidal agents in the waters tested could not be separated from microorganisms. Any treatment that removed or inactivated microorganisms caused loss of virucidal activity. Microbial growth in a sterilized creek water seeded with a small amount of stream water resulted in concomitant production of virucidal activity. When individual bacterial isolates obtained from a stream were grown in this sterilized creek water, most (22 of 27) produced a large amount of virucidal activity, although the amount varied from one isolate to the next. Active and inactive isolates were represented by both gram-positive and gram-negative organisms. Examination of echoviruses inactivated in stream water revealed that loss of infectivity first correlated with a slight decrease in the sedimentation coefficient of virus particles. The cause appeared to be cleavage of viral proteins, most notably, VP-4 and, to a lesser extent, VP-1. Viral RNA associated with particles was also cleaved but the rate was slower than loss of infectivity. These results suggest that proteolytic bacterial enzymes inactivate echovirus particles in fresh water by cleavage of viral proteins, thus exposing the viral RNA to nuclease digestion.

Transmission of viral disease through contaminated water depends on survival of the disease agent for a sufficient period of time in the water to infect a susceptible host. The viruses most likely to be transmitted by this water route are those excreted in large numbers in feces and known to be resistant to inactivation. These enteric viruses cause numerous human diseases, including severe diarrhea and hepatitis, and many cases of human illness due to these agents have been traced to contaminated water (29).

Several studies have been published concerning the survival of enteric viruses in fresh water. The methods used were to seed the water with known concentrations of viruses and measure recoverable PFU as a function of incubation time. Results reported before 1971, as reviewed by Akin et al. (1), suggested that survival times of enteric viruses in both river waters and impounded fresh water varied greatly, even when studied at the same approximate temperatures and with the same virus types. Subsequent studies have supported these original observations (4, 12–14, 17, 19, 30). Although the cause of inactivation was not determined in these experiments, factors such as bacterial enzymes (11), turbidity, and light (8) have been shown to influence the losses of viral infectivity in fresh water. The dominant factor suggested, however, has been temperature (1, 30).

The survival times of enteric viruses in fresh water and factors that affect viral inactivation rates could greatly influence the potential public health hazard associated with the use of this water. The purpose of this study was to measure the inactivation rates of representative enteric viruses in fresh water from different sources and to determine the cause and mechanism of inactivation. A previous study revealed several experimental procedures that should be followed to accurately measure virus inactivation in water samples, including the use of radioactively labeled viruses (27). These procedures were used for the experiments in this report.

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MATERIALS AND METHODS

Viruses and cells. Four enteric viruses were used in this study. Attenuated poliovirus type 1 strain CHAT and coxsackievirus B5 were purchased from the American Type Culture Collection, Rockville, Md. Rotavirus strain SA-11 was provided by M. K. Estes, Baylor College of Medicine, Houston, Tex. Echovirus type 12 (echovirus-12) has been grown in this laboratory for many years. Poliovirus was grown in HeLa cells from a stock retained in this laboratory for several years and plaqued in RD cells purchased from the American Type Culture Collection. Coxsackievirus was grown and plaqued in BGM cells obtained from D. Dahling, U.S. Environmental Protection Agency, Cincinnati, Ohio. Rotavirus was grown and plaqued in MA-104 cells provided by E. A. Bohl, Ohio Agricultural Research and Development Center, Wooster. Echovirus was grown and plaqued in RD cells. All cell lines were grown as monolayer cultures in either Eagle minimial essential medium (HeLa and BGM cells) or special minimal essential medium-Richter modification (MA-104 and RD cells), each containing antibiotics (100 U of penicillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B per ml) and 10% fetal calf serum. Growth and plaquing methods were described elsewhere for both enteroviruses (25) and rotavirus (26).

Labeling and purification of viruses. Viruses used throughout this study were labeled with either [^{3}H]uridine or [^{35}S]methionine and purified. The methods used to label and purify the enteroviruses with [^{3}H]uridine were the same as those described for poliovirus in a previous publication (27). Labeling of echovirus-12 with [^{35}S]methionine was done in the same fashion except that Eagle minimal essential medium without methionine was used during the incubation and 0.2 mCi of [^{35}S]methionine was added to each flask. Purifi-

TABLE 1. Properties of freshwater samples obtained from sites used in this study

Source of water	Description	Total plate count organisms per ml	Total coliforms per ml	Fecal coliforms per ml	Fecal streptococci per ml	pH	Conductivity (mmhos)	Turbidity (nephelometric turbidity units)
1. Lake Isabella	Small, clear	230	<1	<0.1	<0.1	7.7	0.50	2.6
2. Deep well	Depth >30 ft	210	10	<0.01	<0.01	7.3	0.38	12
3. Ohio River	Large, polluted river	3,100	16	1	1,000	7.2	0.38	70
4. Clough Creek	Small, residential stream	5,800	57	0.9	0.1	8.0	0.59	3.8
5. Cattle Creek	Small, rural stream	10,000	12	<1	2	7.9	0.48	5.8
6. Maintenance Creek	Small, semirural stream	280	11	1	1.2	8.2	0.59	4.2
7. Sycamore Creek	Small, residential stream	180	<0.1	<0.1	<0.1	8.0	0.67	3.0
8. Bracken Creek	Small, polluted stream	100,000	980	2	3	7.5	0.57	14
9. Mill Creek	Large, polluted stream	91,000	6,500	4	1,000	7.7	0.74	112

cation was by the same methods as described for poliovirus. Rotavirus SA-11 was labeled with [^{3}H]uridine and purified by the same procedure with the following exceptions: (i) viral lysates were not blended with Freon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) prior to the initial centrifugation; (ii) rotavius was banded in the CsCl cushion by 1 h of centrifugation at 24,000 rpm in an SW27 centrifuge rotor; (iii) the density of the CsCl was adjusted to 1.36 g/ml; and (iv) the virus was dialyzed against phosphate-buffered saline containing 10 mM $CaCl_2$.

Analyses of water samples. Freshwater samples obtained for this study included a lake, large river, deep well, and several streams with varying degrees of fecal and other pollutants. These waters were analyzed by biological (total plate count organisms, total coliforms, fecal coliforms, and fecal streptococci), chemical (pH and conductivity), and physical (turbidity) methods. The procedures used for these analyses were described previously (27) or were performed according to standard methods (3).

Measurement of viral inactivation rates. The methods used to measure virus inactivation in fresh water were developed as described in a previous publication (27). In short, water samples were obtained from the different sites and stored at 4°C. Within 24 h, the biological, physical, and chemical analyses described above were performed. To measure the effects of these waters on virus survival, purified labeled viruses were diluted 1,000-fold into 20 ml of each water sample (treated or untreated) in a 125-ml polypropylene, cotton-stoppered flask. After mixing, a 1-ml sample was removed, diluted into an equal volume of 2× tryptose phosphate broth, and stored frozen at −20°C. This was the zero-time sample. The flasks were then held stationary at the appropriate temperatures, and 1-ml samples were collected at the specified times and frozen in 2× tryptose phosphate broth. The specific infectivity of virus in each sample was determined by plaque analysis and measurement of recoverable radioactivity.

TABLE 2. Effect of different waters on specific infectivity of [^{3}H]uridine-labeled poliovirus

Water source	Specific infectivity (% relative to day 0)	
	Day 2	Day 4
1. Lake Isabella	3.0	0.3
2. Deep well	0.1	0.05
3. Ohio River	8.0	1.6
4. Clough Creek	4.0	0.2
5. Cattle Creek	0.4	0.03
6. Maintenance Creek	2.0	0.04
7. Sycamore Creek	4.0	0.4
8. Bracken Creek	10.0	1.2
9. Mill Creek	0.8	0.07
10. Distilled water	74.0	62.0

Water treatment to remove or inactivate microorganisms. **(i) Heat.** Water samples (20 ml in polypropylene flasks) were immersed for 1 h at temperatures of 40 to 60°C and then cooled rapidly in an ice bath.

(ii) Filtration. Water samples (25 to 100 ml) were passed through either of two 0.2-μm membrane filters composed of cellulose polymers (Millipore Corp., Bedford, Mass., or Nalge Co., Rochester, N.Y.) or a 0.2-μm nylon filter (Nalge Co.).

(iii) Hypochlorous acid. Water samples (20 ml) were treated with 32 ppm (32 μg/ml) of hypochlorous acid for 30 min followed by addition of sodium thiosulfate to 500 ppm. The thiosulfate, which completely neutralized the effects of the hypochlorous acid, was shown to have no effect on the virucidal activity of the waters.

(iv) Sodium azide. Water samples were incubated at 26°C for 19 h following the addition of sodium azide to a concentration of 1%. At this concentration, sodium azide was shown to have no detectable effects on the viability of echovirus-12.

(v) UV light. Water samples (25 ml) were placed in a glass dish with a magnetic stirring bar and exposed to a germicidal UV lamp for 1 h at a distance of 5 cm while being stirred.

All treated waters were seeded with virus and tested for total plate count organisms. Total plate count was measured again after 2 days of incubation at 26°C.

Sedimentation analysis of viral particles. [^{3}H]uridine- and [^{35}S]methionine-labeled echovirus-12 particles (0.1 ml) were mixed separately with 0.9-ml samples of untreated or autoclaved Sycamore Creek water and incubated at 26°C for 0, 8, 16, 24, and 32 h. Each sample was then mixed with an equal volume of 2× tryptose phosphate broth and frozen before being assayed for recoverable specific infectivity. A portion of each sample (100 μl) was mixed with 10 μl of purified echovirus-12 labeled with the opposite isotope as a marker. These samples were layered onto 15 to 30% glycerol gradients in 0.1 M NaCl–0.01 M Tris (pH 7.5)–0.001 M EDTA (NTE) and centrifuged (SW50.1 rotor) at 38,000 rpm for 100 min at 5°C. Fractions were collected and analyzed for both ^{35}S and ^{3}H radioactivity.

Extraction and sedimentation analysis of viral RNA. [^{3}H]uridine-labeled echovirus-12 particles were treated as described above, and 0.15 ml of each was extracted with 0.2 ml of phenol, 0.1 ml of chloroform, 20 μl of 10% sodium dodecyl sulfate, and 5 μl of tRNA (10 mg/ml) by shaking at

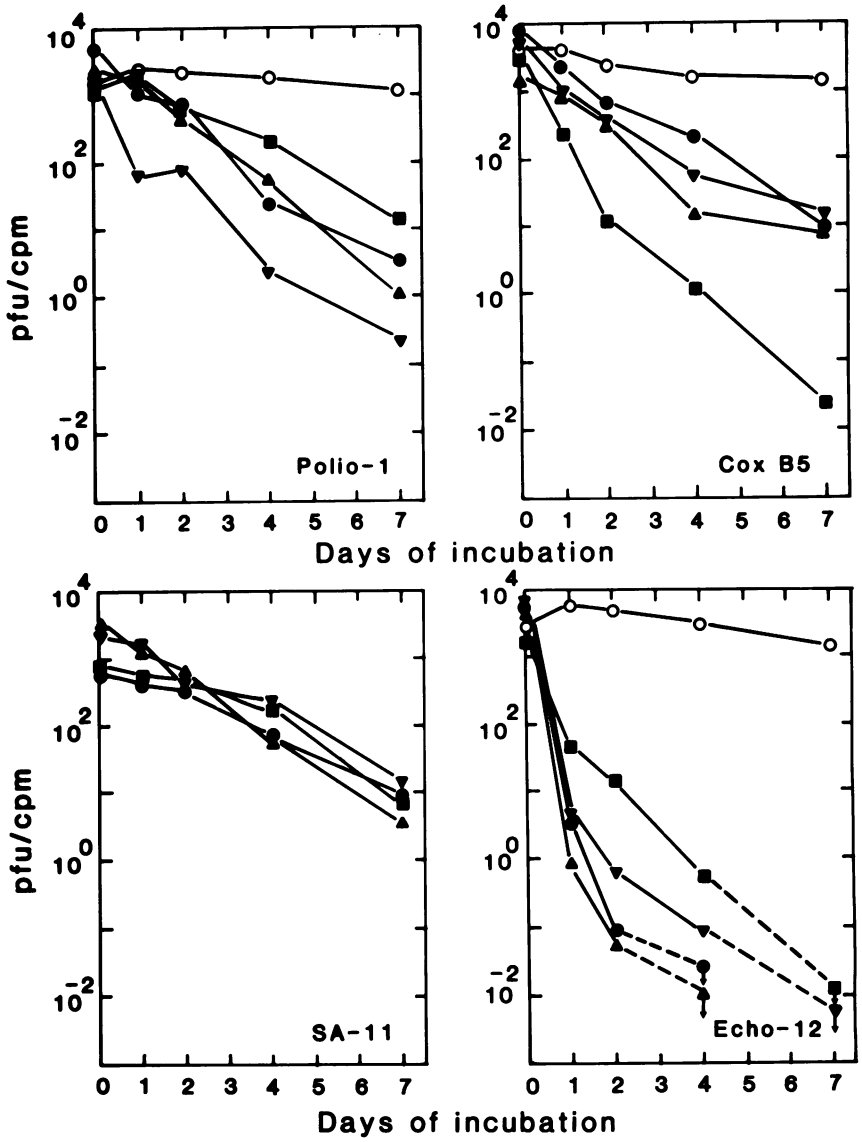

FIG. 1. Inactivation rates (27°C) of four enteric viruses in fresh water from Sycamore Creek (●), Bracken Creek (▲), the Ohio River (■), and a deep well (▼). Inactivation in sterile distilled water (○) is included as a control for the enteroviruses.

room temperature for 10 min. After phase separation by centrifugation, the aqueous phase was collected and recoverable radioactivity in a 10-μl aliquot was measured. The remainder of the sample was layered onto a 5 to 30% glycerol gradient in NTE buffer and centrifuged at 45,000 rpm for 3 h at 5°C in an SW50.1 rotor. Gradient fractions were collected and analyzed for recoverable radioactivity.

Gel analysis of echovirus proteins. [^{35}S]methionine-labeled echovirus particles were incubated in Sycamore Creek water (untreated or autoclaved) as described above, and 50 μl of each sample was diluted in an equal volume of particle disruption buffer (8 M urea, 2.85 M 2-mercaptoethanol, 0.25 M Tris [pH 6.8], 1% sodium dodecyl sulfate, 0.005% bromophenol blue) instead of tryptose phosphate broth before being frozen. After thawing, these samples were heated in boiling water for 90 s. A portion (40 μl) of each was layered onto polyacrylamide slab gels (1-mm thickness) and separated by electrophoresis at a constant current (40 mA) as described by Laemmli (15). The positions of the protein bands were determined by fluorography (16) after impregnation of the gels with 2,5-diphenyloxazole.

RESULTS

Inactivation of poliovirus type 1 in fresh water from different sources. Initial experiments were performed to compare virus inactivation rates in fresh water from nine sources within the Cincinnati area and to determine whether these rates could be correlated with any measured physical, chemical, or biological property of these waters as listed in Table 1. The virus used for this first study was poliovirus type 1, which has been the standard for most environmental studies. Waters were seeded with radioactively labeled poliovirus, and decreases in specific infectivity (PFU per counts per minute) were measured as a function of incubation time at 27°C. A constant temperature was chosen so that direct comparisons could be made between the waters without the influence of differences in temperatures; 27°C was chosen to

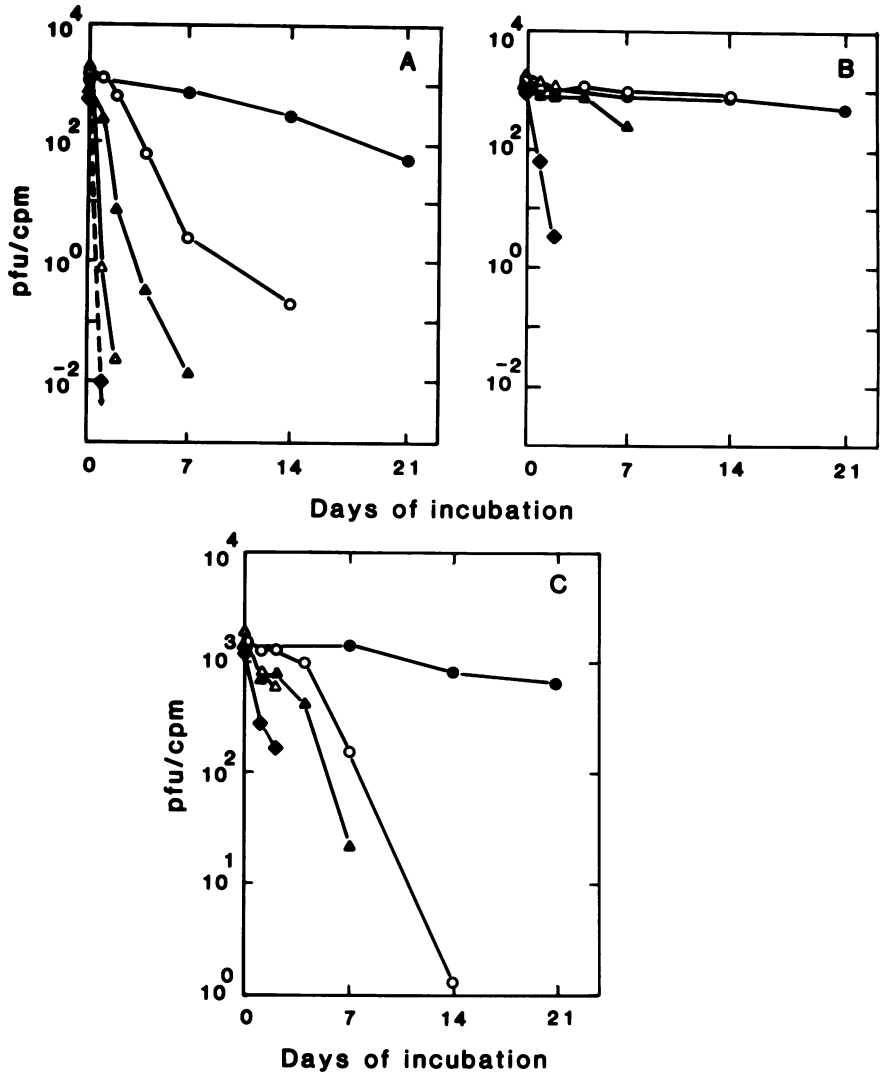

FIG. 2. Effect of temperature on inactivation rates of echovirus-12 in Sycamore Creek (A) and distilled (B) water and of rotavirus SA-11 in Sycamore Creek water (C). The temperatures of incubation were 4°C (●), 16°C (○), 23°C (▲), 29°C (△), and 37°C (◆).

maximize the rates of inactivation while avoiding most inactivation due directly to heat.

The specific infectivity of poliovirus decreased between 90% (Bracken Creek) and 99.9% (deep well) during 2 days and between 98.4% (Ohio River) and 99.97% (Cattle Creek) during 4 days of incubation (Table 2). Only a slight loss of infectivity occurred during this time in sterile distilled water, which shows that little inactivation was caused by the direct effects of heat. Comparisons between virucidal activities and other water properties (Table 1) revealed no statistically significant association ($P > 0.05$). It is concluded, therefore, that all nine environmental waters contained unidentified factors that caused inactiviation of poliovirus.

Comparative inactivation rates of different enteric viruses in fresh water. To establish the general nature of the virucidal action of fresh water, the inactivation rates of three other enteric viruses were compared with those of poliovirus. The viruses chosen (echovirus-12, coxsackievirus B5, and rotavirus SA-11) all represent different genera of enteric viruses, each could be grown to high titers and purified by available methods, and all could be readily plaqued on established cell lines. Four freshwater samples were chosen for this comparative study, two representatives of polluted sources (Bracken Creek and Ohio River) and two from relatively unpolluted sources (deep well and Sycamore Creek). Inactivation rates were again measured at 27°C, and loss of infectivity in distilled water was included as a control for all viruses except SA-11 which was rapidly inactivated under this condition, probably because of the scarcity of calcium and other ions needed to stabilize the rotavirus capsid (21). Addition of 10 mM $CaCl_2$ to distilled water partially but not totally overcame this effect (results not shown).

Echovirus-12 was found to be the most rapidly inactivated of the viruses tested (Fig. 1). Its specific infectivity decreased >99.999% during 7 days of incubation in all four waters. Rotavirus SA-11 was found to be the most stable, but its specific infectivity decreased about 99% during 7 days in all four waters tested. These results suggest that enteric viruses will consistently be inactivated when seeded into natural freshwater sources but the rate of inactivation will depend on the specific virus and water studied.

Temperature effects on virus inactivation in fresh water. Inactivation rates of enteric viruses in fresh water were

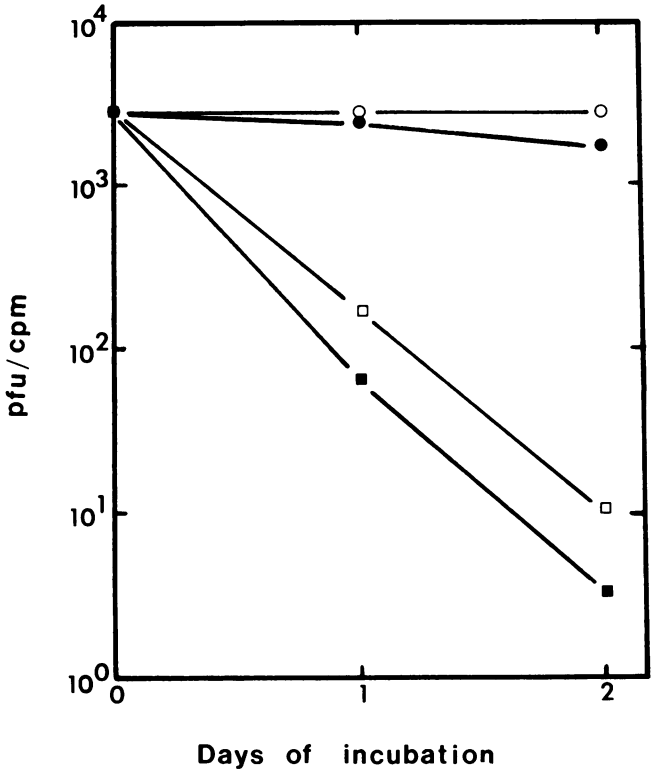

FIG. 3. Effect of heat treatment on virucidal activities of creek water. Aliquots of four creek waters were either left untreated (■) or heated for 1 h at 40°C (□), 50°C (●), or 60°C (○) before being seeded with [^{3}H]uridine-labeled echovirus-12. Samples were taken and specific infectivities were determined after different times of incubation at 23°C. Results from the four water samples were averaged and the geometric mean titers were presented.

determined at 27°C in all experiments presented above. Although the results clearly established the virucidal properties of these waters, this temperature of incubation is greater than expected for most fresh water during the majority fo the year in temperate climates. It is known that water temperature can have dramatic effects on virus inactivation rates (1, 30). To verify that virus inactivation will occur at other temperatures, virus survival was measured over a range of temperatures in several freshwater samples. The viruses chosen for this study were echovirus-12 and rotavirus SA-11, which had been found to be the least and most stable, respectively, of the viruses examined (Fig. 1).

Waters used for this study were samples collected from Sycamore Creek on two different dates (July and October 1984) and from Clough Creek in November 1984. Because waters from all three sites had quite similar effects on virus survival at the temperatures tested, the results from only one site are presented (Fig. 2). In addition, the rates of inactivation of echovirus-12 in distilled water at the same temperatures are presented for comparative purposes. Although both viruses were inactivated slowly in the creek waters at 4°C, incubation at 16°C caused ≥99.9% of each virus type to become inactivated within 14 days. Because echovirus-12 was inactivated much more slowly in distilled water than in creek water at the same temperatures, loss of infectivity was primarily due to properties of the environmental waters. These results suggest that enteric viruses will become inactivated when seeded into environmental waters, even at relatively low temperatures.

Identification of the causative agent of virus inactivation in fresh water. Several investigators have attempted to identify the agents that cause loss of infectious viruses in either fresh or marine water. A recurrent but not always confirmed suggestion is that inactivation is caused by indigenous microorganisms. Experiments were, therefore, designed to test possible associations between microorganisms and the observed virucidal activities of fresh water.

Initial experiments were done to test the effects of heat on virucidal activity because of the heat sensitivity of microorganisms and microbial products. The enteric virus used in this and remaining studies was echovirus-12 since it was the most rapidly inactivated of the viruses studied, thus permitting more experiments to be performed in a shorter period of time. Three samples of Sycamore Creek water (obtained at different times) and one sample of Clough Creek water were aliquoted and each aliquot was heated for 1 h at temperatures ranging from 40 to 60°C. Radioactively labeled echovirus-12 was then added to each and decreases in specific infectivity were measured as a function of incubation time at 23°C. Unheated creek waters were simultaneously seeded with virus and treated in the same manner.

The results with all four water samples were comparable. Therefore, the geometric mean titer of the four water samples were determined at each time point (Fig. 3). Heating at 40°C for 1 h caused partial loss of virucidal activity and 1 h at 50°C almost completely destroyed this activity. Waters obtained from a deep well, the Ohio River, and Bracken Creek responded in the same manner when heated for 1 h at 60°C (results not shown). These heat sensitivity results suggested that microorganisms may cause the loss of virus infectivity in fresh water.

A series of experiments was designed to determine whether removal or inactivation of microorganisms consistently resulted in loss of virucidal activity. The water chosen for these experiments was obtained from Sycamore Creek because it had the most consistent properties throughout the year of the surface waters studied. The treatments used for these experiments are described in Materials and Methods and listed in Table 3. In every case, treatments that removed or inactivated microorganisms caused a concomitant loss of virucidal activity.

If viable microorganisms are responsible for virucidal activity in fresh water as suggested by these experiments, sterile fresh water without virucidal activity should acquire

TABLE 3. Effects of different treatments on virucidal activity of Sycamore Creek water[a]

Water treatment	Total plate count (CFU/ml)		Virus survival (%)
	Immediately after treatment	2 days after virus added	
None	3×10^3	4×10^4	0.004
Filtration (Millipore filter)	$<1 \times 10^0$	$<1 \times 10^0$	20
Filtration (nylon filter)	$<1 \times 10^0$	$<1 \times 10^0$	16
Hypochlorous acid	$<1 \times 10^0$	$<1 \times 10^0$	9
Sodium azide	$<1 \times 10^0$	$<1 \times 10^0$	3
UV irradiation	$<1 \times 10^0$	$<1 \times 10^0$	16
Sterile distilled water (control)	$<1 \times 10^0$	$<1 \times 10^0$	14

[a] After aliquots of a large sample of Sycamore Creek water were treated as described in Materials and Methods, echovirus-12 ([^{3}H]uridine labeled) was added (20 μl to 20 ml of water), and a sample (2 ml) was collected to measure virus-specific infectivity and total plate count. After incubation of the remainder of each aliquot (2 days, 26°C), a second sample was collected and analyzed. Specific infectivities of the virus-containing samples at 2 days were determined relative to those found at time zero.

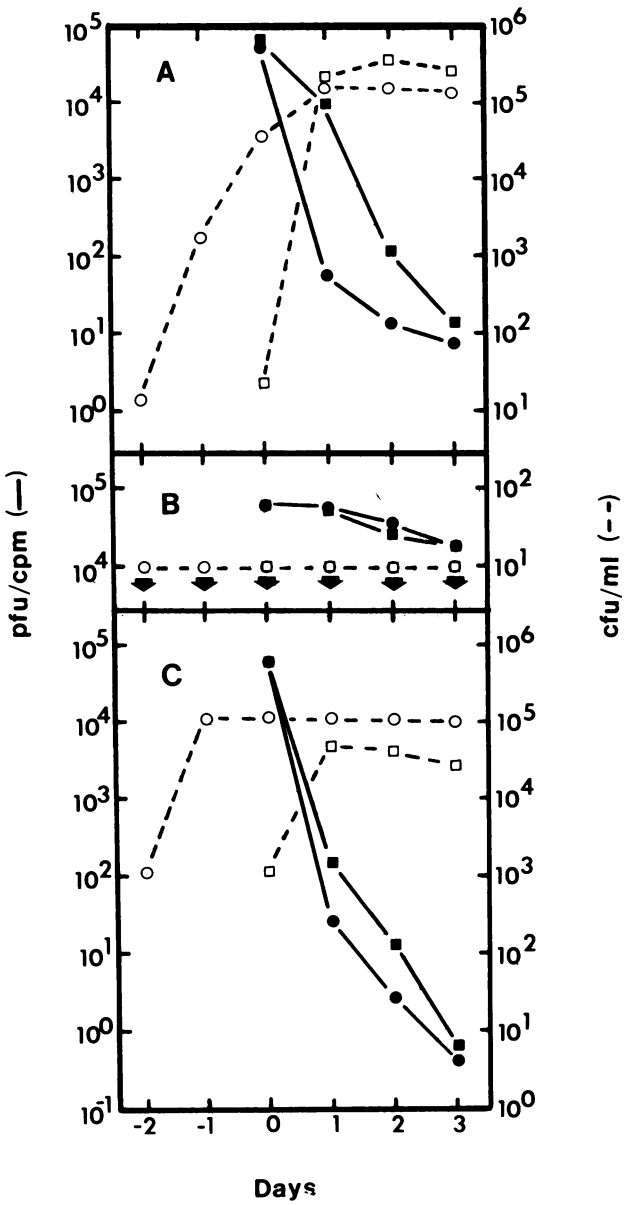

FIG. 4. Production of virucidal activity in sterile creek water. (A) Aliquots of Sycamore Creek water were autoclaved, seeded with 0.01 volume of untreated Sycamore Creek water, and incubated at 26°C. [^{3}H]uridine-labeled echovirus-12 was added either after 2 days of incubation (circles) or at the time of addition of the untreated water (squares). Total plate counts were determined in both samples (open symbols), as was the specific infectivity of the virus after the time of its addition (filled symbols). (B) Same experiment as (A) except that the autoclaved water was not seeded with untreated water. Arrows indicate "less than" values. (C) Same experiment as (B) except that untreated creek water was used in place of the sterile creek water.

this activity if freshwater microorganisms are permitted to grow in these waters. To determine whether this occurs, Sycamore Creek water was sterilized by autoclaving, seeded with 0.01 volume of untreated Sycamore Creek water, and incubated at 26°C. Echovirus-12 was added to the water at the time of seeding or after 2 days of incubation. Total plate counts were determined each day as well as the specific infectivity of the virus following its addition. Virus survival and microbial growth were also measured in untreated and autoclaved (not seeded with untreated water) Sycamore Creek waters as controls.

Addition of a small amount of untreated water to sterile creek water caused microbial concentrations to increase from approximately 10^1 to 10^5 CFU/ml within 1 to 2 days at 26°C (Fig. 4A). Little virucidal activity was observed in this water during the first 24 h of microbial growth, but significantly more activity was detected during the second 24-h period. The autoclaved water sample that was preincubated for 2 days after seeding with untreated water before virus addition caused a large decrease in virus-specific infectivity within 24 h. The specific infectivity of echovirus-12 decreased very little in sterile creek water in the absence of detectable microbial growth (Fig. 4B). Microbial concentrations in the untreated water increased from approximately 10^3 to 10^5 CFU/ml during 24 h of incubation at 26°C (Fig. 4C). This growth appeared to increase virucidal activity; i.e., the water sample incubated for 2 days before virus addition had greater virucidal activity than the sample that was not incubated before virus was added.

These data indicate that growth of microorganisms in creek water results in a concomitant increase in virucidal activity. The next experiment, therefore, was to determine whether individual microbial isolates could be grown in creek water and produce this activity. Microbial colonies were isolated from Sycamore Creek water by plating on agar and were then seeded into autoclaved Sycamore Creek water. After 2 days of incubation at 26°C, echovirus-12 was added to the water samples and the rate of decrease in specific infectivity was measured. Gram stains were also performed with each isolate to determine whether virucidal activity was related to this bacterial cell wall property (3).

TABLE 4. Virucidal activities of bacterial isolates grown in sterile creek water

Isolate	Gram stain	Virus survival (%) after 2 days, 26°C
1	+	86.0
2	−	0.2
3	−	7.4
4	+	2.0
5	+	0.2
6	−	0.03
7	−	0.7
8	−	0.03
9	+	0.05
10	−	0.3
11	−	3.8
12	−	0.2
13	−	0.1
14	−	0.07
15	+	0.08
16	+	0.06
17	−	8.7
18	−	0.2
19	+	24.0
20	−	0.1
21	−	0.2
22	+	102.0
23	−	56.0
24	−	0.02
25	+	0.03
26	−	37.0
27	−	0.2
Control (sterile distilled water)		51.0

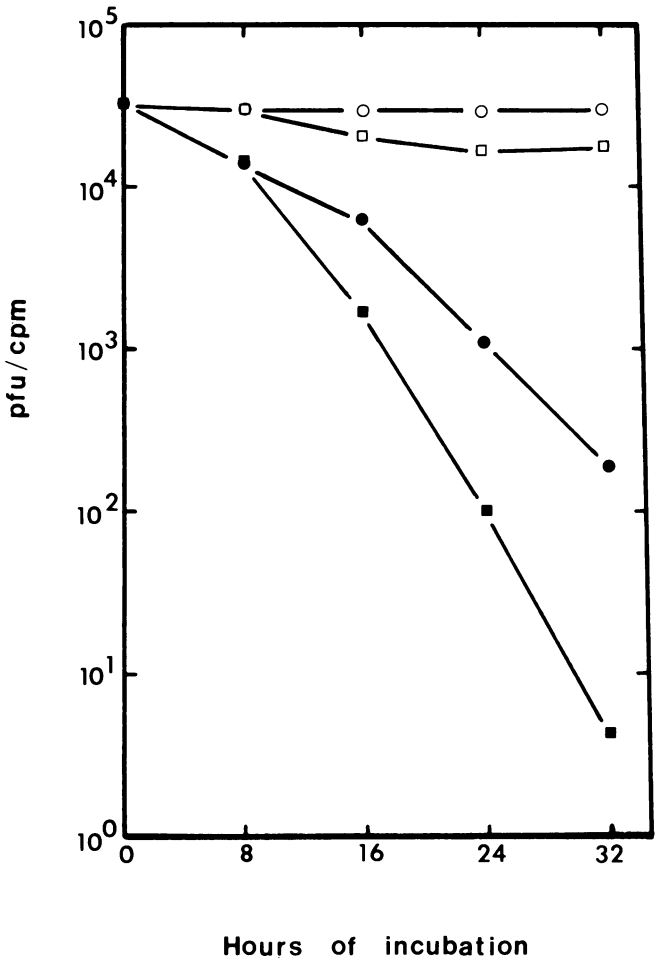

FIG. 5. Inactivation of [^{3}H]uridine- (squares) and [^{35}S]methionine- (circles) labeled echovirus-12 in untreated (filled symbols) and autoclaved (open symbols) Sycamore Creek waters during incubation at 26°C.

Although all isolates were rod-shaped bacteria of various shapes and sizes, most were distinguishable from one another based on colony morphology in agar. The majority (22 of 27) of the cultures caused at least a 90% decrease in specific infectivity within 24 h after addition of echovirus and several caused a decrease of >99.9% during this time period (Table 4). Virucidal activity appeared to be unrelated to whether the bacterium was gram positive or gram negative. Virucidal activity was also not solely dependent upon final bacterial densities. For example, isolates 6, 22, 25, and 26 all contained about 10^6 CFU/ml at the time of virus addition (results not shown), but two (6 and 25) displayed high virucidal activity and two (22 and 26) had little or no activity.

Other investigators have also reported the isolation of bacteria with antiviral activity from either marine or fresh water (7, 9, 22, 23). However, Shuval et al. (22), after making multiple passages in their laboratory, found that marine isolates lost activity. No loss of activity was observed in our isolates when tested after seven passages of growth on nutrient agar at 26°C (results not shown). Thus, these isolates appeared to retain virucidal activity when grown under laboratory conditions.

If these bacterial isolates produced the same type of virucidal agent as those found in fresh water, the virucidal activities of these isolates should be eliminated by heat treatment at 60°C for 1 h. The viability of each isolate along with its virucidal activity were found to be destroyed by this treatment (results not shown).

Once microorganisms were shown to be the cause of the virucidal activity in a freshwater sample, attempts were made to identify the microbial component that caused virus inactivation. To do this, the active component must be separated from other microbial components. As already shown in Table 3, however, treatments that either removed or inactivated microorganisms were all found to eliminate virucidal activity in the water samples. Virucidal activity was also not recovered in dialysis bags when immersed in fresh water (results not shown). Attempts to solubilize and recover the active agent by treatment of freshwater samples with urea or guanidine were similarly unsuccessful. It was observed, however, that the protease inhibitor phenylmethylsulfonyl fluoride caused a large reduction in virucidal activity when added to a sample of creek water (results not shown). This finding suggested the possibility that the virucidal agents are proteolytic bacterial enzymes, a possibility that was further investigated.

Mechanism of echovirus-12 inactivation in fresh water. Another possible method to identify the virucidal components of microorganisms is to determine their effects on viral particles. Echovirus-12, like other picornaviruses, is composed of one single-stranded RNA molecule encapsulated in a particle containing 60 molecules each of four distinct proteins designated VP1 through VP4 (20). Loss of infectivity must be caused by damage to at least one of these virion components. Identification of the component(s) and the type of damage it receives could help to define the nature of the virucidal agents associated with microorganisms. The approach was to examine the effects of incubation in a freshwater sample on the different components of echovirus particles as a function of incubation time at 26°C.

For this experiment, both [^{35}S]methionine- and [^{3}H]uridine-labeled echoviruses were purified and added individually to samples of Sycamore Creek water. Loss of specific infectivity was somewhat faster for the ^{3}H-labeled viruses but >99% of the infectivity was lost for both virus preparations within 32 h of incubation (Fig. 5). Little reduction in specific infectivity of either virus preparation occurred during this time period in autoclaved Sycamore Creek water.

Examination of the sedimentation coefficients of the viral particles revealed that loss of infectivity resulted in a corresponding loss in particles with normal sedimentation coefficients (Fig. 6). The initial change was to particles with slighty reduced S values. With time, however, these particles broke down, and both labeled components (RNA and protein) were found at the tops of their respective gradients after sedimentation.

The individual components of echovirus particles were next examined in an attempt to identify the alteration that was responsible for loss of infectivity. Virion proteins were examined by polyacrylamide gel electrophoresis of the samples after different times of incubation (Fig. 7). Gel patterns revealed that five proteins were present in purified echovirus-12 particles, one of which was VP0, the precursor to VP2 and VP4 (20). No qualitative change in the electrophoretic pattern of echovirus proteins was detected after 8 h of incubation (lane 2) when the specific infectivity had decreased 61%. The relative amount of VP4 appeared, however, to be reduced in comparison to other viral proteins. After 16 h of incubation (lane 3), when the specific infectivity was reduced by 80%, a large portion of VP1 was

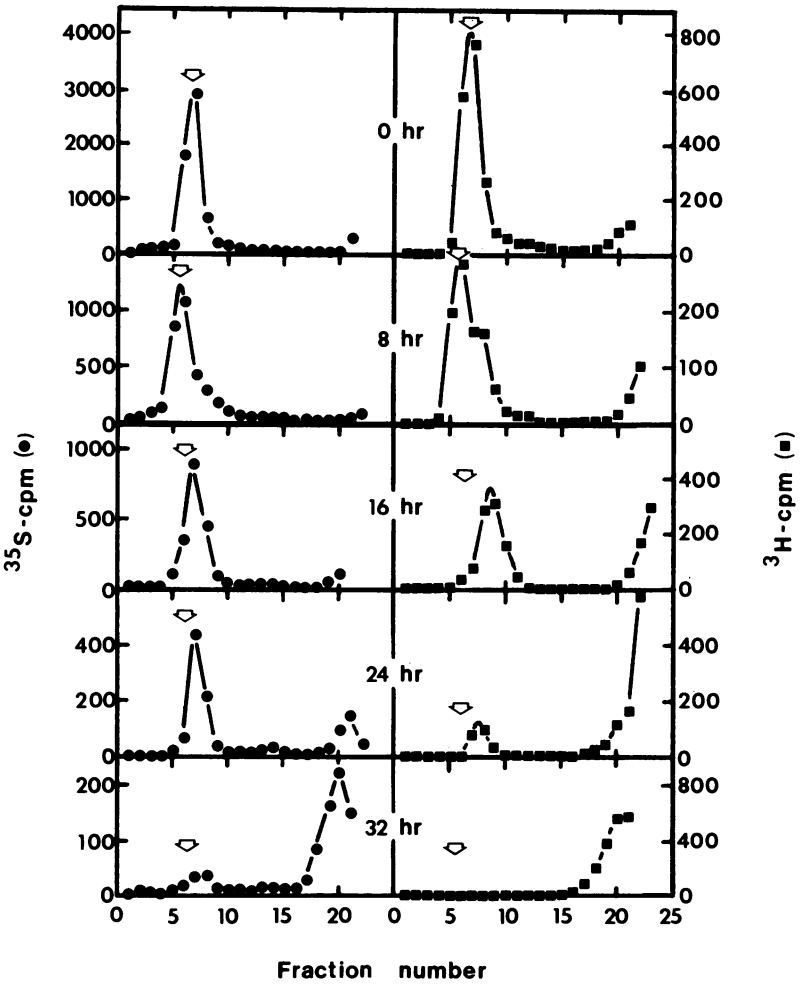

FIG. 6. Changes in sedimentation profiles of [^{35}S]methionine- (circles) and [^{3}H]uridine- (squares) labeled echovirus-12 particles in Sycamore Creek water as a function of incubation time at 26°C. Sedimentation is from right to left in all glycerol gradients. Arrow denotes the sedimentation position of untreated particles of the opposite label included as controls in each gradient.

visibly cleaved and a band migrating between VP1 and VP2 was observable. Also, almost all of VP4 had been cleaved. Since no additional viral protein was found sedimenting at the tops of the glycerol gradients at this time (Fig. 6), the cleaved proteins apparently remained particle associated. After 24 h of incubation (lane 5), when 97% of the particles were no longer infectious and less than one-third of the viral protein was found at the top of the gradient, VP1 was almost totally cleaved and little VP2 or VP3 remained intact. After 32 h (lane 6), almost no viral protein remained intact, although approximately 24% of the virion protein still sedimented in association with particles.

Sedimentation values of echovirus RNA extracted from samples at these different incubation times were also determined. After 8 h of incubation, when the specific infectivity of the [^{3}H]uridine-labeled particles had decreased 59%, the S value of 36% of virion RNA had been modified (Fig. 8). After 16 h, 95% of these viruses were inactivated but only 74% of the viral RNA was detectably altered. Even after 24 h of incubation, when 99.7% of the infectivity was lost, approximately 12% of the RNA appeared to sediment like infectious RNA.

Taken together, these results suggest that the initial damage to echovirus particles in these water samples is cleavage of viral proteins, specifically VP4 and, to a lesser extent, VP1. This is followed by cleavage of viral RNA.

DISCUSSION

The ability to cause inactivation of enteric viruses appears to be a general property of fresh water. Although waters collected from different sources have been shown in this report and by other investigators (4, 12–14, 17, 19, 30) to have different amounts of virucidal activity, the only property of fresh water that has been consistently related to virucidal activity is temperature (1, 30). Even though inactivation rates decrease with reduction in temperature, those ≥16°C caused rapid loss of infectivity of enteric viruses belonging to different genera in the fresh water tested in this study.

If viruses that contaminate fresh water under natural conditions are inactivated at rates comparable to those observed in this and other studies, their survival times should be of limited duration at these temperatures in environmental water. The simplistic approach used to investigate virus survival in fresh water under laboratory conditions, however, may provide poor approximations of true inactivation rates in nature. Although the types of virucidal

effects of fresh water on seeded viruses are anticipated to be similar to those that affect viruses under natural conditions, the magnitude of these effects could be very different. Many of the viruses in fresh water are expected to be associated with particulates (18, 28), and this association is known to protect viruses (6, 10). Therefore, this type of study can only be used to qualitatively describe the virucidal effects of fresh water.

The results of this study indicate that virucidal activity of a freshwater sample could be directly correlated with the presence of viable microorganisms. Treatments that removed the organisms (filtration) or either physically (heat, UV irradiation) or chemically (hypochlorous acid, sodium azide) caused their inactivation all considerably reduced virucidal activity. Furthermore, the temperatures at which heat treatment caused loss of this activity were consistent with those known to cause inactivation of microorganisms, i.e., 40 to 60°C.

Other investigators have suggested that viable microorganisms are needed for virus inactivation in both marine and fresh water (7, 9, 22). In contrast, several investigators have indicated that these waters could retain virucidal activity after filtration (2, 19, 23). Although virus inactivation could have been caused by microbial products in these latter studies, filtration should have eliminated viable microorganisms. Our experiments indicate that filtration of creek water consistently removed its virucidal activity, whether the filter material was a cellulose polymer or nylon. Attempts made to block potential binding sites on the filters by pretreatment with calf serum, non-fat dry milk solution, or broth did not allow passage of the virucidal material. Likewise, the virucidal activity of the filtered water did not increase with the volume of water processed. These results suggest that the active agent was removed by filtration because of its size and not its binding properties.

It is possible that retention of virucidal activity in filtered water reported by some investigators was due to a microbial product not present in water such as that investigated in this study. It is also possible that the virucidal activities they measured did not have a microbial source. It should be noted, however, that slight contamination of sterile water can result in rapid recolonization with subsequent production of virucidal activity, a result observed in the present study. Furthermore, the organisms that cause recolonization may not grow in standard medium and, therefore, remain undetected. Thus, it is possible that viable microorganisms

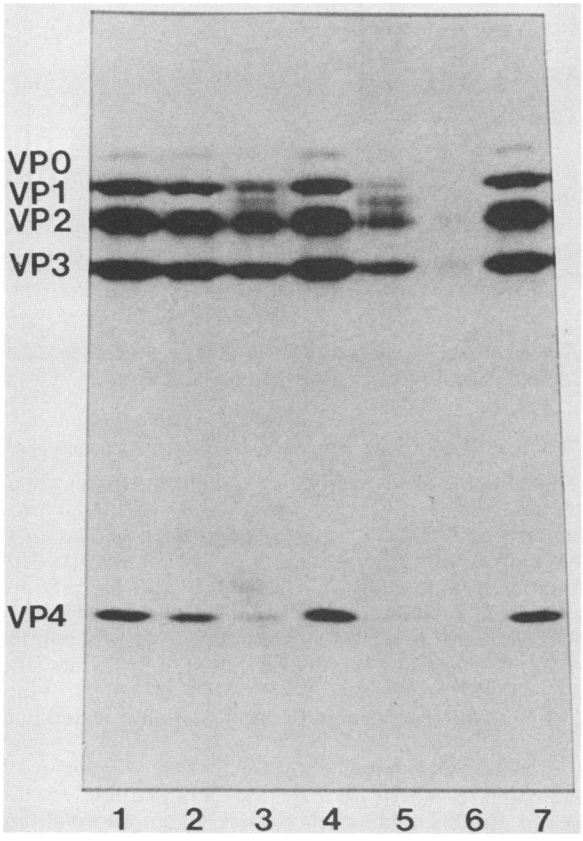

FIG. 7. Electrophoretic profiles of echovirus-12 proteins after various times of incubation of [^{35}S]methionine-labeled virions in Sycamore Creek water. (Lanes 1, 2, 3, 5, 6) Echovirus-12 particles incubated in untreated Sycamore Creek water for 0, 8, 16, 24, and 32 h, respectively. (Lanes 4, 7) Untreated echovirus particles.

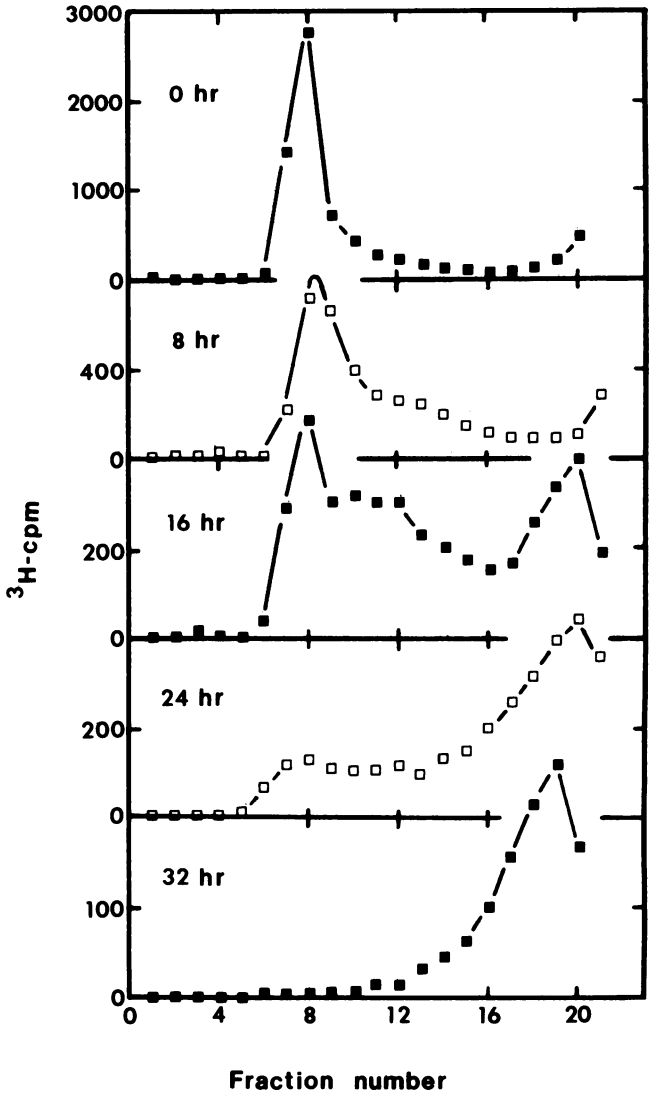

FIG. 8. Sedimentation profiles of echovirus-12 RNA molecules extracted from [^{3}H]uridine-labeled virions incubated at 26°C for various times in Sycamore Creek water. Sedimentation is from right to left.

were present and caused the inactivation of viruses even in filtered water.

The strict association between viable microorganisms and virucidal activity observed in this study indicates that the active agent either remains associated with the microorganisms or has a very short lifetime or both. The same conclusion was reached by Shuval et al. (22) during their studies on the virucidal activity of seawater. Microorganisms contain components that remain membrane bound and also those with short half-lives, especially certain enzymes. Thus, these observed results are not unexpected.

Loss of virus infectivity in the fresh water examined in this study was not due to uptake of virus because labeled virus components did not sediment with microorganisms after virus inactivation (Fig. 6). Therefore, inactivation must have occurred outside of microorganisms, possibly during the time of physical contact between the viruses and microorganisms. Further clues regarding the nature of the virucidal agent(s) were, therefore, obtained by analysis of the inactivated virus particles. These studies, conducted with echovirus-12, revealed that loss of infectivity occurred in proportion to a slight reduction in the sedimentation rates of virus particles. At the same time, a considerable amount of virus protein, especially VP4, appeared to be broken down. Some cleavage of viral RNA was also detected but not in proportion to loss of infectivity. Greater amounts of inactivation with longer incubation periods resulted in further cleavage of viral protein and RNA in inactivated particles, leading to an eventual disintegration of these particles.

Because echovirus particles contain only 1 molecule of viral RNA but 60 molecules of each of four viral proteins (20), cleavage of viral RNA should be much more easily detected if its cleavage is the cause of viral inactivation. The finding that total VP4 appeared to be cleaved as rapidly as viral RNA suggests that protein breakdown precedes RNA cleavage. Breindl (5) showed that release of VP4 from poliovirus by heat treatment exposed the encapsulated viral RNA to RNase digestion. Toranzo et al. (24) also showed that inactivation of poliovirus in filtrates of seawater permitted nuclease digestion of the viral RNA. Thus, exposure of echovirus RNA to exonucleases could have occurred following cleavage of virion proteins within the intact particle. Herrmann and Cliver (11) found that proteolytic enzymes obtained from certain bacteria caused inactivation of coxsackievirus A9. Taken together, these results support the hypothesis that echovirus-12 inactivation in fresh water occurred as the result of cleavage of viral protein by short-lived or microorganism-associated proteolytic enzymes, thus resulting in exposure and breakdown of viral RNA.

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