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Identification of the Virucidal Agent in Wastewater Sludge

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Anaerobically digested sludge contains an agent that causes irreversible inactivation of poliovirus. It has now been shown that the agent responsible for this activity is ammonia. The effect of ammonia on poliovirus appears to be typical for picornaviruses, but reovirus, an enteric virus of another group, is quite resistant to this compound. Because ammonia is not virucidal in its charged state, it expresses significant activity only at pH values greater than 8. Therefore, increasing the pH of sludge should cause rapid inactivation of indigenous picornaviruses.

The presence of an agent with virucidal activity against poliovirus has recently been demonstrated in anaerobically digested sludge, primarily associated with the liquid fraction (2, 3). Although the exact mechanism of viral inactivation by this agent has not yet been determined, both the ribonucleic acid and the two largest proteins of poliovirus are cleaved during incubation of viral particles in digested sludge. Ribonuclease and proteases present in sludge do not appear to be solely responsible because these enzymes have no detectable effect on viral infectivity in the absence of sludge. Furthermore, the virucidal agent was not detected in raw sludge, which should contain an abundance of these enzymes. It was, therefore, suggested that this agent may be produced during anaerobic digestion. The identity of this agent and the reason for its detection in digested but not raw sludge have now been determined.

MATERIALS AND METHODS

Virus and cells. The strain of poliovirus used to identify the virucidal agent was the attenuated type 1 strain CHAT purchased from the American Type Culture Collection. Other virus types used in these experiments were the type 1 poliovirus strain Mahoney and the type 2 poliovirus strain 712-Ch-2ab, both gifts of K. Lonberg-Holm (Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia), the B1 and A13 types of coxsackievirus and echovirus 11, all gifts of L. C. McLaren (Department of Microbiology, School of Medicine, University of New Mexico, Albuquerque), and the type 3 reovirus strain Dearing, a gift of A. J. Shatkin (Roche Institute of Molecular Biology, Nutley, N. J.).

Both the growth and plaquing of virus were carried out with a line of cells sensitive to the particular virus type. HeLa cells, a gift of R. Radloff (Department of Microbiology, School of Medicine, University of New Mexico, Albuquerque), were used for polio- and coxsackieviruses, RD cells, also a gift of L. C. McLaren, were used for echovirus, and L-929 cells, purchased from Microbiological Associates, Bethesda, Md., were used for reovirus. All cells were grown in monolayer cultures in Eagle medium containing either 5% newborn calf serum (HeLa cells) or 5% fetal calf serum (RD cells and L-cells).

Treatment of virus in sludge or sludge components. Both the raw and anaerobically digested sludges used for these experiments were obtained from Albuquerque Sewage Treatment Plant. To measure the virucidal activity of any fraction of these sludges, a 10-fold dilution of virus was made directly into the material to be tested and mixed for 15 min at 21°C. Samples were then incubated at 21 or 28°C for the times specified. After incubation, the samples were prepared for analysis by sonication after the addition of sodium dodecyl sulfate to a concentration of 0.1%. The sodium dodecyl sulfate-sonication procedure was used to break up possible viral aggregates and disrupt binding of viruses to dissolved and suspended solids. This procedure causes no detectable reduction of infectious virus as determined by the plaque assay (2).

RESULTS AND DISCUSSION

General properties of the virucidal agent. To identify the virucidal agent of digested sludge, initial experiments were performed to define some of its general properties. This agent appears to be quite small, as its activity is removed from the liquid fraction of digested sludge upon dialysis but remains after high-speed centrifugation (Table 1). Furthermore, it is heat stable because autoclaving not only does not destroy its activity, but increases it. The probable explanations for this latter finding are that autoclaving either inactivates a competitor of the virucidal agent or converts the agent into a more active form, perhaps as a result of

TABLE 1. *Virucidal activity against poliovirus present in digested sludge after various treatments*

Sample[a]	Recovery of PFU/ml
PBS	7.1×10^7
Anaerobically digested sludge treated as follows:	
Low-speed centrifugation (18,000 × *g*, 20 min)	3.5×10^5
Low-speed centrifugation + dialysis (12,000 mol wt cut-off)	7.1×10^7
High-speed centrifugation (200,000 × *g*, 18 h, 4°C)	1.5×10^5
Low-speed centrifugation + autoclaving (121°C, 20 min)	$<5 \times 10^2$

[a] Samples containing virus were incubated for 48 h at 28°C in phosphate-buffered saline (PBS), or in sludge treated in the manner specified, and assayed for infectious particles. The original titer of the virus preparation was 1.4×10^8 plaque-forming units (PFU)/ml.

causing the pH of the sludge to increase from about 8 to 9. The validity of the second explanation was confirmed by the experiments that follow.

Isolation of the virucidal agent in the distillate of digested sludge. An attempt was made to partially purify the agent by dialysis from the liquid fraction of digested sludge into a small volume of deionized water using dialysis tubing of various pore sizes. The agent was found to readily pass through all dialysis tubing used (molecular weight cut-off of the smallest pore size was 3,500), and its activity was found in the deionized water. However, when the volume of water was reduced by heating at 50°C under a stream of nitrogen in an attempt to concentrate the agent, the virucidal activity disappeared.

Because the virucidal agent survives autoclaving, it is clearly not the temperature that causes loss of activity during the concentration procedure. The most probable explanation is that the agent came off with the water vapor. To test this hypothesis, digested sludge was distilled and more than 95% of the virucidal activity was recovered in the initial 15% of the distillate (Fig. 1). It should be pointed out that the temperatures at which all fractions distilled in this experiment were close to that of water. This suggests that water, and not the virucidal agent, represents the main component of all distillation fractions.

Identification of the agent as ammonia. At this point it seemed probable that the virucidal agent was a small, volatile, organic compound. If this were the case, it should be removable from the distillate with activated charcoal. Therefore, a fraction of the first 10% of the distillate from digested sludge was treated with activated charcoal and assayed for virucidal activity. No loss of activity was observed. However, charcoal did remove some component of the distillate because the pungent, distasteful smell associated with this material disappeared, leaving a single clearly identifiable odor, the odor of ammonia.

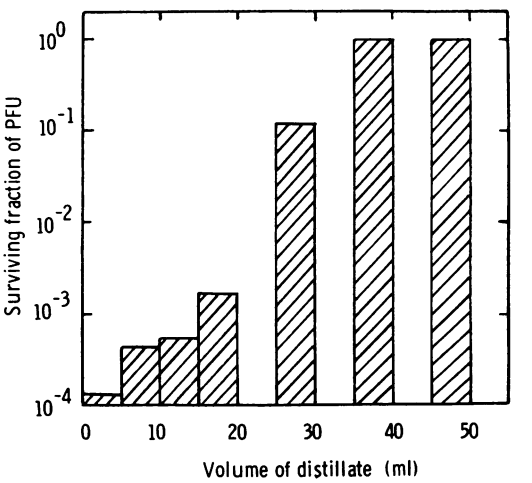

FIG. 1. *Virucidal activity in fractions distilled from digested sludge. Anaerobically digested sludge was centrifuged for 20 min at 18,000 × g and the supernatant (250 ml) was distilled. The distillate was collected in 5-ml fractions, which were separately assayed for virucidal activity. For this, poliovirus was diluted 10-fold into distillate, incubated for 4 h at 21°C, and tested for infectious particles. The titer of the untreated virus was 1.8×10^8 plaque-forming units (PFU)/ml and the surviving fraction of PFU was determined relative to this value.*

The concentration of ammonia in the liquid fraction of digested sludge, in the first 10% of the distillate from this sludge and in this same distillate after treatment with activated charcoal, was then determined using a specific ion probe (Orion Research Inc.). Digested sludge was found to contain 0.055 M ammonia, and most was recoverable in the first 10% of the distillate where the concentration was determined to be 0.475 M. Furthermore, no decrease in ammonia concentration occurred during charcoal treatment of this distillate. Certain other molecules, notably primary amines, can be falsely measured as ammonia with a specific ion probe. Therefore, the pK of the substance(s) in digested sludge that was recorded as ammonia was compared to that of a known ammonia-

containing compounds, NH_4Cl. Because their pK values were found to be identical (i.e., 9.18), the concentrations of ammonia given above are assumed to be correct.

But is ammonia virucidal, and, if so, is it virucidal in the charged or uncharged state? To answer these questions, the virucidal activity of ammonium chloride was determined over a pH range of 7 to 10 in solutions buffered with tris(hydroxymethyl)aminomethane. At pH 7, more than 99% of ammonia in NH_4Cl is in the charged state; this value decreases to 95% at pH 8, 59% at pH 9, and 13% at pH 10. The ammonium ion has no detectable virucidal activity against poliovirus, but the uncharged form of ammonia is extremely active (Table 2). To eliminate the possibility that the observed activity was due merely to pH or salt concentration in this experiment, poliovirus was incubated in solutions containing only buffer or buffer and NaCl at the same concentrations and over the same range of pH values. No loss of poliovirus infectivity was observed in these solutions (Table 2). Therefore, the loss of infectious virus found at the higher pH values is due to the uncharged form of ammonia.

If the virucidal agent in the distillate from digested sludge is ammonia, the activity of this distillate should be quantitatively similar to that of NH_4Cl at the same pH and ammonia concentration. This is indeed the case (Fig. 2). The slight difference in the activities of NH_4Cl and distillate is probably due to a substance(s) in the distillate that partially interferes with the virucidal activity of ammonia. This suggestion is supported by the finding that the activity of NH_4Cl is similarly suppressed when added to ammonia-free distillate of raw sludge and tested under the same conditions (data not shown). The essential feature of the results shown in Fig. 2, however, is that the relative activities of NH_4Cl and the distillate of digested sludge are the same at all pH values studied. This result shows that ammonia is the component of the distillate that causes the inactivation of poliovirus.

TABLE 2. *Virucidal activity of ammonium chloride as a function of pH*

Sample[a]	Recovery of PFU/ml
Control (PBS)	3.1×10^8
Tris, pH 9	3.2×10^8
Tris, pH 10	2.6×10^8
Tris + NaCl, pH 7	2.8×10^8
Tris + NaCl, pH 8	3.1×10^8
Tris + NaCl, pH 9	2.9×10^8
Tris + NaCl, pH 10	3.1×10^8
Tris + NH_4Cl, pH 7	2.8×10^8
Tris + NH_4Cl, pH 8	1.4×10^8
Tris + NH_4Cl, pH 9	3.8×10^4
Tris + NH_4Cl, pH 10	$< 5 \times 10^2$

[a] Poliovirus was diluted 10-fold into either phosphate-buffered saline (PBS), tris(hydroxymethyl)-aminomethane (Tris) (0.1 M), Tris plus NaCl (0.5 M), or Tris plus NH_4Cl (0.5 M), incubated for 4 h at 21°C at the pH values specified, and assayed for infectious particles.

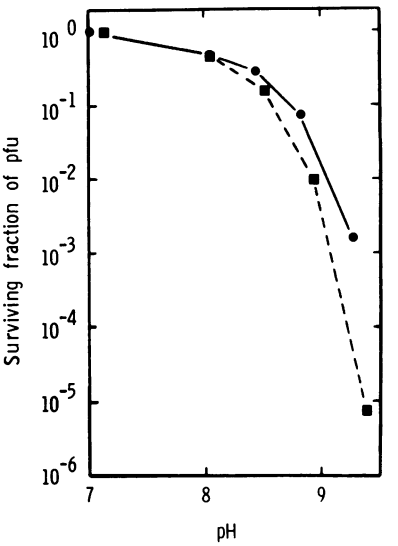

FIG. 2. *Comparative virucidal activities of NH_4Cl and the distillate of anaerobically digested sludge as a function of pH. The initial 10% of distillate from digested sludge was diluted 10-fold with 0.11 M tris (hydroxymethyl)aminomethane and divided into several portions. These portions were adjusted to pH values ranging between 7 and 9.5. Solutions of NH_4Cl containing the same final concentration of ammonia as those of the distillate (47.5 mM) were made up in an identical manner. Poliovirus strain CHAT was then diluted 10-fold into samples of all buffered solutions and incubated for 24 h at 21°C. The pH of each sample was then measured and, finally, the number of infectious viruses was determined after sonication in 0.1% SDS. Symbols: ●, samples with distillate; ■, samples with NH_4Cl.*

The experiments presented so far demonstrate that ammonia is one virucidal agent of digested sludge. There could be more. If other virucides with significant activities against poliovirus are present, the relative virucidal activity of digested sludge from one pH value to the next should be significantly different than that of NH_4Cl. It is not. No detectable activity is found in digested sludge between pH values of 4.5 and 7.5, but, as the ammonium ions in the sludge are converted into free ammonia by increasing the pH, the expected increase in virucidal activity occurs (Fig. 3). Furthermore, the

activity of NH_4Cl increases with pH in the same manner as that of digested sludge when compared at the same ammonia concentration. However, NH_4Cl is somewhat more virucidal. Again, this is probably due to the presence of a protective substance in the sludge. Figure 3 also shows that raw sludge expresses virucidal activity with the same dependence on pH. This activity was not previously detected in raw sludge because its natural pH is about 6.0, approximately two full pH units less than that of anaerobically digested sludge. From these results, it appears that ammonia is the only major virucidal agent in either raw or digested sludge that inactivates poliovirus at pH values between 4.5 and 9.5. It should be possible to take full advantage of this agent by merely increasing the pH of either type of sludge.

Although the toxicity of ammonia against a number of organisms has been shown to display the same pH dependence as that described here for poliovirus (cf. references 1, 4) we know of no similar toxic effects that have been demonstrated with viruses. One brief report published in 1975 suggests that ammonia at pH 7 has some virucidal activity against poliovirus (J. M. Reed, J. M. Fenters, and C. Lue Hing, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, Q10, p. 206). Because less than 1% of ammonia is in the uncharged state at this pH, very little of its potential activity should have been expressed under these conditions.

Effect of ammonia on other enteric viruses. Poliovirus is only one of several types of viruses that have been found in sludge but, up to this time, only one strain of poliovirus has been shown to be sensitive to ammonia, the type 1 strain CHAT. Therefore, other types of enteric viruses along with other strains of poliovirus were examined for their sensitivities to ammonia. Table 3 shows that other strains of polio-

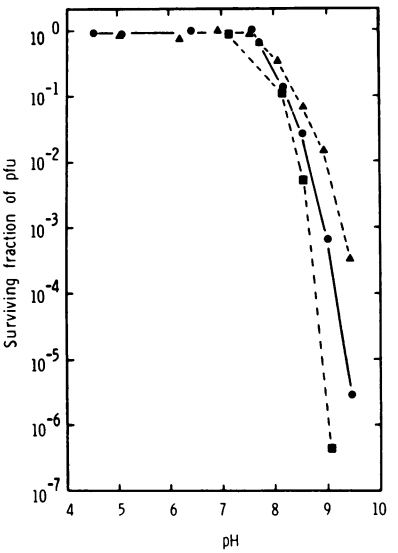

FIG. 3. *Comparative virucidal activities of NH_4Cl, digested sludge, and raw sludge as a function of pH. Samples of anaerobically digested and raw sludge were centrifuged at 18,000 × g for 20 min, and the ammonia concentrations in the supernatant fractions were measured and found to be 55 and 32 mM, respectively. A 20-fold dilution of either 2 M acetate, phosphate, or tris(hydroxymethyl)-aminomethane (Tris) was then made into samples of each of these sludge fractions. Solutions containing 55 mM NH_4Cl were treated in the same manner and the pH values of all samples were adjusted. Samples with acetate buffer were adjusted to pH values of 4 and 5, those with phosphate buffer to values of 6 and 7, and those with Tris buffer to pH values between 7.5 and 9.5. Poliovirus strain CHAT was next diluted 10-fold into these samples and incubated at 21°C for 72 h. The final pH of each sample was then determined, and the number of infectious viruses was measured by the plaque assay. Symbols: ●, samples with digested sludge; ▲, samples with raw sludge; ■, samples with NH_4Cl.*

TABLE 3. *Effect of ammonia on infectivities of several strains of enteric viruses*[a]

Virus	Recovery (%) of PFU			
	PBS	Tris (pH 9.5)	NH_4Cl (pH 7)	NH_4Cl (pH 9.5)
Poliovirus type 1 strain CHAT	103	63	53	<0.000035
Poliovirus type 1 strain Mahoney	108	100	68	<0.000014
Poliovirus type 2 strain 712	86	60	24	<0.000044
Coxsackievirus A13	58	28	35	<0.00012
Coxsackievirus B1	107	100	52	<0.000046
Echovirus 11	61	9.6	34	<0.00015
Reovirus type 3	55	9.8	62	3.1

[a] Virus was diluted 10-fold into phosphate-buffered saline (PBS), pH 7.0, 0.1 M tris(hydroxymethyl)-aminomethane (Tris), pH 9.5, 0.1 M Tris-0.5 M NH_4Cl, pH 7.0, or 0.1 M Tris-0.5 M NH_4Cl, pH 9.5, and incubated at 21°C for 24 h. The final pH values of the samples were then determined and each sample was titered after sonication in 0.1% sodium dodecyl sulfate. Percent recoveries were determined relative to a control sample left frozen during the period of incubation.

virus, coxsackieviruses A13 and B1, and echovirus 11 are all readily inactivated by ammonia. These viruses all belong to the picornavirus group, indicating that the virucidal effect of ammonia is a general property for viruses of this group. Reovirus, a common enteric virus of a different group, is quite insensitive to ammonia under these conditions. These results suggest that reovirus may be a more suitable indicator of viral disinfection than poliovirus during wastewater treatment. Further studies on the mechanism of viral inactivation by ammonium should help establish a feasible and effective method of ridding wastewater sludges of infectious viruses. The possibility of extending these results to the treatment of wastewater and other materials contaminated with viruses is also under consideration.

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