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Characterization of Virucidal Agents in Activated Sludge

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A comprehensive study was carried out to determine the properties of agents responsible for loss of virus infectivity in mixed-liquor suspended solids (MLSS) of activated sludge. Initial experiments revealed that model enteric viruses (poliovirus-1 and rotavirus SA-11) were irreversibly inactivated in MLSS and released their RNA genomes. Enteric viruses belonging to other genera (echovirus-12, coxsackievirus A13, reovirus-3) were also shown to lose infectivity in MLSS. Although the virucidal activity decreased at reduced temperatures, MLSS still retained significant activity at 4°C. The virucidal agents in MLSS were stable for months at 4°C, but their activity decreased approximately 50% during 4 days of aeration at 26°C. Primary effluent, the nutrient source for activated sludge, also contained virucidal activity. After centrifugation of MLSS, almost all virucidal activity was found in the particulate fraction because of inhibitory substances retained in the supernatant fraction. Decreasing or increasing the solids concentration of the particulate fraction did not increase the virucidal activity of the fraction. The effects of heat and antibiotics on the virucidal activity of MLSS, coupled with the finding that the activity can be produced in autoclaved primary effluent seeded with MLSS, strongly support the conclusion that microorganisms are responsible for this activity. Attempts to characterize the virucidal microbial components of MLSS indicated that treatments that resulted in the inactivation or removal of microorganisms also caused a loss of virucidal activity. Thus, it appears that the virucidal components of microorganisms are either short-lived or active only while bound to the organisms themselves.

Sewage treatment processes that remove or inactivate indigenous pathogens reduce the potential health risks associated with human contact with wastewater effluents. One process known to reduce the concentrations of detectable enteric pathogens is activated sludge treatment. Initially, it was suggested that virus loss during this treatment is due to association with wastewater particulates which subsequently settle and become components of sludge (5, 7, 14, 17–19). Other evidence indicates that the observed disappearance of infectious viruses is due to inactivation (11, 22). As previously reported by one of the authors of this article, a plaque assay showed that poliovirus seeded into mixed-liquor suspended solids (MLSS) of activated sludge loses infectivity (27), a fact which supports the suggestion that virus loss is due to inactivation.

Accelerated loss of viral infectivity has also been reported for other water environments such as wastewater sludge (26), fresh water (1, 6, 12, 13, 15, 21, 24, 32, 33), and marine water (2, 3, 16, 20, 23). Loss of viral infectivity in these waters has been associated with various factors such as ammonia (29), ionic detergents (30), light (9), and heat (1, 33). A number of studies have suggested that microorganisms also have a role in causing the disappearance of infectious viruses from environmental waters (8, 10, 25, 32) as well as in activated sludge (27). This possibility was examined as part of an in-depth study of the inactivation of enteric viruses in MLSS of activated sludge.

MATERIALS AND METHODS

Activated sludge samples. MLSS of activated sludge were obtained from the Sycamore Sewage Treatment Plant in Cincinnati, Ohio. For this facility, which handles 5×10^6 gal (2×10^7 liters) per day, the influent is >90% domestic. Activated sludge treatment is a secondary treatment at this plant and follows primary sedimentation of incoming sewage. The process is contact stabilization, in which an average retention time of 5 to 8 h is expected. Grab samples of MLSS were obtained and transported directly to the laboratory, where they were kept at 4°C. Total plate count (TPC) of organisms and suspended solids concentration were measured on each sample within 24 h by standard methods (4).

Viruses and cells. Five enteric viruses were used in this study. Attenuated poliovirus type 1 CHAT, coxsackievirus A13, and reovirus-3 Dearing were purchased from the American Type Culture Collection, Rockville, Md. Rotavirus SA-11 was provided by M. K. Estes, Baylor College of Medicine, Houston, Tex. Echovirus-12 was from our laboratory. Poliovirus and coxsackievirus were grown in HeLa cells. The titers of coxsackievirus were determined by plaque assay in the same kind of cells. Echovirus was grown and the titers of echovirus and poliovirus were determined by plaque assay in RD cells. Rotavirus and reovirus were grown and their titers were determined in MA-104 cells and L-929 cells, respectively. All cell lines were grown as monolayer cultures in either Eagle minimal essential medium (HeLa and L-cells) or special minimal essential medium, Richter modification (MA-104 and RD cells), each containing antibiotics and 10% fetal calf serum. Methods for growing, labeling, and purifying the viruses and methods for conducting plaque assays have been described previously (28, 31, 32).

General experimental procedure. Except for specific changes which are described for each set of experiments, the following protocol was used throughout the study. MLSS of activated sludge (20 ml in a 125-ml cotton-stoppered flask) were seeded with a 100-fold dilution of virus (purified or from a clarified lysate) and incubated on a rotating platform (180 rpm), usually at 26°C. Samples were collected at the start of the experiment (0 h) and after different times of incubation. These samples were diluted twofold with a 2× concentration of tryptose phosphate broth and stored frozen at −70°C. This broth was particularly important in preserving the infectivity of rotavirus and reovirus during freezing in

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TABLE 1. Effects of MLSS on recoverable poliovirus infectivity[a]

Sample and incubation time (h)	Recoverable poliovirus infectivity (PFU/ml)
MLSS	
0	2.4×10^8
48	1.3×10^5 (0.05%)
MLSS (filtered)	
0	2.0×10^8
48	1.4×10^8 (70%)
Control (broth)	
0	4.0×10^8
48	3.5×10^8 (88%)

[a] [^{3}H]uridine-labeled poliovirus was added (100-fold dilution) to MLSS, filter-sterilized (0.2-μm pore size) MLSS, or tryptose yeast extract broth and incubated at 26°C on a rotating platform. A portion of each collected sample was immediately analyzed by glycerol gradient centrifugation (Fig. 1), and the remainder was measured for recoverable PFU.

MLSS. The infectivity of each virus sample was determined by plaque assay. The initial virus titer in every experiment was between 10^6 and 10^8 PFU/ml. All experiments were done at least twice. Little variation was observed in replicate experiments; therefore, only the results of one experiment from each replication are presented.

RESULTS

Effects of activated sludge on poliovirus particles. Previous results (27) showed that the titer of infectious poliovirus as determined by a plaque assay decreases rapidly in activated sludge. It was not determined, however, whether loss of infectivity was due to true inactivation or masking of infectivity by sludge components. To make this determination, the effect of the MLSS of activated sludge on the sedimentation coefficient of purified [^{3}H]uridine-labeled poliovirus particles was measured by glycerol gradient centrifugation after incubation at 26°C. The effect of filter-sterilized MLSS on virus stability, which had previously been shown to have no detectable effect on the rate of poliovirus inactivation (27), was measured as a control.

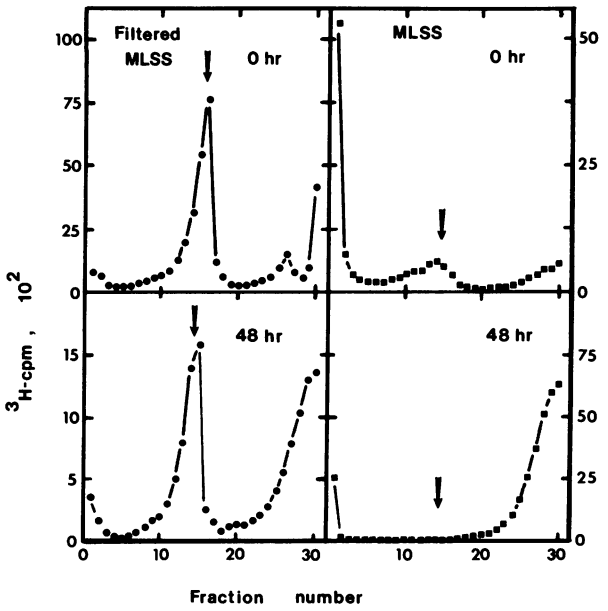

FIG. 1. Sedimentation analysis of [^{3}H]uridine-labeled poliovirus in 15 to 30% glycerol gradients after incubation (26°C) for 0 or 48 h in either filter-sterilized or untreated MLSS. Centrifugation was for 3 h at 25,000 rpm in an SW27 rotor. The direction of sedimentation was from right to left. The arrow denotes the position of [^{14}C]uridine-labeled polioviruses added to the samples as markers just before gradient analysis.

As expected, incubation of poliovirus in filtered MLSS for 48 h at 26°C had little effect on poliovirus infectivity, while the untreated MLSS caused recoverable infectivity to decrease >99.9% under the same conditions (Table 1). Likewise, the filtered MLSS caused <50% of poliovirus particles to break down and release their RNA genomes during this time (Fig. 1). In contrast, most poliovirus particles in untreated MLSS became associated with particulates and sedimented to the bottom of the glycerol gradient. After incubation for 48 h at 26°C, however, these particles were disrupted, as shown by the fact that their RNA remained near the top of the gradient after centrifugation. Thus, incubation in untreated MLSS caused irreversible inactivation of poliovirus particles.

Effects of activated sludge on the infectivity of other enteric viruses. To determine whether loss of infectivity in activated sludge is a general property of viruses commonly found in sewage, the inactivation rates of enteric viruses representative of different genera were measured in MLSS. In addition, the effects of filtration (0.2-μm pore size) and heat (autoclaving) on the virucidal properties of MLSS were determined. These treatments were previously found to destroy virucidal activity against poliovirus (27). Inactivation rates in broth, which is very protective of viruses, were measured as controls.

Infectivities of all viruses tested (i.e., coxsackievirus A13, echovirus-12, rotavirus SA-11, and reovirus-3) were found to be reduced between 98.8 and 99.8% within 24 h in MLSS at 26°C (Table 2). Little or no reduction occurred during this time in filtered or autoclaved MLSS. The effects of MLSS and filtered MLSS on purified [^{3}H]uridine-labeled SA-11 particles were also measured by sedimentation analysis in glycerol gradients, and the results were similar to those found with poliovirus (Fig. 1). Thus, inactivation in MLSS appears to be a general property of enteric viruses.

TABLE 2. Inactivation of enteric viruses from different genera in MLSS

Virus and incubation time (h)	% Survival of PFU of enteric virus in:			
	MLSS	Filtered MLSS	Autoclaved MLSS	Broth
Coxsackievirus A13				
0	100	100	100	100
24	0.3	68	51	100
48	0.3	35	31	81
Echovirus-12				
0	100	100	100	100
24	0.2	80	83	47
48	0.1	47	88	31
Rotavirus SA-11				
0	100	100	100	100
24	1.2	76	100	90
48	0.5	37	100	76
Reovirus-3				
0	100	100	100	100
24	0.2	46	100	100
48	0.04	21	21	92

Effect of incubation temperature on virucidal activity of activated sludge. Because the temperature of wastewater during activated sludge treatment in most plants is <26°C during most of the year, the effect of lower temperatures on the virucidal activity of MLSS against poliovirus was examined. Activity at 15°C was found to be nearly equivalent to that at 26°C, but poliovirus inactivation was very slow at 4°C (Table 3). However, relative to that found in broth at 4°C, significant activity was still displayed by the MLSS. Thus, activated sludge continued to accelerate virus inactivation even at temperatures near the freezing point of water.

Stability of virucidal components of activated sludge. In the experiments described above, MLSS were treated for total suspended solids and TPC of organisms on the day they were obtained and then stored at 4°C until used for experimentation. Some differences in the virucidal properties of MLSS obtained on different dates were observed, but these differences were unrelated to the small variations in total suspended solids or TPC. No loss of virucidal activity was found during a 4-month storage period for any MLSS sample tested (results not shown). Incubation of an MLSS sample at 26°C with aeration, however, was shown to cause a large reduction in virucidal activity against poliovirus (Fig. 2). This reduction was approximately 50% within 4 days. Thus, the virucidal components of MLSS were found to be unstable for extended periods at 26 but not at 4°C.

Virucidal activity of primary effluent. Since the major portion of MLSS used in this study originated from primary effluent of settled raw sewage, it was of interest to determine whether this source material contained virucidal activity. The addition of poliovirus to primary effluent from the Sycamore Treatment Plant was found to cause loss of infectivity at 26°C at a rate nearly comparable to that normally found in MLSS (results not shown). Filtration of this material removed the virucidal components, as was also found with MLSS. Therefore, the virucidal activity of MLSS was present in the source material.

Virucidal activities of concentrated and diluted activated sludge. Although in the previous study (27) some residual virucidal activity was found in the supernatant fraction of MLSS after centrifugation at moderate speeds (27,000 × *g*; 20 min), the MLSS samples examined in the study reported here retained little activity in the supernatant fraction after centrifugation at 11,000 × *g* for 20 min (Table 4). Suspension of the pelleted fraction in distilled water to a volume equal to that of the original MLSS, however, permitted nearly full recovery of virucidal activity. To determine whether the activity of the pelleted fraction could be increased above that found in MLSS, this fraction was also suspended at different concentrations in distilled water and tested for virucidal activity against poliovirus. Comparable activities were found at solids concentrations ranging from 5 to 0.1 times that present in the source MLSS, but little activity remained at a concentration 0.01 times that of MLSS (Table 4). A similar result was found with rotavirus SA-11, except that a large decrease in virucidal activity was found at the 0.1× concentration (results not shown). These results indicated that suspension of pelleted particulates to a solids concentration normally present in MLSS permitted nearly optimal virucidal activity.

Identification of virucidal agents in activated sludge. Evidence has been presented previously that microorganisms in activated sludge are responsible for virus inactivation (27).

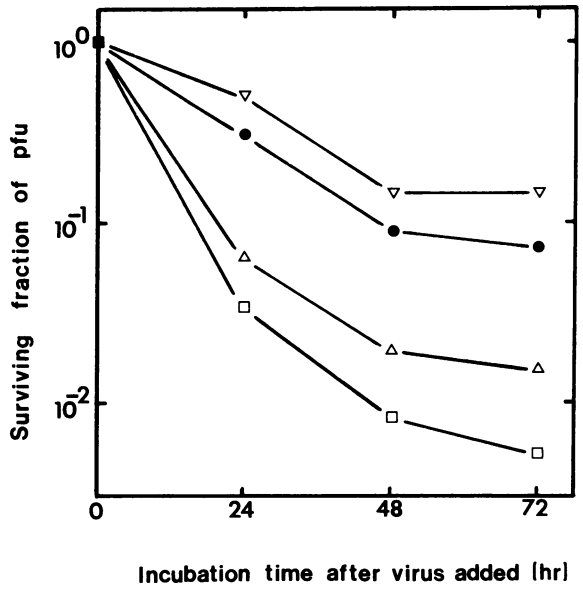

FIG. 2. Effect of incubation time on the virucidal activity of MLSS against poliovirus. MLSS were incubated for 0 (□), 2 (△), 4 (●), or 6 (▽) days at 26°C with aeration; seeded with poliovirus; and tested for virucidal activity.

TABLE 3. Effects of incubation temperature on the inactivation rate of poliovirus in MLSS

Incubation temp (°C)	Incubation time (h)	% Survival of PFU of poliovirus in:	
		MLSS	Broth
26	0	100	100
	24	3.9	100
	48	0.8	95
	72	0.7	90
15	0	100	100
	24	4.6	100
	48	1.9	100
	72	1.6	100
4	0	100	100
	24	84	100
	48	73	100
	96	34	100
	144	26	100

TABLE 4. Virucidal activities of MLSS particulates against poliovirus

Sample	% Survival of poliovirus PFU[a]
MLSS	0.8
Distilled water	71
MLSS supernatant after centrifugation[b]	18
MLSS particulates suspended in distilled water at concn of:	
5×	6.5
2×	2.8
1×	1.9
0.5×	2.1
0.1×	2.1
0.01×	38
0.001×	77

[a] After incubation at 26°C for 48 h.
[b] At 11,000 × *g* for 20 min.

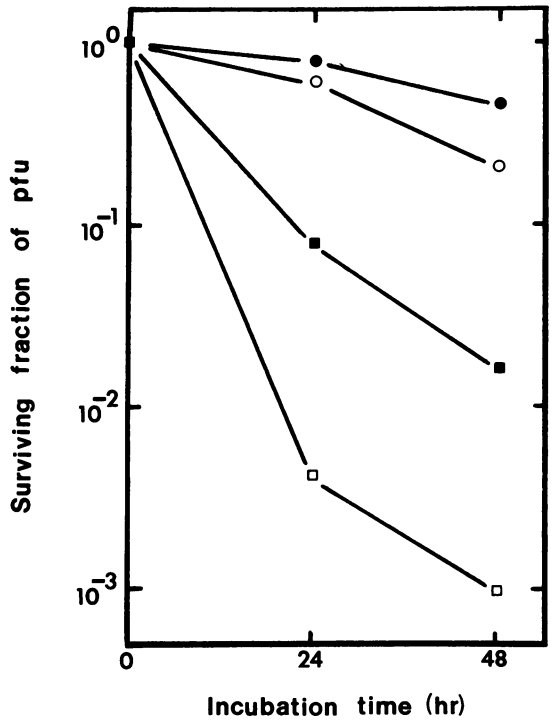

FIG. 3. Effect of heat treatment on the virucidal activity of MLSS on rotavirus SA-11. MLSS were incubated at room temperature (□), 40°C (■), 50°C (○), or 60°C (●) for 1 h before being seeded with poliovirus. Loss of infectivity was determined by plaque assay.

The strongest support for this suggestion was the finding that broth seeded with activated sludge acquired virucidal activity against poliovirus after incubation at 26°C. It appeared, therefore, that the microorganisms responsible for the virucidal activity in MLSS could readily be grown in broth, isolated, and identified. During our investigation it became apparent, however, that the active agent in seeded broth had properties different from those of the active agent in activated sludge. For example, the agent in broth retained full virucidal activity against poliovirus after autoclaving or filtration and had increasingly greater activity at higher pH values (results not shown). Ammonia, previously identified as the virucidal agent against enteroviruses in anaerobically digested sludge, had these same properties (29). The concentration of ammonia in the broth cultures was measured and found to be identical to that predicted from the virucidal activities of the cultures. Therefore, broth cultures could not be used for identification of the virucidal agents in sludge. To avoid possible effects from ammonia, all further experiments were conducted with rotavirus SA-11 as the model virus, because it was not found to be inactivated by this compound.

A study recently completed in our laboratory showed that microorganisms were responsible for virucidal activity in fresh water (32). It was found that virucidal activity of fresh water was destroyed during 1 h of heat treatment at 60°C. If microorganisms are also responsible for the virucidal activity of activated sludge, this activity would be expected to respond in a comparable manner to heat treatment. It was found that 1 h at temperatures between 40 and 60°C had the same effects on the virucidal activity of activated sludge as was found with fresh water (Fig. 3). This finding supports the suggestion that microorganisms also produce the virucidal activity in activated sludge.

The suggestion was further supported by studies of the effects of different antibiotics on the virucidal activity of activated sludge. A 24-h incubation period with inhibitory concentrations of gentamicin essentially eliminated virucidal activity. Streptomycin and chloramphenicol considerably reduced this activity, while penicillin and tetracycline both caused a slight decrease in virucidal activity of MLSS (Fig. 4). Cycloheximide, which is used specifically to block protein synthesis in eucaryotic organisms, had no effect on this activity.

The following experiment confirmed that microorganisms can produce virucidal activity in activated sludge. MLSS were added (10-fold dilution) to autoclaved primary effluent and incubated at 26°C for 4 days with aeration. The product was subcultured four times in the same manner and tested for virucidal activity. Although the activity of the subcultured material was less than that of the original activated sludge, it was much greater than that of the control sample, i.e., autoclaved MLSS seeded into autoclaved primary effluent but otherwise treated in the same manner (Fig. 5). Furthermore, heat treatment (60°C, 1 h) sharply reduced the virucidal activity of the subcultured product, as it had reduced that of activated sludge (Fig. 3). Since the original MLSS had been diluted 100,000-fold during this experiment and a dilution of ≥100-fold was found to eliminate detectable virucidal activity (results not shown), the virucidal agent must have been produced by microbial growth in the autoclaved primary effluent.

Additional experiments revealed that if the suspended solids of the subcultured product were collected by centrif-

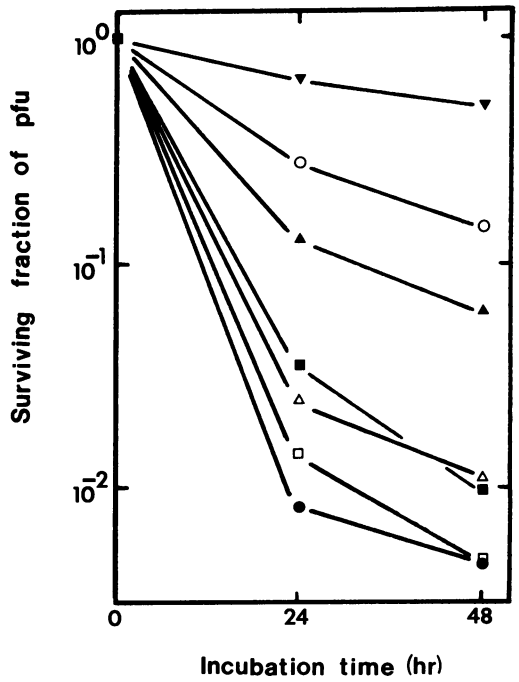

FIG. 4. Effect of antibiotics on the virucidal activity of MLSS against rotavirus SA-11. MLSS were incubated at 26°C with aeration for 24 h with (per milliliter) no antibiotic (□), cycloheximide (500 μg) (●), tetracycline (25 μg) (△), penicillin (400 U) (■), chloramphenicol (125 μg) (▲), streptomycin (400 μg) (○), or gentamicin (100 μg (▼). Each treated sample was seeded with virus, and loss of infectivity was measured after further incubation at 26°C.

ugation and subcultured in autoclaved primary effluent every 24 h over a 5-day period, the new subcultured material developed virucidal activity comparable to that of the original MLSS and displayed the same heat sensitivity profile (results not shown). It was concluded, therefore, that microorganisms in activated sludge could be grown in autoclaved primary effluent and would produce the same kind of virucidal activity as was present in the original activated sludge. Because of the difficulties associated with producing significant activity under these conditions, the specific microorganisms responsible for activity were not identified. A previous study with fresh water indicated that many bacterial species could have this property (32).

Attempts to characterize virucidal components of microorganisms. Identification of the microbial components responsible for virucidal activity requires that they be separable from the other components of activated sludge and that they retain their activities after separation. Initial attempts to isolate the virucidal components of activated sludge were made by differential centrifugation. Although centrifugation at 11,000 × *g* for 30 min pelleted the active material from MLSS, as has been shown (Table 4), it was possible that virucidal activity could be retained in the supernatant fraction after blending of MLSS and removal of most suspended solids by centrifugation at much lower speeds. It was found, however, that centrifugation of blended MLSS at 60 × *g* for 5 min, conditions that pelleted only 59% of the TPC of organisms, caused almost complete removal of virucidal activity (results not shown).

Further experiments revealed that lack of activity in the supernatant fraction centrifuged at 60 × *g* and containing large numbers of viable organisms was due to inhibitory substances. Centrifugation of this supernatant at 11,000 × *g* for 30 min followed by two washes of the resulting pellet with distilled water allowed greater virucidal activity to be

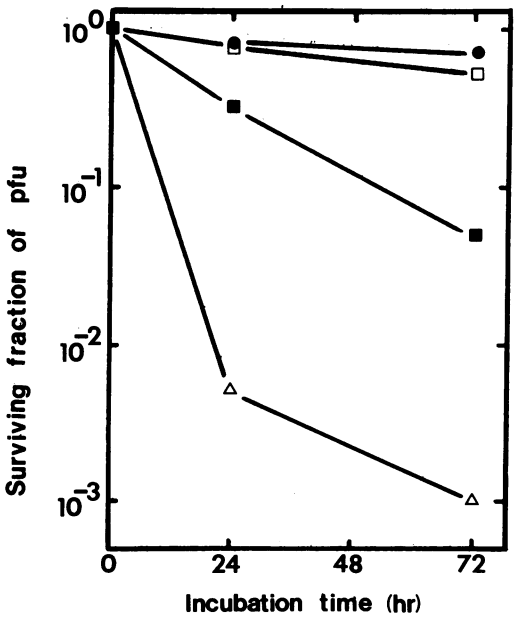

FIG. 5. Production of virucidal activity in autoclaved primary effluent seeded with MLSS. The experimental protocol is described in the text. Rotavirus SA-11 inactivation was measured at 26°C in the original MLSS (△), in MLSS seeded into autoclaved primary effluent and subcultured four times (■), in the same material heated for 1 h at 60°C (□), and in autoclaved MLSS seeded into autoclaved primary effluent and subcultured four times (●).

TABLE 5. Release or loss of virucidal activity from the pellet fraction of MLSS after blending with chemical solubilizers, centrifuging at low speed, and washing

Chemical (concn)	TPC/ml	% Survival of SA-11 PFU[a]
None	1.3×10^7	0.02
Sodium dodecyl sulfate (0.1%)	2.6×10^6	0.04
Tween 20 (0.1%)	8.9×10^6	0.07
Tween 80 (0.1%)	6.4×10^6	0.10
Triton X-100 (0.1%)	1.3×10^7	0.10
Trypsin (10 mg/ml)	5.4×10^6	0.07
Urea (6 M)	5.8×10^4	62

[a] After incubation at 26°C for 48 h.

expressed than was found in the original MLSS (results not shown). Inhibitory substances could also be washed from the original pellet after centrifugation at 60 × *g* for 5 min, thus permitting the expression of greater virucidal activity in this MLSS fraction as well. The active agents in both washed fractions were largely inactivated by 1 h of heat treatment at 60°C, as were the active agents in the original MLSS. These results show that MLSS of activated sludge contained components with competing activities regarding viral inactivation rates.

To avoid the effects of these inhibitory substances, attempts were made to release the virucidal agents from the sludge particulate fraction by treatment with solubilizing chemicals. MLSS were blended in the presence of these chemicals and centrifuged (60 × *g*; 5 min). The pellet was then washed twice with distilled water to remove the chemicals as well as inhibitory substances and examined for TPC of organisms and virucidal activity (Table 5). The only chemical tested that caused a large release or loss of virucidal activity from this pellet was urea, which also was the only chemical that caused a >90% reduction in the TPC of organisms in this pelleted fraction compared with that in the untreated control.

If urea causes release of virucidal components from the particulate fraction of activated sludge rather than destruction of the active agents, the activities of these components should be detectable in the released material. Accordingly, MLSS were blended and then washed four times to remove inhibitory substances. A portion of the final pellet was tested for virucidal activity. The remainder was blended with 6 M urea, dialyzed (8,000-molecular-weight cutoff) to remove urea, and tested for virucidal activity. Virucidal activity present in the washed pellet of MLSS was not recovered after urea treatment and dialysis (results not shown). Therefore, unless the virucidal component of the MLSS pellet fraction was removed during dialysis, it must have been inactivated during urea treatment together with >99% of the TPC of organisms.

DISCUSSION

The results of this study demonstrate that enteric viruses are irreversibly inactivated when seeded into MLSS of activated sludge. Although the rate of inactivation was shown to increase with temperature, MLSS caused virus inactivation even at 4°C. Thus, if viruses in sewage were exposed for a sufficient amount of time to the virucidal components of MLSS, they should eventually be inactivated, even at temperatures near the freezing point of water. Further studies were performed to determine the mechanism of virus inactivation in MLSS. With this information, it

should be possible to find methods to accelerate the inactivation process.

The cause of virus inactivation in MLSS appears to be microorganism related. Methods that inactivated or removed microorganisms in MLSS consistently resulted in loss of virucidal activity. The same conclusion was reached in a previous study on virus inactivation in fresh water (32). Because the virucidal microbial components in MLSS could not be separated from viable microorganisms, it follows that these components either are short-lived and must be continuously produced or are active only while associated with the microorganisms themselves. It was previously suggested that the virucidal components of freshwater microorganisms are proteolytic enzymes (32). Since no significant difference was found between the properties of the virucidal components of activated sludge and those of fresh water, it is further suggested that proteolytic microbial enzymes may also be the virucidal agents in activated sludge. If this is the case, it may be possible to increase the activities or concentrations of these enzymes in activated sludge, perhaps by seeding with the appropriate microorganisms. This, in turn, should reduce the infectious virus concentration of sewage treatment plant effluents along with the health risks associated with these effluents.

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