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Influence of pH, Salinity, and Organic Matter on the Adsorption of Enteric Viruses to Estuarine Sediment

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This study was designed to determine the degree of adsorption of enteric viruses to marine sediment and factors controlling this association. Adsorption and elution characteristics of several enteroviruses and one rotavirus to estuarine sediments were studied under varying conditions of pH, salinity, and presence of soluble organics. Greater than 99% of the added poliovirus type 1 (LSc), coxsackievirus type B3 (Nancy), echovirus type 7 (Wallace), and rotavirus (SA-11) adsorbed to sediment. Echovirus 1 (Farouk) and a recent isolate typed as coxsackievirus B4 adsorbed significantly less than poliovirus 1 under similar conditions of varying salinity and pH. The presence of soluble organic matter, in the form of secondary sewage effluent or humic acid, did not affect these patterns of adsorption. Only echovirus 1 (Farouk) desorbed when the pH or salinity was altered and then only to a small extent. Three recent isolates of echovirus 1 and echovirus 29 (strain JV-10) also demonstrated varying amounts of adsorption to sediment. These data indicate that enteric viruses can become readily associated with sediment in the estuarine environment and that this association may play a major role in their hydrotransportation and survival.

Fecal contamination of water used for consumption, commercial, or recreational purposes is regarded as a public health hazard because of the presence of pathogenic bacteria and viruses. The continued occurrence of outbreaks of infectious hepatitis (16, 20) and gastroenteritis (1), caused by the consumption of shellfish harvested from sewage-polluted waters, indicates the need for a greater understanding of the fate of enteric viruses in marine waters.

Recent studies have indicated that marine sediments may play a role in the survival and distribution of enteric bacteria and viruses in the marine environment. In laboratory studies, *Escherichia coli* (6) and enteroviruses (24) were shown to survive longer in the presence of sediment than in seawater alone. They have also been detected in high concentrations in sediments of polluted coastal areas (5, 26). From these results it appears that sediment can act as a reservoir of viruses, even though it may not be possible to detect them in the overlying water.

Virus retention on the surface of solids is dependent upon salt concentration, pH, soluble organic matter, type of surface, etc. (2, 10, 26). This study was undertaken to determine whether the changing environmental conditions that occur in estuaries could affect virus adsorption to sediments. Such data would provide further insight into their role in virus transport and survival in nature.

MATERIALS AND METHODS

Virus and virus assays. Enterovirus assays were performed using the BGM cell line, which was passaged, grown, and maintained by previously described methods (19). Enteroviruses used included poliovirus (polio) 1 (strain LSc), echovirus (echo) 1 (Farouk), coxsackievirus (coxsackie) B3 (Nancy), echo 7 (Wallace), and echo 29 (JV-10); one coxsackie B4 and various echo 1 isolates that were isolated and typed in this laboratory were also used. The simian rotavirus SA-11 was also used. Plaque-purified enterovirus stocks were grown in BGM cells, concentrated 10-fold, partially purified by membrane chromatography (13), and stored at −20°C. Rotavirus SA-11 was grown and plaqued in MA-104 cells, a continuous cell line of fetal rhesus monkey kidney, as described previously (E. M. Smith, M. K. Estes, D. Y. Graham, and C. P. Gerba, J. Gen. Virol., in press). Virus samples were diluted in tris(hydroxymethyl)aminomethane-buffered saline which contained 20 mM tris(hydroxymethyl)aminomethane, 140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 6 mM dextrose, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2% fetal calf serum. Virus assays were performed by the plaque-forming unit (PFU) method as used in this laboratory (19). A modified procedure was required to plaque assay rotavirus: 100 μg of diethylaminoethyl (DEAE)-dextran per ml and a 1:60 dilution of Oxoid pancreatin were added to the overlay medium (without serum) (Smith et al., in press).

Salinity. Salinity, determined with an AO T/C refractometer (American Optical Corp., Buffalo, N.Y.), is expressed as grams per kilogram.

pH. pH was determined with a Corning digital 110 pH meter (Corning Glass Works, Corning, N.Y.).

Adsorption experiments. Sediment was obtained from a coastal canal community bordering Galveston Bay, which is located along the upper Texas Gulf Coast. Sediment was collected with an Ekman dredge. The sediment was composed of 20.7% sand, 24.88% clay, and 54.4% silt and contained 3.8% organic matter. It was washed with 2 volumes of deionized water to remove the salts and was then dried in a 350°F drying oven overnight. It has been shown previously (9) that polio 1 (LSc) adsorbs equally well to dried and natural sediment. The present studies were performed with dried sediment and artificial seawater to minimize the number of variables involved. Filter-sterilized (0.22 μm) artificial seawater was prepared by using the values of Dittmar as given by Matossian and Garabedian (17).

Virus stocks were diluted 1:1,000 or 1:10,000 to give approximate final titers from 10^4 to 10^5 PFU/ml in artificial seawater. To study the effects of different parameters on virus adsorption, a 30-ml amount of filter-sterilized artificial seawater containing virus was added to 10 g of washed and oven-dried sediment. The mixture of virus, seawater, and sediment was shaken for 30 min at 250 rpm and then centrifuged at 1,400 × *g* for 5 min. Under these conditions, more than 99.9% of polio 1 (LSc) was adsorbed within 5 min. An adsorption time of 30 min was used to assure maximum virus adsorption. To monitor virus inactivation during the course of each experiment, samples of artificial seawater adjusted to each parameter being tested were taken at the beginning and at the conclusion of each experiment. The amount of virus adsorption to sediment was determined by the following formula: 100% − [(number of viruses in the centrifuged supernatant/number of viruses in the control) × 100%]. No inactivation of viruses was observed in the controls during the course of the experiments. All experimental data shown are averages of two to six experiments.

Elution experiments. In elution experiments, virus was first adsorbed to the sediment in the pH 8.0 artificial seawater diluted to 16-g/kg salinity in distilled water (pH 7.4 to 7.7 after mixing with sediment). These values were chosen because the seawater in the coastal canal community from which the sediment was obtained had an average pH of 8.0 and a salinity of 16 g/kg. The sediment was then pelleted and resuspended in seawater that had been adjusted to the test parameter. The sample was shaken for 30 min and the sediment was removed from suspension by centrifugation. The supernatant was then sampled to determine the amount of virus eluted under the test conditions.

Test conditions. Salinity, pH, and the presence of soluble organic matter were studied to determine their effects on virus adsorption to or elution from sediment. Salinity of filter-sterilized artificial seawater (33-g/kg salinity) was adjusted by dilution with distilled deionized water. The pH was adjusted to the desired value in several samples of artificial seawater diluted to 16-g/kg salinity. Virus was added to each sample, and then 30-ml amounts were added to 10-g amounts of sediment. After the seawater and sediment were mixed, pH was again adjusted to the desired levels since the sediment had a considerable buffering capacity. The pH was adjusted using 1 N HCl, 0.1 N NaOH, or 1 N NaOH. Samples were then shaken for 30 min to allow for virus adsorption.

Two different sources of organic material were used—humic acid and secondary sewage effluent. Humic acid is a normal breakdown product of vegetable matter in soil and is found in almost all natural waters (15, 22). Humic acid was obtained from the Aldrich Chemical Co., Milwaukee, Wis., and the secondary effluent was obtained from a local sewage treatment plant in Houston, Tex. In all experiments in which the pH was not adjusted, the presence of sediment caused the pH to equilibrate in the range of 7.5 to 7.7. All experiments were performed at room temperature (22 to 25°C).

Attempts to elute echo 1 (Farouk). Sediment with adsorbed virus was sequentially resuspended five times in artificial seawater at high salinity (33 g/kg) and pH 8.0. A second sediment sample containing virus was sequentially resuspended five times in artificial seawater at low pH (6.0) and 16-g/kg salinity. The mixture of sediment and seawater was readjusted to pH 6.0 after it was mixed and was then shaken for 30 min in attempts to desorb virus.

Elution of virus from sediment versus direct assay. Virus was suspended in artificial seawater (pH 7.8, 16-g/kg salinity) at a concentration of 10^5 PFU/ml. A 15-ml amount of seawater containing virus was mixed with each of two samples of 10 g of washed and dried sediment and was shaken for 30 min on a shaker table at 250 rpm. The sediment was then pelleted by centrifugation at 1,400 × *g*, and the supernatant was assayed for virus. The two sediment samples were then assayed for virus. One sample was eluted with 0.25 M glycine plus 0.05 M ethylenediaminetetraacetic acid, pH 11.5, for 5 min (9) and pelleted by centrifugation, the supernatant was poured off and neutralized to pH 7.5, and then the sample was diluted in sterile saline for assay. The other sediment sample was resuspended in 10 ml of sterile saline, diluted in sterile saline, and assayed directly. Viruses were assayed on the same day that the experiment was performed, without freezing and thawing of the samples, to avoid virus inactivation.

RESULTS

The salinity of estuarine waters can vary from that of freshwater (<1 g/kg) to almost seawater levels (30 to 35 g/kg) (29). Several viruses were tested for their ability to adsorb to sediment at different salinity levels. Figure 1 shows the effects of different salinities on the adsorption to sediment of polio type 1 (LSc), coxsackie type B3 (Nancy), rotavirus SA-11, and echo type 1 (Farouk). More than 99% of the added polio 1, coxsackie B3, and rotavirus adsorbed at all salinities tested, whereas echo 1 adsorbed to a lesser degree (87 to 93%). The effects of salinity on virus desorption are shown in Fig. 2. All of the viruses studied remained adsorbed to the sediment at the salinities tested, except echo 1, which desorbed slightly at the higher salinities.

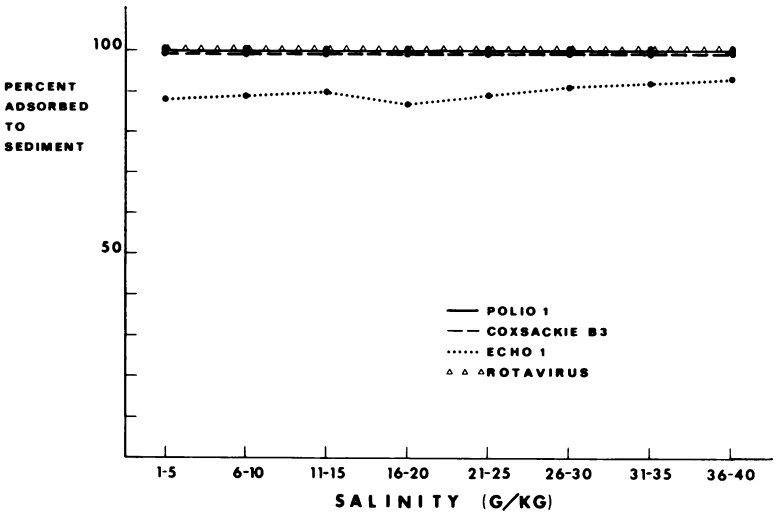

FIG. 1. *Effect of salinity on virus adsorption.*

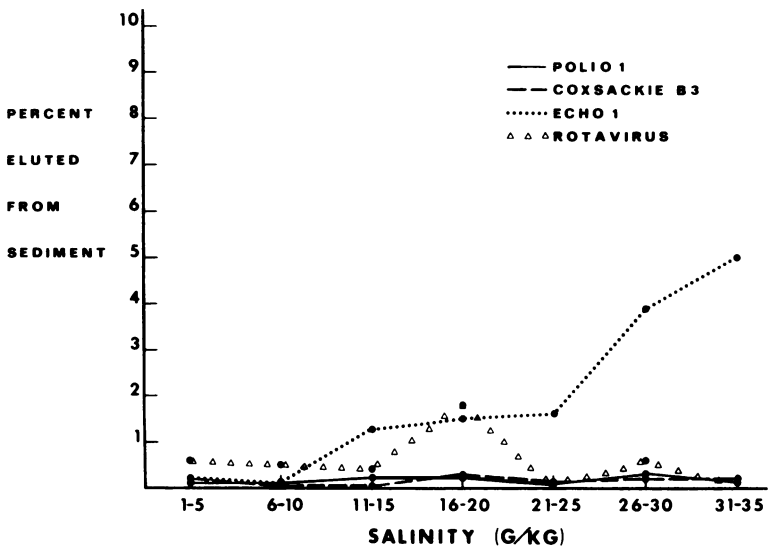

FIG. 2. *Effect of salinity on virus elution from sediment.*

The pH of estuarine environments can vary from 3 to 9 (27) and is related to salinity. Seawater of 30- to 35-g/kg salinity has a pH range of 8.0 to 8.5, whereas less saline waters (1 to 10 g/kg) range from pH 7.0 to 7.5 (11). Of course, local variations can occur, especially in areas which are polluted by waste discharges. Sewage effluent usually has a pH of about 8.0, and acid effluents may be emitted from industrial sources. Figure 3 shows the effect of pH on virus adsorption to sediment at a salinity of 16 g/kg. More than 99% of the added polio 1 (LSc), coxsackie B3 (Nancy), and rotavirus SA-11 adsorbed to the sediment at all pH's studied. Again, echovirus 1 adsorption differed from the other viruses. About 20% of the added virus remained unadsorbed at pH 6.0 to 7.5. Only above pH 7.5 was echo 1 adsorption enhanced.

The effect of pH on virus desorption is shown in Fig. 4. Polio 1, coxsackie B3, and rotavirus remained adsorbed to the sediment at all pH values studied, but echo 1 again exhibited a different behavior. Between pH 6.0 and 7.5, some of the echo 1 desorbed. It is possible that echo 1 desorbs to an even greater extent in the environment where the sediment would be exposed to constantly changing overlying waters. To investigate this possibility, the following experi-

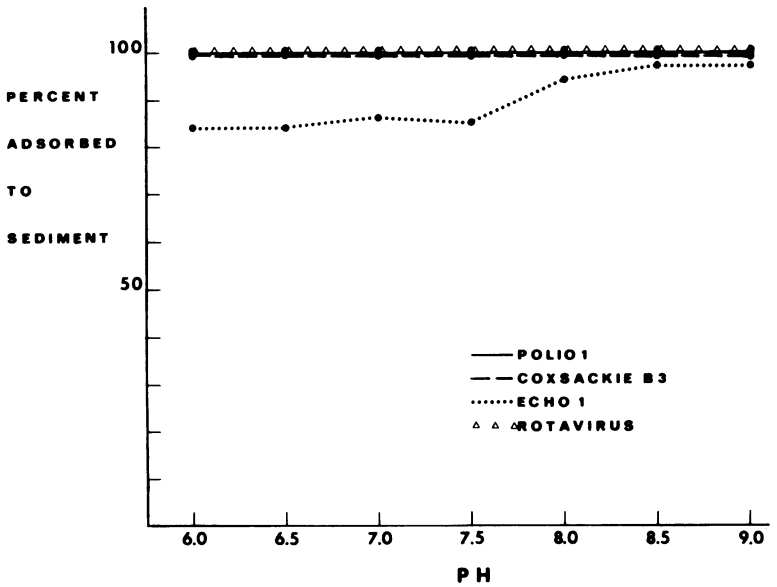

FIG. 3. *Effect of pH on virus adsorption to sediment.*

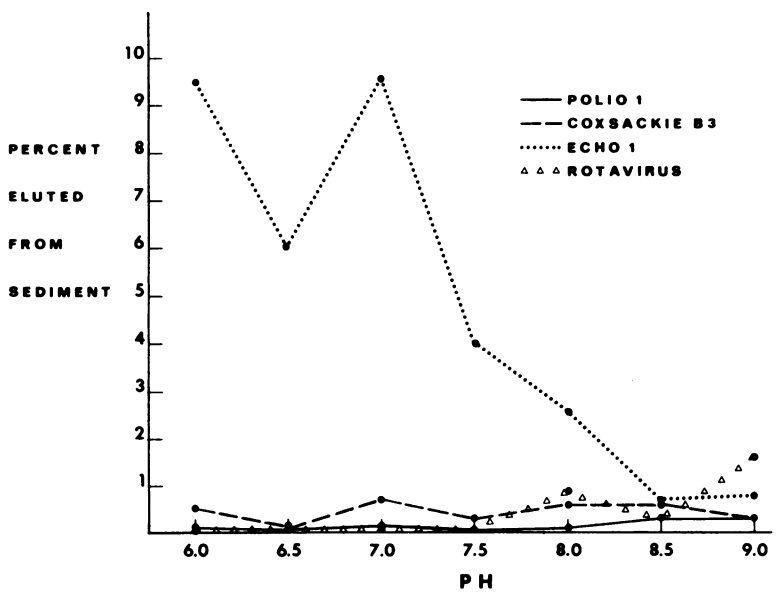

FIG. 4. *Effect of pH on virus elution from sediment.*

ment was conducted. Virus was adsorbed to the sediment in artificial seawater at pH 8.0 and 16-g/kg salinity and was resuspended in artificial seawater that had been adjusted to one of the two conditions that have been shown to cause some elution of echo 1 (high salinity and low pH). This mixture was shaken for 30 min and then centrifuged. The supernatant was then sampled for the presence of virus. This attempt to elute virus was repeated several times. The results (Table 1) demonstrate that echo 1 which has adsorbed cannot be desorbed.

Pollution in the water environment is sometimes indicated by the presence of soluble organic material, such as industrial chemicals, organics found in sewage effluent, and organics derived from soils which have been leached out by runoff water. It has been shown (3, 8) that

the amount of virus which may adsorb to clays can be modified by the presence of organic matter. Therefore, the amount of virus adsorption to sediment in the presence of naturally occurring soluble organics was investigated. Figure 5 shows the effects of various amounts of secondary sewage organics on virus adsorption to sediment. Adsorption of polio 1 and echo 1 was similar to that in artificial seawater without sewage. Figure 6 shows data in which humic acid was used as the source of organic material. Again, the adsorption curves of polio 1 and echo 1 were the same as would be expected in the absence of organics. When the same organic materials were used to elute virus from sediment (data not shown), very little (<0.1%) of polio 1 was eluted, and the amount of echo 1 eluted (0 to 3%) was within the range observed in the other elution attempts shown in Fig. 2 and 4.

The experiments detailed above were all performed with artificial seawater. Experiments using natural seawater were also performed to demonstrate that the results observed were also applicable to field conditions. Figures 7 and 8

TABLE 1. *Elution of echo 1 (Farouk) by repeated resuspension of sediment*[a]

No. of times resuspended	Elution by high salinity[b] (%)	Elution by low pH[c] (%)
1	<0.01	0.8
2	<0.01	0.4
3	<0.01	0.4
4	<0.01	0.2
5	<0.01	<0.01

[a] Data are expressed as percentage of virus added (10^5 PFU/ml) which remained in the supernatant after centrifugation.

[b] Salinity, 33 g/kg; final pH, 7.7.

[c] Salinity, 16 g/kg; final pH, 6.0.

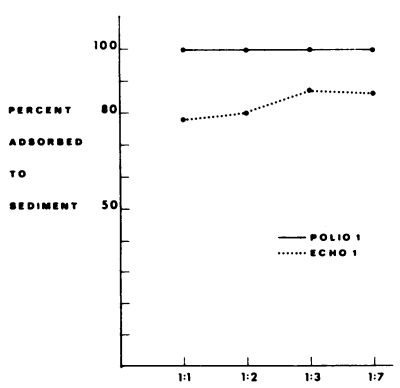

FIG. 5. *Effect of sewage organics on virus adsorption.*

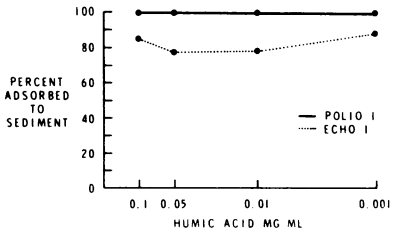

FIG. 6. *Effect of humic acid on virus adsorption.*

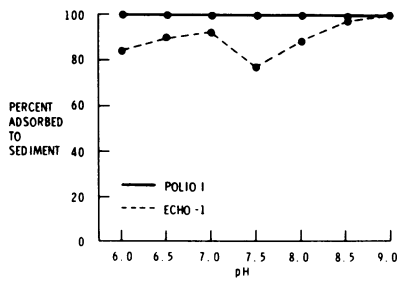

FIG. 7. *Effect of pH on the adsorption of polio 1 and echo 1 to sediment in natural seawater.*

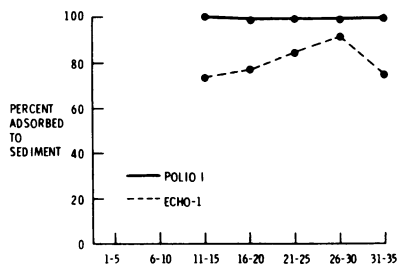

FIG. 8. *Effect of salinity on the adsorption of polio 1 and echo 1 to sediment in natural seawater.*

show the amount of virus adsorption observed under conditions of varying pH and salinity with natural seawater. Polio 1 and echo 1 exhibited adsorption characteristics similar to that seen with artificial seawater. Since one virus (echo 1 [Farouk]) did not behave the same as polio 1, the adsorptive behavior of other echoviruses was investigated. Echo 7 (strain Wallace) and two recent echo 1 isolates were tested for adsorption to sediment. The adsorptive behavior of both echo 7 and echo 1 (isolate V248) was similar to polio 1. Echo 1 (isolate V212) showed some similarity to the Farouk strain in that it adsorbed less at lower pH's (95%) and lower salinities (89%). However, at pH values equal to or greater than 7.0 and at salinities greater than 11, this isolate adsorbed to sediment as well as did the other enteroviruses.

Recently, experiments have been conducted in our laboratory to investigate the adsorption

of enteroviruses to soil (4), and several viruses were found to adsorb significantly less than polio 1. Three of these viruses were tested for their ability to adsorb to sediment. Figure 9 shows the results observed with these viruses. Salinity appeared to have a considerable effect on the amount of virus that will adsorb. Echo 29 (JV-10) did not adsorb well at salinities below 5 g/kg, but did adsorb well at salinities above 5. Coxsackie B4 (isolate V240) displayed an adsorption curve similar to that of echo 1 (Farouk). Echo 1 (isolate V239) adsorbed more than coxsackie B4 (V240) but less than echo 29 (JV-10). Figure 10 shows the results obtained when these viruses were adsorbed to sediment at different pH levels. Echo 29 adsorbed quite well at all pH's, but only about 95% of coxsackie B4 adsorbed at pH's less than or equal to 7.5. Echo 1 (isolate V239) showed even less adsorption to sediment at pH's less than or equal to 7.5.

It appears from these data that the majority of enteric viruses adsorb quite well to sediment under all conditions tested but that some do not adsorb as well as others under similar conditions. Figure 11 shows the different affinities of viruses for sediment. The data were found to conform to a Freundlich isotherm. The Freundlich isotherm, an empirical relationship developed for adsorption phenomena, is expressed as: $y/m = Kc^n$ or $\log y/m = \log K + n \log c$, in which y = the amount of virus adsorbed at equilibrium, m = the weight of the sediment, c = the amount of virus remaining in solution at equilibrium, and n and K are experimental constants. In a plot of the second equation, n and K are the slope and y intercept, respectively. Since a log-log plot of y/m versus c resulted in a straight line, these data conform to the Freundlich model. The data in Fig. 10 show that polio 1 (LSc) adsorbed to a much greater degree than did echo 1 (Farouk), and the steeper slope of the polio data indicates that more virus was adsorbed per milligram of sediment at higher concentrations of polio than at lower concentrations.

In an effort to demonstrate whether virus adsorbed to sediment may be easily resuspended

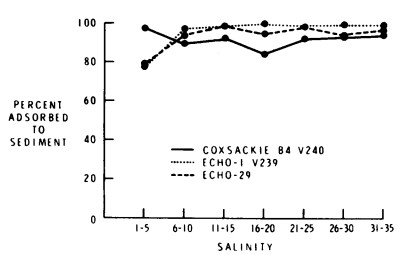

FIG. 9. *Effect of salinity on the adsorption of different enteroviruses to sediment.*

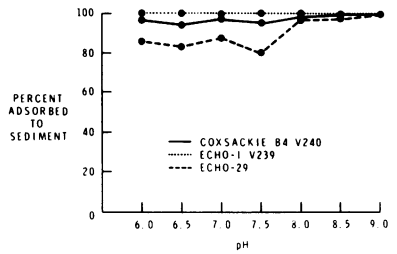

FIG. 10. *Effect of pH on the adsorption of different enteroviruses to sediment.*

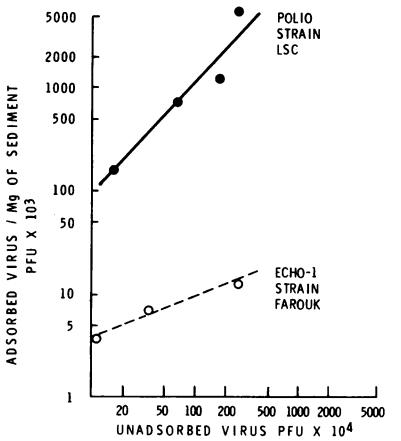

FIG. 11. *Freundlich isotherm plots of polio 1 (LSc) and echo 1 (Farouk) adsorption to sediment. Salinity, 16 g/kg; pH, 7.7.*

by wave motion or other sediment-disturbing factors, and therefore transported while in association with a sediment particle, the following experiment was performed. Polio 1 (LSc) and echo 1 (Farouk) were each adsorbed to sediment in artificial seawater at 16-g/kg salinity and pH 7.6 for 30 min. The samples were shaken at 250 rpm during adsorption. The sediment-virus mixture was then removed from solution by centrifugation at 1,400 × g for 5 min. The overlying seawater was decanted, replaced with an equal volume of artificial seawater, and then resuspended by shaking. The samples were allowed to remain undisturbed while portions were taken from the supernatant periodically as the sediment settled. After 22 h, the samples were gently rocked so that approximately the top 0.5 mm (of about 16 mm of sediment) was resuspended. A sample was then obtained of the resuspended sediment and seawater mixture. The results are shown in Table 2.

It should be noted that when the entire sediment sample was resuspended in the artificial seawater and assayed directly, only 15 and 31% of the original amounts of echo 1 and polio 1,

TABLE 2. *Demonstration of settling and ease of resuspension of virus adsorbed to sediment*

Settling time (h)	% of original titer remaining in the supernatant[a]	
	Polio 1 (LSc)	Echo 1 (Farouk)
3	0.4	0.9
22	0.1	0.3
22 (after gentle agitation)	4.6	0.47

[a] A total of 10^5 PFU/ml added. Salinity, 16 g/kg; final pH, 7.7.

respectively, could be detected by direct assay. Therefore, the amounts resuspended by gentle agitation represent 3.1 and 15.0% of the assayable amounts of echo 1 and polio 1, respectively. If one considers that approximately 5% of the sediment was resuspended by the gentle rocking motion, it becomes evident that most of the assayable virus present was still capable of resuspension. Therefore, it appears that such sediment-virus combinations in the environment could easily be resuspended and transported.

DISCUSSION

The data from Fig. 1 to 11 indicate that most enteroviruses, with some exceptions, readily adsorb to sediments and do not easily desorb. Therefore, transport of most enteric viruses away from contaminated areas is probably dependent upon local conditions that would lead to sediment resuspension and transport, e.g., currents, boating, dredging, swimming, and other activities. Virus adsorption studies indicate that viruses readily adsorb to solids under the proper ionic conditions (2, 3). The molarity of magnesium chloride specified by Dittmar's formula for artificial seawater is approximately 0.05 M, a concentration found to be optimum for enhancement of virus adsorption to nitrocellulose filters and clay (8, 28). Therefore, virus adsorption to sediment in seawater and estuarine waters is not an unexpected event. The present study investigated factors that may exist which would elute viruses to facilitate their transportation. It is apparent from our data that, once most enteroviruses are attached to a sediment particle, they are dependent upon the particle for transport.

We have shown recently (24) that virus survival in seawater is enhanced by the presence of sediment. Schaub and Sagik (21) have shown that virus adsorbed to clay particles is still viable and may be assayed in mice and tissue culture. We have also been able to assay virus adsorbed to sediment by plating directly (Table 3). The percentage of virus recovered by elution was similar to that detected by direct assay for polio 1 (LSc), echo 1 (Farouk), and echo 1 (isolate V212). A higher percentage of coxsackie B3 (Nancy) was detected by direct assay than by elution. It is possible that this virus could not be eluted as well as the other viruses or that it was inactivated by the elution process. It is also obvious that, with the exception of polio 1 (LSc), much of the virus is not recoverable from sediment with these procedures. This may indicate that the virus is so well adsorbed that it cannot be detected by either our elution procedure or direct assay, that virus is inactivated in the process of adsorption or desorption or both, or that multiple viruses adsorb to single particles and are assayed as one infectious unit. We do not know the answer at present.

Based on these observations, the ease and frequency of sediment resuspension appear to be important factors in the transport of virus in estuaries. We have demonstrated (Table 2) that viruses adsorbed to the sediment used in these experiments can be resuspended easily. The current velocities required to resuspend sediment have been studied (14) in areas surrounding deep marine offshore sewage outfalls in California. Hendricks (14) noted that the current speed necessary to resuspend sediment (average, 6 cm/s) near sewage outfalls was much lower than that in areas farther away from the sewage outfall and that current speed estimates extrapolated from wave data would indicate that this current speed (6 cm/s) would be exceeded 39% of the time during the spring of the year. Hendricks also measured the current speed and found that it exceeded 6 cm/s 24% of the time. Thus, it is evident that the sediment around the sewage outfall could be easily resuspended. These data may or may not apply to the situation in canal communities on the Texas coast,

TABLE 3. *Plating efficiency of sediment-adsorbed virus*

Condition	Original titer (%)[a]			
	Polio 1 (LSc)	Echo 1 (Farouk)	Echo 1 (V212)	Coxsackie B3 (Nancy)
Virus adsorption[b]	99.8	98.1	99.6	99.6
Virus recovered by elution[c]	99.9	13.0	5.0	4.5
Virus detected by direct assay of sediment[d]	99.9	18.0	9.0	37.0

[a] Original titer, 10^5 PFU/ml.
[b] Salinity, 16 g/kg; final pH, 7.8.
[c] A solution of 0.25 M glycine plus 0.05 M ethylenediaminetetraacetic acid at a final pH of 11.0 for 5 min.
[d] Resuspended and diluted in sterile saline.

but they are evidence that sediment surrounding sewage outfalls can be easily resuspended by local turbulence. It is highly probable that sediment around a sewage outfall in a coastal canal community would be much more likely to be resuspended by local activities, such as boating and storm runoff, because of its shallow depth.

It was our experience that the sediment surrounding the sewage outfalls in the canal communities was very fluid and easily resuspended. Others have demonstrated that bacteria are resuspended by disturbance of the estuarine environment. Gerba and Schaiberger (7) noted that a sudden peak of increased coliform numbers near Miami, Fla., bathing beaches occurred after heavy rainfall. They proposed that heavy rains resuspended and transported bacteria from polluted sediments in freshwater drainage canals and washed them out to sea. Matson et al. (18) have constructed a model based on their observations in the Shetucket River basin, which indicates that the numbers of microorganisms in the river water will increase with a simultaneous decrease of microorganisms in river sediment soon after a high water runoff due to heavy rainfall. It has also been demonstrated that dredging activities (12) release bacteria from sediment.

Echo 1 adsorbed less than polio 1 in seawater at all of the salinities tested (pH 7.4 to 7.7) and at pH 6.0 to 7.5. However, since repeated desorption attempts eluted very little virus, it is probable that viruses with adsorptive properties similar to those of echo 1 and coxsackie B4 (isolate V240) are also very dependent upon sediment for transport. Differential virus adsorption to bentonite clay at various pH values was previously observed by Shirobokov (23), who proposed that serotypes of coxsackieviruses could be identified by this procedure. Various degrees of adsorption of different viruses to activated sludge and soil have also been observed (C. P. Gerba, S. M. Goyal, C. J. Hurst, and R. L. LaBelle, submitted for publication).

The Freundlich isotherm plot of virus adsorption per gram of sediment demonstrates that there is a great difference in the amount of each virus which will adsorb to a given sediment. This emphasizes the danger in using one virus as a typical example of virus behavior in the environment, since a general application of these observations may not be accurate.

These results strongly indicate that viruses will be carried by currents from sources of pollution while adsorbed to sediment particles. Also, viruses will probably be present in higher numbers in sediment than in seawater, since viruses will adsorb to sediment or other particles and settle to the bottom. These observations indicate that the microbiological quality of sediment should be investigated as part of the evaluation of the public health safety of estuarine areas.

ACKNOWLEDGMENTS

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