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Association of Enteroviruses with Natural and Artificially Introduced Colloidal Solids in Water and Infectivity of Solids-Associated Virions

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Encephalomyocarditis viruses adsorb to introduced organic and inorganic solids in water over a wide range of pH and with various concentrations and species of metal cations. Visible flocculation of solids was not a prerequisite for significant virus association. Virus adsorption to natural solids in various types of natural waters was significant but variable. Clay-adsorbed virus retained its infectivity in tissue culture monolayers. These solids-associated viruses also retained infectivity in mice.

Various enteric viruses are excreted in human feces and urine during infection and may be found in raw domestic sewage. Excellent reviews such as those prepared by Grabow (13) and Berger et al. (2) provide ample evidence that the use of secondary treatment processes such as trickling filtration, activated sludge, and oxidation ponds can reduce the infectious virus titers by varying degrees. The removal of infectious viruses by these processes is not complete, however, and each process appears to give variable results. Even chlorination of the secondary effluents may not disinfect the waste waters. This effluent discharged into receiving waters often introduces enteric viruses to those waters.

Infectious viruses can be carried in the natural resource to points distant from their origin in sewage. Reports indicate that it is not uncommon for fresh and estuarine waters to carry detectable virus several miles (14, 23, 26, 32, 38; O. C. Liu, H. R. Seraichekas, O. A. Brashear, W. P. Heffernan, and V. J. Cabelli, Bacteriol. Proc., p. 151, 1968). Akin et al. (1) have reviewed the literature and have cited ample evidence that enteric viruses can survive for long periods in waters of the natural resource. Domestic sewage and naturally occurring organic materials may prolong virus survival in natural waters (2, 8, 18). On the other hand, certain substances in water such as microbial enzymes or heavy metals may be antagonistic to the viruses (24, 27, 28).

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Laboratory studies such as those by Carlson et al. (5) and by Drewry and Eliassen (9) have shown that various bacteriophages adsorb to clay and soil particles in water or soil columns. They noted that low pH and the addition of metal cations enhanced adsorption. Clearly the association of viruses with suspended materials in natural resource waters may alter the virion survival patterns. Robeck et al. (30) showed that sand filtration would not remove viruses from the water adequately. Lime and alum flocculation with subsequent sedimentation or filtration have been shown to increase the efficiency of the virus removal during water treatment (4, 12, 22). The virus in the flocs, however, are not inactivated and even may be eluted from the flocs with appropriate pH adjustments (6, 12, 36).

The importance of water-borne viruses in human infection has been reviewed recently by Mosley (29) who examined 50 published reports on epidemics of hepatitis. He indicated that at least 30 of the epidemics incriminated water as the route of transmission. Several poliomyelitis outbreaks also were attributed to water dissemination. Other enteric viruses such as the agents of viral gastroenteritis and diarrhea also may be transmitted by contaminated water.

The studies reported here have been designed to add to our understanding of the means of virus transmission via the natural water resource.

MATERIALS AND METHODS

Diluents and media. Hanks balanced salt solution containing 1% fetal calf serum (BSSC-1) generally was used as the standard diluent for virus plaque assays. In certain control studies deionized water plus

10^{-3} M $CaCl_2$ was used as a diluent.

L-cell cultures were grown and maintained in Eagle medium (11) modified by the substitution of Hanks balanced salt solution for Earle salts (Hanks and Wallace [16]) and the utilization of 2× concentrations of all amino acids and vitamins. The medium was supplemented with 10% fetal calf serum (EFC-10). Kantrex (Kanamycin Sulfate, Bristol Laboratories, Syracuse, N.Y.) at a final concentration of 0.1 mg/ml and Mycostatin (nystatin, E. R. Squibb and Sons, Inc., N.Y.) at a final concentration of 50 U/ml were added to the medium.

Virus plaque assays with solid overlay medium contained EFC-10 and 1% Ionagar (Colab). Protamine sulfate (grade 1) was added (0.1 mg/ml) to enhance the size and number of virus plaques.

Cell cultures. L-cell cultures were propagated using EFC-10 medium. Plates for assay were prepared from trypsinized cultures by suspending dilution to approximately 5.0×10^5 cells in 4 ml of EFC-10 and dispensing into 60-mm plates. Virus suspensions were assayed on day 2 after cell seeding, when a confluent monolayer had formed.

Viruses. Two encephalomyocarditis (EMC) viruses (Columbia SK and mengovirus), obtained from Major David J. Giron, U.S. Air Force, School of Aerospace Medicine, Brooks A.F.B., San Antonio, Tex., were used.

Stock virus was prepared in L-cell monolayer plate cultures. After 18 h of incubation at 37 C the liquid overlay was harvested, debris was removed by centrifugation at 2,000 × *g* for 5 min, and the infective supernatant fluids were frozen at −70 C.

Tenfold serial dilutions of virus were made in BSSC-1 and kept in a wet ice bath until L-cell plates were infected. A 1-h attachment period at 37 C in a 2.5% CO_2 in air atmosphere was used. The plates were overlaid with 4 ml of solid overlay medium, inverted, and incubated in a 2.5% CO_2 in air atmosphere at 37 C for 2 days. The plates were flooded with neutral red (0.1 mg/ml) contained in a balanced salt solution for 60 min at 37 C. The excess stain was removed, and the plates were inverted and then held at 37 C for an additional 2 h. Viral plaques could be distinguished as clear areas on a red background. Virus titers were expressed as plaque-forming units (PFU) per ml. Four plates were used for each dilution.

Suspended solids preparation. Montmorillonite clay (inorganic suspended solids) was prepared by grinding in a mortar with a pestle until a fine powder was formed. Daily, standard suspensions were made by adding 200 mg of this clay to 1 liter of deionized water. The material was stirred vigorously for 30 min with a magnetic stirring bar. Heavier material was allowed to settle for 30 min and the upper half of the mixture was decanted and used.

Standard organic suspended solids were prepared by mixing 2.5 g of Gaines Meal dry dog food in 1 liter of water in the same manner as were the inorganic solids, including use of the upper half only. Mixing and sedimentation periods were 1 h.

The organic and inorganic suspended solid materials were analyzed for total and volatile suspended solids by Forrest and Cotton Engineering Consultants, Austin, Tex.

Virus adsorption procedures. Suspended solids (98 ml) were put into screw-cap dilution bottles along with 1-ml portions each of the various cations (100× Stocks) and standard concentrations of EMC virus (10^6 PFU/ml final concentration) contained in 1 ml of deionized water. The mixture was shaken vigorously for 10 s every 5 min during a 30-min adsorption period. Then, 10-ml samples either were centrifuged in a refrigerated Sorvall centrifuge at 12,100 × *g* for 15 min or filtered through a 0.45-μm Gelman cellulose triacetate membrane filter. Centrifuge supernatant or membrane filtrate samples were assayed for unadsorbed virus as described previously. Viruses contained in deionized water with or without cations were utilized as controls. The adsorption kinetics were studied in a similar manner except that samples were centrifuged at 14,500 × *g* for 5 min.

A pH of 5.5 to 6.5 was maintained throughout the tests with addition of HCl or NaOH, as applicable, unless otherwise specified. No buffers were added to the system. In studies designed specifically to measure the effects of pH upon virus adsorption, continuous pH monitoring was used. The pH adjustments were done with HCl and NaOH as required. All other phases of these pH studies were identical with those mentioned previously.

Mouse infection via the oral route. Outbred 7- to 8-day-old suckling mice (Manor Farms, Staatsburg, N.Y.) were used. The mice were starved for 1.5 to 2.0 h before administration of virus. The virus preparations were made in either BSSC-1, deionized water plus 10^{-3} M $CaCl_2$, or in deionized water with Montmorillonite clay containing 10^{-3} M $CaCl_2$. The suspensions contained 2.5% sucrose for palatability. The inoculum was delivered by tuberculin syringe with the needle replaced by sterile intramedic polyethylene tubing (outside diameter = 0.067 inch [ca. 0.17 cm]). A dose of between 0.01 and 0.02 ml of the various mixtures of EMC virus was given after inserting the tubing into the animal's mouth. The infected litters and mothers were kept in plastic metabolism cages at 26 C. The test animals were observed for a 10-day period. All dead mice were disposed of when discovered to prevent secondary infection by cannibalism.

Mouse infection via the cerebral route. Weanling outbred mice (21 to 26 days old) were utilized for intracerebral inoculation of mengovirus contained in the same mixtures as used for oral treatment, except that sucrose was omitted. Each virus dilution was inoculated into unanesthetized mice. The dose of 0.03 ml was injected on the left side of the skull to a depth of approximately 4 mm. The mice were kept in plastic cages with metal tops at room temperature (23 C). They were observed for a 12-day period. All deaths occurring in the first 24 h after inoculation were attributed to inoculation trauma and, therefore, were not considered in the calculations.

Stomach pH determinations. The stomachs of 8-day-old mice were analyzed immediately after nursing and after 2-h starvation periods. The mice were sacrificed by decapitation or etherization and the stomachs were removed and opened. Close range pH paper (pH hydrion) was utilized to determine the pH of both cardiac and pyloric portions of the stomach.

EMC inactivation studies. BSSC-1, 10^{-3} M

$CaCl_2$, and 10^{-3} M $CaCl_2$ plus clay diluents were acidified to pH 3.8 with 0.1 M HCl and acidity was monitored throughout the test period. The temperature was elevated to 37 C and the virus was added to give a final concentration of 10^6 PFU/ml. Initial virus samples in their respective diluents were taken before introduction to the heated, acidified mixtures. Samples were taken at 20, 60, and 120 min. The samples were assayed for infectivity after neutralization of the low pH with NaOH.

Natural resource sampling. Samples of Town Lake water (Austin, Tex.) were collected at the IH 35 bridge crossing at Austin, Tex., in 1-gallon (3.785 liter) bleach bottles which had been continuously rinsed for 24 h with tap water to remove all chlorine. The grab samples were obtained from approximately 1 foot (30.48 cm) below the water surface. The samples were brought to the laboratory immediately and experiments were initiated.

The samples from Red Fish Bay were taken from a site off the main road leading from Aransas Pass to Port Aransas, Tex. The sampling point was a channel where a strong current existed at both ebb and flood tide. Grab samples in washed bleach bottles were taken by wading into the channel and taking the sample at approximately 1 foot (30.48 cm) below the water surface. Samples were iced and sent to Austin where experiments were initiated within 24 h.

The Wichita River samples were sent by bus from a hired local water sampler. The grab samples were collected as described for the other water samples and iced while shipped. Experiments were initiated upon sample arrival.

No preservatives of any kind were added to any of the samples. Duplicate samples also were sent to Forrest and Cotton, Consulting Engineers, for physical and chemical analyses.

RESULTS

Adsorption. The concentration of suspended solids in laboratory test material (organic and inorganic) was determined. The total organic suspended solids standard concentration was 56 mg/liter, of which 100% was volatile and presumed to be organic. The unrefined Montmorillonite standard clay sample concentration was 36 mg/liter of total suspended solids of which only 6.4% was of a volatile nature.

The virus-adsorptive capacity of organic suspended solids was determined at various concentrations of divalent metal cations. Table 1 illustrates the organic solids capacity to adsorb EMC (Columbia SK) virus in the presence of manganese, magnesium, and calcium ions. The presence of these cations at a concentration of 10^{-2} M led to at least 98.8% adsorption. Reduced Mg^{2+} and Ca^{2+} concentrations reduced virus adsorption. The effect of Ca^{2+} concentration on adsorption to Montmorillonite clay is shown in Table 2.

TABLE 1. *EMC virus adsorption onto organic suspended solids*[a]

Sample	Virus absorbed (%)
Solids – no cation	30.00
Solids + 10^{-2} M $McCl_2$	99.91
Solids + 10^{-1} M $MgCl_2$	78.50
Solids + 10^{-2} M $MgCl_2$	98.80
Solids + 10^{-3} M $MgCl_2$	95.80
Solids + 10^{-2} M $CaCl_2$	99.85
Solids + 10^{-3} M $CaCl_2$	84.00
Solids + 10^{-3} M $CaCl_2$	62.00
Solids + 10^{-4} M $CaCl_2$	56.00

[a] Virus at 10^6 PFU/ml was added to organic suspended solids (56 mg/liter) in the presence of various metal cations. After a 30-min adsorption period 10-ml aliquots were centrifuged at 12,000 × *g* for 15 min and the supernatant fluids were examined for infectivity. Percentage of virus adsorbed was calculated from the virus removed along with clay, compared against 30-min controls (deionized water) prepared in the same manner.

TABLE 2. *EMC virus adsorption onto Montmorillonite clay*[a]

Sample	Virus absorbed (%)
Clay + deionized water	32
Clay + 10^{-4} M $CaCl_2$	55
Clay + 10^{-3} M $CaCl_2$	94
Clay + 10^{-3} M $CaCl_2$	98.4
Clay + 10^{-2} M $CaCl_2$	96.3

[a] Virus at 10^6 PFU/ml was added to Montmorillonite clay (36 mg/liter) in the presence of $CaCl_2$. After a 30-min adsorption period 10-ml aliquots were centrifuged at 12,100 × *g* for 15 min and the supernatant fluids were examined for infectivity. Percentage of virus adsorbed was calculated from 30-min deionized water controls run in parallel.

Water from a local man-made lake (Town Lake, Austin) which receives suspended solids from storm runoff and some domestic sewage waste was examined to determine if natural suspended solids would adsorb EMC virus. The data in Table 3 indicate that viruses are adsorbed to natural solids. All samples were compared to controls of lake water from which the solids were removed by 0.45-μm/average pore diameter membrane filtration. During the adsorption period between 15 and 35% of the virus became solids associated. The addition of calcium ion, reduction in pH, or both in combination, significantly enhanced adsorption. The lowest level of adsorption occurred (experiment

TABLE 3. *EMC virus adsorption onto suspended solids in lake water*

Expt	Sample	pH	PFU/ml at 30 min[a]	% Residual virus	% Adsorbed
1	Lake water control—no solids	8.1	2.3×10^5		
	Lake water	8.1	1.5×10^5	65.0	35.0
	Lake water + 10^{-2} M $CaCl_2$	8.1	6.0×10^3	2.6	97.4
2	Lake water control—no solids	7.9	2.7×10^5		
	Lake water	7.9	1.8×10^5	66.6	33.3
	Lake water + 10^{-3} M $CaCl_2$	7.9	8.0×10^4	29.0	71.0
3[b]	Lake water control—no solids	7.9	2.0×10^5		
	Lake water	7.9	1.7×10^5	85.0	15.0
	Lake water—acidified with HCl	6.0	8.2×10^4	41.0	59.0
	Lake water + 10^{-3} M $CaCl_2$	7.9	7.3×10^4	36.0	64.0
	Lake water + 10^{-3} M $CaCl_2$ and acidified with HCl	6.0	5.7×10^4	28.0	72.0

[a] Virus at 10^5 PFU/ml was added to Town Lake water (filtered or with suspended solids present) for a 30-min adsorption period. In some instances calcium chloride was added and the pH acidified to 6.0. All samples were centrifuged at 12,100 × *g* for 15 min. The percentage of residual virus was calculated from lake water controls.

[b] Heavy rains caused high turbidity.

number three) after heavy rains. Although turbidity (suspended solids content) was very high, the cation content was reduced by dilution. Data provided by the U.S. Department of Interior, Geologic Survey (39), are given in Table 4 and indicate that low divalent metal cation concentrations, hardness and conductivity are found ordinarily at this sampling point. During periods of high mean water discharge these concentrations are even lower.

Kinetics of adsorption. The kinetics of EMC virus adsorption to Montmorillonite clay in 10^{-3} M $CaCl_2$ was studied. A control consisting of virus in 10^{-3} M $CaCl_2$ minus clay was tested in a parallel series. Samples from each mixture were taken at 0, 1, 10, 30, and 60 min. Samples were centrifuged at 14,500 × *g* for 5 min immediately after and the supernatant fluids were assayed for infectivity. Figure 1 illustrates the rapid rate of virus adsorption to the clay. Ninety-two percent of the virus adsorbed to clay within 1 min. A visible floc was noted only after the bulk of the virus was adsorbed, suggesting that virus adsorption is not dependent on visible floc formation. In the absence of clay, there was an initial decrease in free virus (probably due to attachment to glass), but this stabilized within 10 min.

Effect of pH on adsorption. The effects of pH on virus adsorption to Montmorillonite clay in the presence of the cation was studied (Table 5). The greatest degree of adsorption occurred at pH 5.5; at pH 3.5 adsorption was poorest. Adsorption also decreased at pH 9.5. Relatively more virus was detected in the pH 9.5 controls than at lower pH, suggesting the possible disaggregation of small virus clumps.

Interference with viral adsorption. Experiments were designed to determine the extent of interference with virus adsorption to inorganic suspended material by soluble proteins. Table 6 shows that serum protein at a final concentration of 0.6 mg/ml in deionized water containing 10^{-3} M $CaCl_2$ inhibited virus adsorption markedly. The addition of this concentration of serum to the clay plus cation mixture permitted only 16% of the virus to adsorb. It appears, therefore, that the virus can be excluded from particulate association if a suitable competitor is present.

Infectivity of adsorbed virus for cell monolayers. Tests were performed to determine the tissue culture infectivity of suspended solids-bound viruses. Controls consisted of centrifuged supernatant samples of virions suspended in filtered water from the natural resource and also of virus in deionized water containing 10^{-3} M cation. The infectivity of viruses absorbed to the natural resource solids was determined by plating the virus-solids mixture directly. The infectivity of the virus-solids mixture was compared with the controls containing no solids. Table 7 shows that samples of EMC virus adsorbed to standard Montmorillonite clay or to natural solids are more infectious than is virus in waters from which the solids were removed. Centrifugation effectively reduced the virus titer, indicating the large quantity of solids-bound viruses.

Animal infectivity studies. The next series of experiments was undertaken to determine whether virions adsorbed to solids were infective in test animals. The standard Montmorillonite clay was chosen for the tests because of its

TABLE 4. *Colorado River basin, Colorado River at Austin*[a]

Chemical analyses (mg/liter), water year October 1967 to September 1968

Date	Mean discharge (CFS)	Silica (SiO_2)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Sodium (Na^+)	Potassium (K^+)	Bicarbonate (HCO_3^{2-})	Carbonate (CO_4^{2-})	Sulfate (SO_4^{2-})	Date	Chloride (Cl^-)	Fluoride (F^-)	Nitrate (NO_3^{2-})	Dissolved solved solids (sum of constituents)	Hardness (Ca Mg)	Noncarbonate hardness	Specific conductance (micromhos)	pH
Oct.	457	8.3	55	16	17	3.1	215	0	24	Oct.	28	0.2	4.2	262	203	27	455	7.8
Nov.	753	9.2	66	18	14	1.8	260	0	24	Nov.	23	0.2	6.3	286	238	33	494	7.7
Dec.	386	8.6	64	18	15	1.7	244	0	26	Dec.	25	0.1	3.0	281	234	34	496	7.8
Jan.	3630	7.8	52	17	20	3.0	203	0	29	Jan.	34	0.2	2.2	265	200	33	496	7.7
Feb.	6070	6.6	43	18	27		174	0	29	Feb.	46	0.3	1.0	257	180	37	461	7.6
Mar.	5690	7.1	43	15	31		172	0	26	Mar.	47	0.2	.9	255	169	28	457	8.1
Apr.	5860	7.3	43	14	24	3.6	169	0	25	Apr.	40	0.2	1.1	241	165	26	438	8.1
May	7250	7.2	45	14	27		172	0	23	May	44	0.3	1.9	247	170	29	454	8.1
June	5640	7.2	46	14	29		174	0	25	June	45	0.3	1.8	254	172	30	476	8.1
July	2740	8.2	50	14	25	3.2	188	0	23	July	43	0.3	1.6	260	182	28	464	8.2
Aug.	2000	8.6	51	15	27		194	0	24	Aug.	43	0.3	1.0	265	188	30	475	7.9
Sept.	1230	8.2	50	15	26		190	0	22	Sept.	44	0.3	.5	259	186	31	478	8.2
Wtd. avg. time		7.4	46	15	26		180	0	25	Wtd. avg. time	43	0.3	1.5	254	178	30	463	8.0
Wtd. avg. tons	3,460	7.9	51	16	23		196	0	25	Wtd. avg. tons	38	0.2	2.1	261	191	30	470	7.9
Per day		69	433	142	245		1680	0	238	Per day	398	2.4	14					

[a] Location: Latitude 30° 18′ 53″, longitude 97° 47′ 10″, Travis County, at raw water intake at Austin City Waterplant, just downstream from Lamar Boulevard bridge in Austin, 0.5 miles (ca. 8.05 km) downstream from Barton Creek, and 4.5 miles (ca. 7.01 km) upstream from gauging station at Montopolis Bridge on U. S. Highway 183. (U. S. Dept. of Interior, Geological Survey-Water Resources Division, Water Resources Data for Texas 1968.)

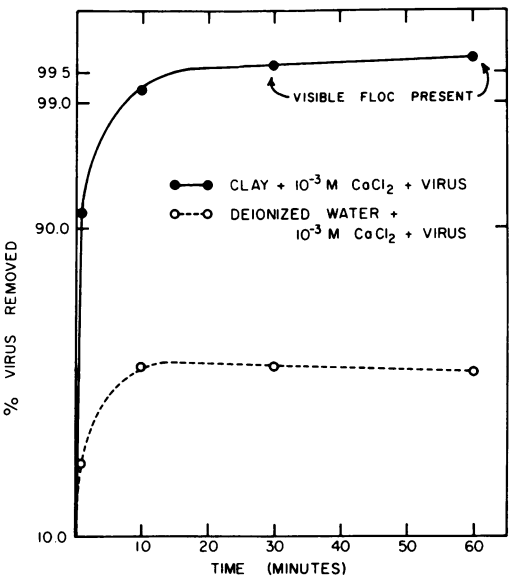

FIG. 1. *Kinetics of EMC virus adsorption. Virus (10⁵ PFU/ml) was adsorbed onto Montmorillonite clay in the presence of 10⁻³ M $CaCl_2$. After 1, 10, 30, and 60 min, 10-ml aliquots were centrifuged at 14,500 × g for 5 min and centrifuge supernatant fluids were assayed for residual virus infectivity. The same procedure was performed on a parallel series of virus in deionized water containing 10⁻³ M $CaCl_2$. The percentage of virus removed (adsorbed) was calculated from zero time centrifuged controls.*

TABLE 5. *Effect of pH on EMC virus adsorption to Montmorillonite clay in the presence of $CaCl_2$*[a]

Sample	pH	PFU/ml in supernatant	% Virus adsorbed at each pH
Control – no clay	3.5	2.2×10^5	
Clay + 10^{-3} M $CaCl_2$	3.5	1.6×10^4	92.7
Control – no clay	5.5	2.6×10^5	
Clay + 10^{-3} M $CaCl_2$	5.5	2.0×10^3	99.2
Control – no clay	9.5	3.0×10^5	
Clay + 10^{-3} M $CaCl_2$	9.5	9.8×10^3	96.7

[a] All samples were either acidified with HCl or made basic with NaOH. Both controls and clay samples contained 10^{-3} M $CaCl_2$. Virus (10^5 PFU/ml initial concentration) was then added and 10-ml aliquots were centrifuged at 12,100 × g for 15 min after a 30-min adsorption period. The percentage of virus adsorbed to the clay at each pH was determined from 30-min controls at that pH.

widespread natural occurrence in solids and sediments and also because it gave uniform virus adsorption under standard conditions.

TABLE 6. *Inhibition of virus adsorption to clay by serum protein*[a]

Sample	PFU/ml	Virus adsorbed (%)
Deionized H_2O at time zero	1.9×10^5	
Deionized H_2O + 10^{-3} M $CaCl_2$	1.0×10^5	48
Deionized H_2O + 10^{-3} M $CaCl_2$, then 0.6 mg of serum protein per ml	1.9×10^5	0.0
Deionized H_2O + clay + 10^{-3} M $CaCl_2$	5.0×10^2	99.74
Deionized H_2O + clay + 10^{-3} M $CaCl_2$, then 0.6 mg of serum protein per ml	1.6×10^5	16

[a] Duplicate samples of 10^{-3} M $CaCl_2$ in deionized water and duplicate samples of Montmorillonite clay in 10^{-3} M $CaCl_2$ were prepared. To one of each duplicate sample fetal calf serum (0.6 mg of protein per ml) was added. The virus (10^5 PFU/ml) was then mixed with these samples for 30 min and then centrifuged at 12,100 × g for 15 min. Centrifuge supernatant fluids were assayed for residual infectivity. The percentage of virus adsorbed was calculated from a deionized water zero time control prepared in the same manner.

The oral route of infection was utilized as this obviously is the most probable route in virus transmission via water. Mengovirus (EMC group virus with greater mouse infectivity than Columbia SK) was suspended with clay in deionized water with 10^{-3} M $CaCl_2$. Virus controls were prepared in BSSC-1 and in 10^{-3} M $CaCl_2$ in deionized water. Seven- to 8-day-old suckling outbred white mice, not allowed to nurse for 1.5 to 2 h before infection, were given an oral dose of between 0.01 and 0.02 ml per mouse. Table 8 shows that the approximate mean lethal dose (LD_{50}) for the BSSC-1 control was between 10^3 and 10^4 PFU/mouse. The amount of virus required to provide an equivalent LD_{50} in the $CaCl_2$ control group was significantly higher (10^5 PFU/mouse). Virus adsorbed to clay and administered at 10^5 PFU/mouse was approximately as infective as that dose in the $CaCl_2$ control. Almost all of the virions in the clay-$CaCl_2$ mixture were clay bound, as shown by the fact that less than 0.001 of the virions would pass through a 0.45-μm membrane filter. The free virus level thus was several orders of magnitude less than that necessary to approximate the LD_{50} in $CaCl_2$.

To explain the different mortality rates observed when virions were diluted in BSSC-1, $CaCl_2$ and clay plus $CaCl_2$, the per os data were compared with the results obtained by in-

TABLE 7. *Infectivity of adsorbed viruses for cell monolayers*[a]

Sample	Disposition of sample	PFU/ml	Direct plate control (%)
Deionized H_2O + 10^{-3} M $CaCl_2$ control	Direct plate	3.2×10^5	
Deionized H_2O + 10^{-3} M $CaCl_2$ + clay	Direct plate	4.3×10^5	134
Deionized H_2O + 10^{-3} M $CaCl_2$ + clay	Centrifuged	1.3×10^4	4.7
Filtered Town Lake control	Direct plate	3.0×10^5	
Filtered Town Lake + clay	Direct plate	3.2×10^5	107
Filtered Town Lake + clay	Centrifuged	2.4×10^5	60
Filtered Wichita River control	Direct plate	6.4×10^5	
Wichita River with natural solids	Direct plate	7.7×10^5	121
Wichita River with natural solids	Centrifuged	1.6×10^4	2.5
Filtered Red Fish Bay control	Direct plate	4.7×10^5	
Red Fish Bay with natural solids	Direct plate	6.5×10^5	138
Red Fish Bay with natural solids	Centrifuged	1.5×10^5	32
Filtered Town Lake control	Direct plate	8.0×10^5	
Town Lake with natural solids	Direct plate	7.0×10^5	87
Town Lake with natural solids	Centrifuged	5.5×10^5	68

[a] The various samples with or without suspended solids were inoculated with virus (10^6 PFU/ml) and allowed 30 min for adsorption. The control samples without solids were plated on L-cell monolayers directly. Samples with suspended solids were assayed in parallel by direct plating on L-cell monolayers or by assay of 12,100 × *g* centrifuge supernatants. Solids containing samples were compared against the direct plate controls.

TABLE 8. *Infectivity of EMC virus by ingestion in suckling mice*[a]

Suspending menstruum	PFU/ mouse	No. of mice tested	No. of mice dead[a]	Mortality (%)
BSSC-1	10^5	19	17	89
BSSC-1	10^4	20	14	70
BSSC-1	10^3	19	8	44
BSSC-1	10^2	10	0	0
10^{-3} M $CaCl_2$	10^5	8	4	50
10^{-3} M $CaCl_2$	10^4	7	0	0
Clay + 10^{-3} M $CaCl_2$	10^5	25	10	40

[a] All deaths between 2 and 10 days were recorded. Suckling mice 7 to 8 days old were given mengovirus by ingestion of the virus in the virus in the various suspending menstruums. Viruses were administered in a dose of 0.01 to 0.02 ml by a tuberculin syringe, the needle being replaced by small-diameter catheter tubing.

tracerebral inoculation. This route eliminated some of the variability observed with the oral route. For this study, young weanling mice were utilized.

No major differences in infectivity were noted among mice inoculated intracerebrally in any of the test suspensions (Table 9). The approximate LD_{50} for the virus in BSSC-1, $CaCl_2$ and clay plus $CaCl_2$ was between 10 and 10^1 PFU/mouse. In comparison, it may be seen that a filter mat derived from the clay-bound virus in $CaCl_2$ subjected to 0.45-μm Gelman filtration retained infectivity when resuspended and introduced intracerebrally. The membrane filtrates collected from the initial filtration procedure had no infectivity even when the initial suspension contained 10^3 PFU/mouse inoculum volume.

Investigation of the suckling mouse stomach revealed that the stomach pH after 2 h of starvation was between 3.0 and 4.0. Because food does not pass from the semipartitioned stomach for at least 2 to 3 h it was thought that this long duration of low pH could be responsible for the differences observed by inoculation by the oral route. To test the effects of low pH at body temperature the three test mixtures (BSSC-1, 10^{-3} M $CaCl_2$, and clay plus 10^{-3} M $CaCl_2$) were acidified to pH 3.8 with HCl and heated to 37 C. Samples taken over a 2-h time period indicated identical virus inactivation rates. It is probable that proteolytic enzymes of the mouse digestive system may be responsible for the differences noted: the serum-containing BSSC-1 protected virus infectivity through the stomach.

DISCUSSION

This study has demonstrated that both organic and inorganic solids suspended in water can adsorb enteroviruses. Adsorption depends

TABLE 9. *Infectivity of EMC virus by i.c. inoculation of weanling mice*[a]

Suspending menstruum	PFU/ mouse	No. of mice tested	No. of mice dead[b]	Mortality (%)
BSSC-1		12	12	100
$CaCl_2$		10	10	100
Clay + $CaCl_2$	10^2	10	10	100
Clay filter mat		10	5	50
Clay filtrate		10	0	0[c]
BSSC-1		14	12	85
$CaCl_2$		10	4	40
Clay + $CaCl_2$	10^1	10	5	50
Clay filter mat		10	3	30
Clay filtrate				
BSSC-1		15	7	46
$CaCl_2$		10	2	20
Clay + $CaCl_2$	10^0	10	1	10
Clay filter mat		10	1	10
Clay filtrate				

[a] Suckling mice were inoculated intracerebrally (i.c.) with the various mengovirus samples by a tuberculin syringe. The dose of virus contained in 0.03 ml was injected into the skull to a depth of one-fourth inch (ca. 0.63 cm). Virus adsorbed to clay was also examined after 0.45-μm Gelman membrane filtration. Clay with adsorbed virus was removed from the membranes in 10^{-3} M $CaCl_2$, equivalent to the volume originally filtered. This and the original filtrates were both tested in mice in the same manner as virus contained in BSSC-1, 10^{-3} M $CaCl_2$, and clay + 10^{-3} M $CaCl_2$.

[b] All deaths between 2 and 10 days were recorded.

[c] There was no mortality in mice inoculated with filtrate of samples originally containing 10^3 PFU/ mouse.

on the concentration and nature of the adsorbent material, metal cation and, also, the degree of natural interference.

Most proteinaceous materials, and many other organic materials, have a net electronegative charge at neutral and basic pH. Clays such as Illite, Kaolinite, and Montmorillonite are also electronegatively charged at neutral and basic pH (40). Because both the artificial and the natural materials containing particulate organics flocculated in the presence of cations in a manner similar to clays, we suggest that under our study conditions virus adsorption is best explained in terms of colloids chemistry.

The Montmorillonite clay which served as our standard test clay is a three-layer expanding clay and a hydrophobic colloid. It undergoes interlayer swelling when introduced to water. A notable characteristic of this clay is its relatively high cation capacity of 80 to 100 meg/100 g. Flocculation of this clay follows the Schulz-Hardy rule in the presence of cations: the flocculating power of inert electrolytes (calcium, magnesium, and sodium) is primarily determined by their valence. We believe that, within the general range of pH and cation concentrations used in these studies, the organic as well as inorganic solids obeyed this rule.

Colloids in water develop electrical charges around them due either to dissociation of ionizable groups on the colloid or to adsorption of ions to the colloid surface. Because of a net negative charge (diffuse double layer) on their surfaces at a pH of approximate neutrality and the lack of metal cation, the colloids (clay, virus or organic solids) remain stable. In other words they remain in suspension and do not coagulate; or, in the case of viruses, they do not adsorb to the suspended solids surface.

Coagulation (adsorption) of similarly charged particles depends on the difference in kinetic energy (Brownian movement or turbulent mixing) and resultant interaction energy. Coagulation can be improved by reducing the interaction energy (defined as the net value of Coulombic electrostatic repulsive energy and attractive Van der Waals energy). Coagulation of colloidal systems with simple nonhydrated ions such as Na^+, Ca^{2+}, Mn^{2+} as used in this study is generally due to the compression of the double-layer thickness surrounding the particles due to incorporation of these nonhydrated ions (counter ions) into the diffuse double layer (33).

Our studies suggested that virus entrapment within the building floc was not a significant mechanism for additional removal of virus from suspension. Previously formed flocs, when redispersed, were able to adsorb virus to the same degree as flocs initially formed in the presence of virus. This indicates that there was no significant decrease in the number of sites available to the virus in this system. Thayer and Sproul (37) and Wentworth et al. (41) found that preformed chemical flocs of $Mg(OH)_2$ or $CaCO_3$ were not good virus adsorbents. These studies may not be comparable, however, as the authors did not use mineral solids, did not disperse the chemical flocs, and relied on settling and subsequent analysis of supernatants after settling for determination of residual suspended virus.

It is likely that, as the ratio of viruses to a limited number of adsorptive sites becomes increasingly high, then solids coagulation before contact with virus may reduce total adsorption and later adsorption kinetics. This may be due to the inaccessibility of adsorption sites because of their enclosure in the floc. Also metal

ion-to-virus-to-solids bridging may be significantly reduced.

The results of our studies with organic solids, with natural mixtures of organic and inorganic materials in lake water, and with unrefined Montmorillonite clay are in agreement with data indicating virus adsorption to organic or inorganic suspended solids of raw and activated sludge-treated sewage (3, 7, 19, 20, 21, 25). This adsorption was also noted on pond water solids and algae in sewage stabilization ponds (41). In most of these instances, adsorbed viruses were noted to retain at least some residual infectivity, although quantification was not done. G. Berg (personal communication) has also noted significant virus association with sediments in river water.

High natural turbidity (marine silt) was shown to adsorb large amounts of virus but this did not enhance virus accumulation in shellfish (17). Hamblet et al. (15) also observed that poliovirus adsorbed to marine silt in seawater. They suggest that this may be important to virus survival and hydro transportation.

Dunn and Hitchborn (10) observed that plant viruses would adsorb to technical grade magnesium bentonite in the presence of metal salts ($MgSO_4$) in conjunction with sodium or potassium phosphate buffers. The ratio of magnesium ions to the buffer ions affected adsorption. All viruses studied would adsorb but with differing efficiencies. Sproul (35) found that bacteriophage T-4 adsorbed to various mineral surfaces (silicas). In general, progressive reduction in pH increased virus adsorption within normal water pH ranges. There appeared to be a relationship between decreasing electrophoretic mobility of the mineral and increased adsorption. Shirobokov (31) observed that Coxsackieviruses A and B adsorbed to bentonite clay. Adsorption of the various strains varied with pH. In general lower pH provided best adsorption.

Carlson et al. (5) earlier found clays to adsorb coliphage and poliovirus. They noted that virus adsorption was greater with divalent cations than with an identical molar concentration of monovalent cations and suggested that the primary function of the cations is to effect a proper charge distribution, thus allowing the formation of clay-cation-virus bridges. To establish interaction of this sort it would likely be necessary to depress the electric double layer. It is interesting to note that while monovalent cations enhanced adsorption there was no appreciable alteration in the zeta potential of the clays. Divalent metal cations, on the other hand, significantly lowered the zeta potential of the clays.

Drewry and Eliassen (9) obtained similar coliphage adsorption when using various soils. In batch and column tests, metal cations reduced the repulsive electrostatic potentials on the soil and the bacteriophage particles, thereby enhancing adsorption. In studies performed without cation the adsorptive capacity of most soils tested diminished at a basic pH. They suggested that at a higher pH more phage carboxy groups were ionized and the soil particles were more electronegatively charged.

The metal cation concentration determines, to a great extent, the time required for a given amount of clay or other similarly charged cations to coagulate. Generally, as the cation concentration increased, less time was needed for coagulation.

The particular size of the particles to be adsorbed or coagulated also is an important consideration. It is likely that clay-to-clay particle attraction in natural systems is influenced greatly by shearing forces, whereas the relatively small virus particle has a much greater chance of remaining attached to the larger particles of clay or other solids. This was seen in our Montmorillonite clay system where flocculation is not noticeable until approximately 30 min after cation addition (10^{-3} M $CaCl_2$). By this time essentially all of the EMC virus has been adsorbed. The clay floc containing virus is readily dispersed by agitation, but the virus particles remain adsorbed.

In this study in the presence of 10^{-3} M $CaCl_2$, pH was not an important factor in adsorption. Adsorption to clay in the presence of metal cation was good over the pH range from 3.5 to 9.5. This is not surprising, as metal cations lower the net electronegative ion atmosphere by ion incorporation, an affect which is duplicated by lowering the pH. It seems likely that in a natural water resource virus adsorption would occur throughout the normal water pH range if cations were present in adequate concentration. Because of these features it is obvious that virus adsorption to suspended material in water may affect virus movement in the natural resource. The adsorbed virus may be carried only to a point where the water velocity slows sufficiently to allow the virus-laden particles to settle or coagulate and settle.

Our studies indicate that it may not be prudent to speak of solids-adsorbed viruses in natural systems as being inactivated either temporarily or permanently. We observed that enteric viruses adsorbed to either Montmorillo-

nite clay or other naturally occurring solids were completely infective and even assayed with elevated titer in cell culture. The clay solids may function by allowing the virus to establish better proximity to the cells.

Finally, our experiments indicate that EMC virus retained animal infectivity in laboratory test animals while adsorbed to clay.

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