Adsorption of Reovirus to Clay Minerals: Effects of Cation-Exchange Capacity, Cation Saturation, and Surface Area

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The adsorption of reovirus to clay minerals has been reported by several investigators, but the mechanisms defining this association have been studied only minimally. The purpose of this investigation was to elucidate the mechanisms involved with this interaction. More reovirus type 3 was adsorbed, in both distilled and synthetic estuarine water, by low concentrations of montmorillonite than by comparable concentrations of kaolinite containing a mixed complement of cations on the exchange complex. Adsorption to the clays was essentially immediate and was correlated with the cation-exchange capacity of the clays, indicating that adsorption was primarily to negatively charged sites on the clays. Adsorption was greater with low concentrations of clays in estuarine water than in distilled water, as the higher ionic strength of the estuarine water reduced the electrokinetic potential of both clay and virus particles. The addition of cations (as chloride salts) to distilled water enhanced adsorption, with divalent cations being more effective than monovalent cations and 10^{-2} M resulting in more adsorption than 10^{-3} M. Potassium ions suppressed reovirus adsorption to montmorillonite, probably by collapsing the clay lattices and preventing the expression of the interlayer-derived cation-exchange capacity. More virus was adsorbed by montmorillonite made homoionic to various mono-, di-, and trivalent cations (except by montmorillonite homoionic to potassium) than by comparable concentrations of kaolinite homoionic to the same cations. The sequence of the amount of adsorption to homoionic montmorillonite was Al > Ca > Mg > Na > K; the sequence of adsorption to kaolinite was Na > Al > Ca > Mg > K. The constant partition-type adsorption isotherms obtained when the clay concentration was maintained constant and the virus concentration was varied indicated that a fixed proportion of the added virus population was adsorbed, regardless of the concentration of infectious particles. A heterogeneity within the reovirus population was indicated.

Human enteric viruses (e.g., poliovirus, coxsackievirus, reovirus) have been isolated from marine (34), estuarine (19), and river (11) waters and from edible shellfish (25). The transmission to humans of hepatitis A virus (42), Norwalk virus, and rotavirus (25, 65), either directly or indirectly through sewage-contaminated potable or recreational waters and shellfish, indicates the threat of water-borne viruses to public health. The inability of wastewater treatment processes to inactivate viruses present in sewage (29) suggests that viruses are readily passed into the environment during the disposal of effluents and sludges.

Human enteric viruses have been isolated in higher numbers from estuarine and marine sediments than from the water column (31, 49), and laboratory studies showed that the survival of enteric viruses (51; S. M. Lipson and G.

Stotzky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N51, p. 188), bacteriophages (20; M. Schiffenbauer and G. Stotzky, in preparation), and herpesvirus type 1 (B. H. Yu and G. Stotzky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N53, p. 188) is enhanced when the viruses are complexed with naturally occurring inorganic particulates such as clay minerals.

The mechanism(s) by which animal viruses adsorb to particulates has only been minimally investigated. Schaub and Sagik (51), for example, suggested that adsorption of virus to clay minerals and organic particulates occurs by physical means, as a result of van der Waals forces and hydrogen bonding, whereas Carlson et al. (10) suggested that adsorption occurs through the formation of a cation bridge between net negatively charged viruses and net negatively charged clay minerals.

Most studies investigating the interactions between animal viruses and particulates have dealt with viruses of the family Picornaviridae, primarily the enterovirus group, and few studies have dealt with other environmentally important viruses of the family Reoviridae. Reovirus is a common infectious agent in sewage (35) and appears to be endemic to urban and suburban populations, as suggested by the persistent contamination of San Diego Bay (15) and the coastal New Hampshire (37) estuarine regions. England (15) suggested that subclinically infected persons excrete large quantities of reoviruses and that these viruses eventually enter local receiving waters through sewage effluents and sludge. Consequently, we studied the possible mechanisms involved in the adsorption of reovirus type 3 to the hydrous aluminosilicate clay minerals, kaolinite (K) and montmorillonite (M).

MATERIALS AND METHODS

Virus. Reovirus type 3 was kindly supplied by S. Dales (University of Western Ontario, Ontario, Canada). Seed virus was inoculated onto mouse fibroblast (L-929) cells grown in 25-cm^2 plastic tissue culture flasks (Corning Glass Wares). At the onset of maximum cytopathic effect, the cells and medium were frozen and thawed three times, followed by centrifugation at 7,710 × g for 10 min (Sorval RC2-B, SS-34 rotor). The virus was freed from nonviral proteins and lipids with Genesolv-D (trichlorotrifluoroethane; Refrigeration Sales Co.) by adding the supernatants to an equal volume of reagent, consisting of 10 ml McIlvaine's buffer (diluted 1:50 in distilled water) and 5 ml of Genesolv-D, homogenizing the suspension for 10 min (VirTis "23" homogenizer, medium speed), and then centrifuging the homogenate at 750 × g for 10 min. The aqueous supernatant, which contained the purified virus, was placed into a cellulose dialysis bag and concentrated for 5 h against a 70% solution of polyethylene glycol (molecular weight, 20,000), which extracts water by osmotic ultrafiltration.

Cell culture. L-929 cells were kindly supplied by P. Gomatos (Sloan-Kettering Institute, New York, N.Y.). The growth medium consisted of Hanks minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories), 1% L-glutamine, and 100 U of penicillin, 100 μg of streptomycin, and 1.0 μg of amphotericin B per ml. The maintenance medium was the same as the growth medium, except that 2% fetal bovine serum was employed. Stock cultures were maintained at 37°C in 25-cm^2 plastic flasks, and the cells were digested with a 0.2% trypsin solution in Hanks balanced salt solution without Ca and Mg. At the onset of monolayer disintegration, the cells were suspended in maintenance medium, pelleted at 270 × g for 5 min, suspended in growth medium, and appropriately diluted for seeding. The buffer capacity of Hanks balanced salt solution obviated the need for a 5% CO_2 atmosphere.

Virus assay. A 0.2-ml suspension of trypsinized L-929 cells (approximately 2 × 10^5 cells per ml of growth medium) was seeded into each of 88 wells of a flat-bottom clear plastic microtitration plate (Costar). Vi-

rus stock and experimental samples were serially diluted in maintenance medium from 10^{-1} to 10^{-8}, and cell cultures that had grown into monolayers (3- to 4-day incubation) were inoculated with 0.025 ml of the virus suspension. Cells were observed for cytopathic effect with an inverted microscope (100× magnification) at 48-h intervals, and final readings were made after 7 days. Endpoints were determined by the statistical procedure of Reed and Muench (45) and expressed as 50% tissue culture infective doses (TCID$_{50}$) per 0.025 ml. The cell culture maintenance medium was changed by aspiration after 3 and 6 days.

Clay minerals. The Wyoming bentonite (Fisher Chemical Co.) contained primarily M, with small amounts of mica (biotite), quartz, feldspar, and zeolite-type minerals. The kaolin (Fisher Chemical Co.) contained primarily K, with small amounts of anauxite, dickite, and nacrite (from X-ray diffraction analysis and personal communication with A. M. Langer). The majority of the particulates had a diameter from 0.1 to 1.0 μm, as measured with a calibrated ocular micrometer, although each clay population contained some particles greater than 10 μm and ranging to 200 μm. The cation-exchange capacity (CEC) and the cations saturating the exchange complex of K and M are listed in Table 1.

(i) Clays with a natural mixed complement of cations. Stock suspensions of K and M were prepared by three washings of each clay in distilled or synthetic estuarine water by centrifugation (10,000 × g at 4°C for 10 min). The clays were used either immediately or after temporary (i.e., <48 h) storage at 4°C.

(ii) Homoionic clays. Homoionic clays (clay minerals containing only one type of cation on the exchange complex) were prepared by the procedures of Dashman and Stotzky (12) and Harter and Stotzky (27): K and M were suspended (4 g/100 ml) in freshly prepared 0.4 N Na$_2$CO$_3$ (pH 9.5), centrifuged at 60 and 160 × g, respectively, the greater than 5-μm fraction (containing the mineral impurities) was discarded, and the clay remaining in the supernatant was washed at least three times by centrifugation (40,000 × g for 10 min) with the appropriate chloride salt (0.5 N) to make the clays homoionic to Na, K, Ca, Mg, or Al. The homoionic clays were then washed (at least seven times) with distilled water until the supernatants were chloride negative, as determined with AgNO$_3$. The size of the homoionic clay particles ranged from 0.6 to ≤ 2.0 μm in diameter (30), and they were used shortly after their preparation.

Synthetic estuarine water. Instant Ocean (Aquarium Systems, Inc.) synthetic sea salts (24.8 g of synthetic

TABLE 1. CEC and exchangeable cations of M and K^a

Clay mineral^b	CEC	Exchangeable cations (meq/100 g of clay)				
		H	K	Na	Ca	Mg
M	97.7	8.5	1.5	49.9	41.9	10.3
K	5.8	0.3	0.05	0.01	4.2	0.07

^{a} Data from reference 57.
^{b} Clay minerals were distributed as bentonite and kaolin by Fisher Scientific Co.

sea salts per liter plus 0.31 g of trace elements per liter) were added to distilled water to yield a salinity of 25 ppt, determined from standard conversion tables (59) based on water density measurements with a hydrometer. Samples were autoclaved (121°C at 15 lb/in^2), and the pH was adjusted to 7.2 when necessary.

Adsorption kinetics. A 0.1-ml sample of virus stock was added to 0.9 ml of distilled water containing 5 mg of M or 20 mg of K, and the virus-clay suspensions were permitted to react for 0, 5, 10, 20, and 30 min, agitated every 5 min, followed by centrifugation at 10,000 × g for 10 min. The control consisted of 0.1 ml of virus and 0.9 ml of distilled water.

Construction of equilibrium adsorption isotherms. (i) Constant virus versus varied clay concentrations. A 0.1-ml sample of stock virus, diluted to 3.0 × 10^5 or 3.1 × 10^4 TCID$_{50}$/0.025 ml, was mixed with 5 ml of distilled or synthetic estuarine water containing different concentrations of K or M with a natural mixed complement of cations at room temperature (23 ± 2°C) for 30 min, with agitation on a Genie Vortex mixer (medium speed) at 5-min intervals. The control consisted of 5 ml of distilled water and 0.1 ml of virus, as preliminary studies showed no significant differences in the survival of the virus between estuarine and distilled water during the 30-min adsorption period. The suspensions were centrifuged for 10 min at 7,710 × g, and the supernatants were serially diluted and inoculated onto cell cultures. The centrifuged virus-clay pellets were kept in an ice bath during the 30 to 60 min required for preparation of serial dilutions and inoculations.

Similar studies were conducted with 0.1 ml of virus (1.64 × 10^5 TCID$_{50}$/0.025 ml) added to 0.9 ml of distilled water containing various concentrations of K or M homoionic to Na, K, Ca, Mg, or Al. The control consisted of 0.1 ml of virus and 0.9 ml of distilled water.

The number of virus particles adsorbed was calculated by subtracting the titer of viruses in the experimental (clay) supernatants from the control (no clay) supernatants. The percentage of virus adsorbed was determined from the ratio (× 100) of virus adsorbed in the experimental and control tubes.

(ii) Constant clay versus varied virus concentrations. Reovirus stock was diluted (serial, 10-fold) in maintenance medium, and 1 ml of each virus dilution was added to 1 ml of distilled water containing pelleted K or M, which was suspended by blending in a Vortex mixer. The 2-ml virus-clay suspensions contained final virus titers ranging from 4.2 × 10^0 to 2.1 × 10^6 TCID$_{50}$/0.025 ml. The data represent a composite of 11 experiments with clay concentrations ranging from 0.5 to 3.0 mg of M and from 10 to 40 mg of K per 2 ml. Controls consisted of 1 ml of distilled water and 1 ml of maintenance medium containing the appropriate virus concentrations. The suspensions were agitated every 5 min for 30 min at room temperature and centrifuged at 10,000 × g for 10 min, and the supernatants were titered for infectious virus.

Adsorption of nonadsorbed virus by fresh clay. A 0.1-ml sample of stock virus was added to 0.9 ml of distilled water containing 1 mg of K or M. The suspensions were agitated every 5 min for 30 min and centrifuged at 10,000 × g for 10 min at 4°C, and infectious virus titers were determined in the supernatants. The supernatants containing unadsorbed reovirus were added to 1 mg of pellets of freshly prepared K

or M, agitated, and treated as described above. The supernatants from the second clay-virus mixtures were added to another 1-mg pellet of fresh K or M, followed by titration of the resultant supernatants for infectious virus. The amounts of reovirus adsorbed by sequential 1-mg samples of fresh clay were calculated on the basis of virus present in the previous supernatants and do not represent cumulative titers.

Effect of cations on adsorption of reovirus by clays containing a natural mixed complement of cations. Samples (5.0 ml) of distilled water containing 10^{-2} or 10^{-3} M Na, K, Ca, or Mg as the chloride salt and either 5 mg of K or 0.4 mg of M were mixed with 0.1 ml of virus (1.1 × 10^8 TCID$_{50}$/5.1 ml) for 30 min (with agitation every 5 min) and centrifuged at 10,000 × g for 10 min, and virus titers were determined in the supernatants. The control tubes were identical to the experimental tubes, except that no cations were added. Preliminary experiments, in agreement with the results of Floyd and Sharp (16), indicated that the concentrations of cations used did not affect virus infectivity.

Statistics. Experiments were performed in triplicate, and virus titrations were performed in duplicate or triplicate. The data are presented as the arithmetic mean ± standard error of the mean. On logarithmic plots, the vertical bars indicate the value of the standard error below the mean. Control and experimental means were compared by the Student's t test; P < 0.05 was assumed to be statistically significant. Computations were performed on a Tektronix 31 programmable calculator.

RESULTS AND DISCUSSION

Adsorption kinetics. Depending upon the clay mineral and the virus type, adsorption may be immediate (e.g., herpes simplex virus type 1; Yu and Stotzky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N53, p. 188) or may require approximately 15 min (e.g., encephalomyocarditis [51], poliovirus, and reovirus [55]), 20 min (e.g., coliphage T2 [10]), or 45 min (e.g., coliphage MS-2 [56]) or as long as 18 h (e.g., coliphage T2 by a Cherkassy M [46]). Maximum adsorption of reovirus type 3 by K and M was essentially instantaneous (Table 2), but an adsorption period of 30 min was used to facilitate

TABLE 2. Kinetics of reovirus adsorption to clay minerals[a]

Time (min)	Virus adsorbed (% of control, mean ± SEM)	
	M[b]	K[c]
0	89.7 ± 2.9	92.7 ± 2.7
5	92.7 ± 2.7	93.2 ± 0.6
10	91.3 ± 1.3	93.2 ± 0.6
20	94.0 ± 1.4	91.8 ± 1.8
30	96.2 ± 0.8	89.5 ± 3.1

[a] The following concentrations were used: 5 mg of M and 20 mg of K per ml.
[b] Control: 1.3 × 10^5 ± 0.01 × 10^5 TCID$_{50}$/ml.
[c] Control: 1.1 × 10^5 ± 0.02 × 10^5 TCID$_{50}$/ml.

handling (e.g., inoculation, agitation) of the numerous preparations.

Equilibrium adsorption isotherms. (i) Constant concentrations of reovirus and varied concentrations of clay. Adsorption isotherms of reovirus were constructed from data obtained with K and M containing a natural mixed cation complement in distilled water and synthetic estuarine water. At low clay concentrations, M adsorbed more virus than did K; this occurred both in distilled and synthetic estuarine water and with virus concentrations of 3.1×10^4 and 3.0×10^5 $TCID_{50}/0.025$ ml (Fig. 1 and 2). In distilled water, a concentration of K approximately 20 times greater than that of M was required to adsorb an equal amount of virus, but this difference in the concentration of K and M was not as large (approximately 10 times more K than M was required) in estuarine water. In synthetic estuarine water, the adsorption of reovirus occurred at lower concentrations of both K and M.

Adsorption of reovirus by K and M in distilled water (Fig. 3) or synthetic estuarine water (Fig. 4) appeared to be related to the CEC of the clays, indicating that adsorption occurred primarily to the negatively charged sites on the clays. The correlation between the adsorption of the virus and the CEC was independent of the type of water or the concentration of virus used, as a single isotherm was obtained when the percentage of added virus adsorbed in both waters was plotted against CEC (Fig. 5). Adsorption by soils of coliphages ϕX174 (8) and T1, T2, and f2 (14, 18) and of a bacteriophage of *Arthrobacter* sp. (40) by M and Dowex 50 (a cation-exchange resin) was also correlated, in part, with the CEC of the adsorbents. Conversely, Goyal and Gerba (22) found no relationship between the CEC of nine soil types and the adsorption of a number of enteroviruses, coli-
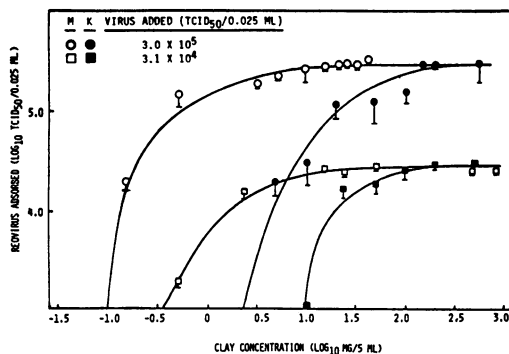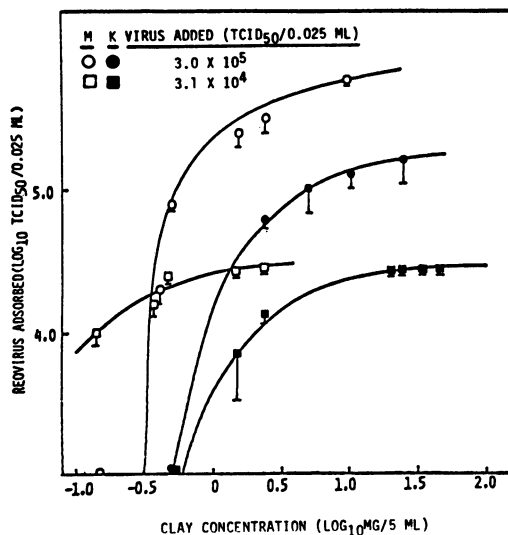

FIG. 2. Effect of clay concentration on the adsorption of two concentrations of reovirus by M and K with a natural mixed complement of cations in synthetic estuarine water. Mean titer ± standard error of the mean.

phages, and a simian rotavirus, but their conclusions may have been inaccurate, as their centrifugation speed ($2,500 \times g$ for 5 min) may have been too low to remove virus-soil particulates from the supernatant. Consequently, their inoculation of cell cultures with supernatants containing particulate-associated viral particles may have enhanced (54), decreased (60), or had no effect (52) on infectivity, depending on the biophysical characteristics of the viruses and the physicochemical characteristics of the soils studied (S. M. Lipson and G. Stotzky, *in* A. Misra, ed., *Virus Ecology*, in press). The adsorption of coliphage T1 and, to a lesser extent, T7 by K and M was related to the anion-exchange capacity of the clays, as blockage of positively charged sites by pretreatment of the clays with sodium metaphosphate decreased virus adsorption (53).

The exterior of a reovirus is composed of a protein coat (capsid), which, in turn, is composed of individual polypeptide capsomers. Consequently, variations in the net charge of the virion follow the laws of protein chemistry: at pH values below the pK_R (the average ionization constant of the amino acid side chains) of the surface proteins, protonation of the ionizable groups will result in net positive charge; at pH values above the pK_R, deprotonation predominates and the virion has a net negative charge. Glutamic ($pK_R = 4.25$) and aspartic ($pK_R = 3.86$) acids are the predominant amino acids in the polypeptides of the reovirus capsid (41),

FIG. 1. Effect of clay concentration on the adsorption of two concentrations of reovirus by M and K with a natural mixed complement of cations in distilled water. Mean titer ± standard error of the mean.
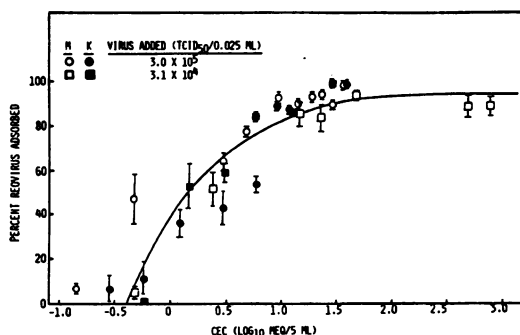
FIG. 3. Effect of CEC on the percent adsorption of two concentrations of reovirus by M and K with a natural mixed complement of cations in distilled water. Mean titer ± standard error of the mean.

which probably account for the low isoelectric point (pI) of the virus (pI = pH 3.8 [55]).

The surface pH (pH$_s$) of clay minerals is presumably 3 to 4 units lower than the bulk pH (pH$_b$) of the suspension (6, 61), which may explain, for example, the requirement for a higher pH$_b$ for the same microbial activity in soil than in solution (36) and, at pH$_b$ = 6.5, the decreased infectivity titer of a clay-associated actinophage (60). The acidity at the clay surface (pH$_s$) probably resulted in proton (H$^+$) transfer (protonation) from the surface of the clay to the reovirus, followed by cation exchange between the net negatively charged clay and the now net positively charged virus.

van der Waals forces of attraction and hydrogen bonding may also have been involved in the adsorption of the virus to the clay. Greenland et al. (24), for example, emphasized the significance of van der Waals forces in the adsorption of amino acids at or near their pI to clays in the absence of proton or cation exchange, and Stotzky (58) pointed out the importance of hydrogen bonds in the binding of proteins to clays at pH$_b$ values above the pI of the proteins.

(ii) **Effect of surface area and surface charge density of the clays on adsorption of reovirus.** The adsorption of reovirus by clay minerals with a natural mixed cation complement was plotted as a function of the surface area of K (15 m^2/g) and the external (82 m^2/g) and total (750 m^2/g) surface area of M (63) (Fig. 6). Adsorption was not related to surface area, regardless of whether the clays were suspended in synthetic estuarine or distilled water, as the isotherms for each clay and in each water were discrete and the points for the two clays did not fall on the same isotherms. Adsorption was also not related to surface charge density (CEC per specific surface) of the clays, which was 0.0037 meq/m^2 for K and 0.0119 (external) and 0.0013 (total)
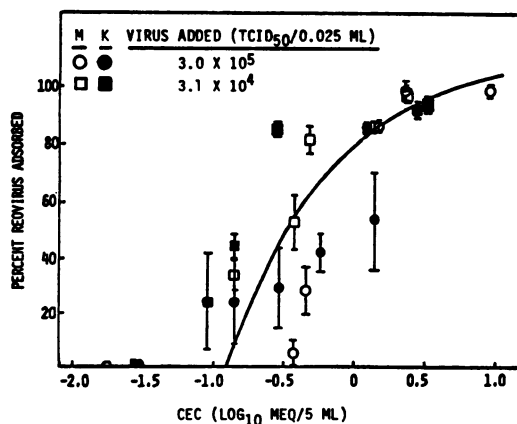

FIG. 4. Effect of CEC on the percent adsorption of two concentrations of reovirus by M and K with a natural mixed complement of cations in synthetic estuarine water. Mean titer ± standard error of the mean.

meq/m^2 for M. These data indicate further that CEC was the major characteristic of the clays involved in the adsorption of the virus.

Equilibrium adsorption isotherms constructed from data obtained with homoionic M and K showed that more reovirus was adsorbed at lower clay concentrations by M homoionic to Ca, Al, Na, or Mg than by comparable quantities of K homoionic to the same cations (Fig. 7). The sequence in the amount of adsorption to homoionic M was Al > Ca > Mg > Na > K, and the sequence of adsorption to K was Na > Al > Ca > Mg > K.

The reovirus may have been better able to compete with monovalent Na ions for exchange sites on K, as cations of higher valence are bonded with a higher energy and cannot be as easily replaced (27). Although the relatively low
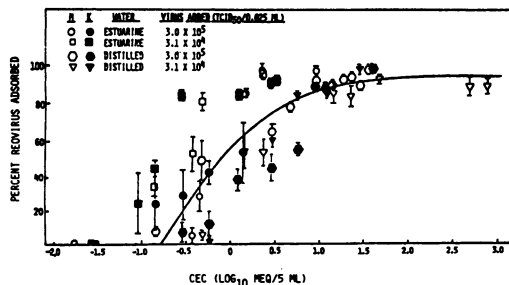

FIG. 5. Effect of CEC on the percent adsorption of two concentrations of reovirus by M and K with a natural mixed complement of cations in synthetic estuarine and distilled water. Mean titer ± standard error of the mean.

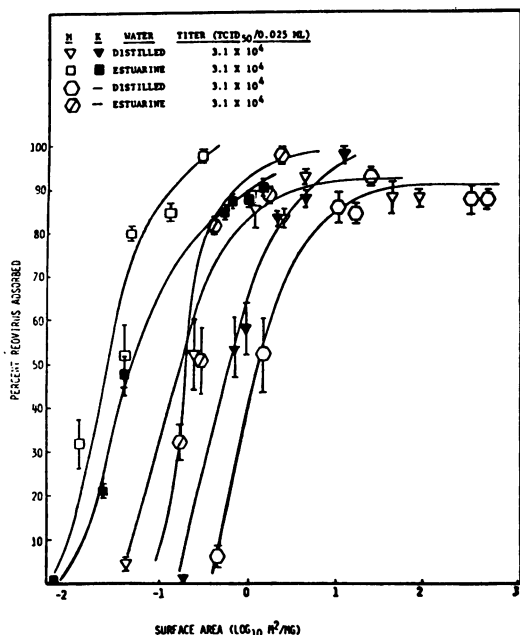| M	M̄	WATER	TITER (TCID₅₀/0.025 ML)
▽	▼	DISTILLED	3.1 X 10⁴
□	■	ESTUARINE	3.1 X 10⁴
○	—	DISTILLED	3.1 X 10⁴
⊘	—	ESTUARINE	3.1 X 10⁴

FIG. 6. Effect of (specific) surface area on the percent adsorption of reovirus by M and K with a natural mixed complement of cations in synthetic estuarine and distilled water. Symbols: △ and □, external surface area of M; ○ and ⊘ total surface area of M. Mean ± standard error of the mean.

adsorption of K homoionic to K ions may appear anomalous, this was probably the result of the higher bonding energy of K ions, as a result of their relatively smaller hydrated ionic radius (64), than of the other cations. Similar data were obtained by Sykes and Williams (60), in that adsorption of actinophage f6 of *Streptomyces* sp. was greater to K homoionic to Na than to K homoionic to Ca or Al.

The ionic size and geometric configuration of K ions results in their entrapment between the basal oxygen sheets of expanding 2:1 clays (5), and the low adsorption of reovirus to M homoionic to K ions was probably the result of the collapsing of the clay lattices, which prevented expression of the interlayer-derived CEC. The high amount of adsorption of reovirus to M homoionic to Al may have been related to the sufficient reduction of the electrokinetic potential of the particles by the polyvalent cation, in accordance with the Schulze-Hardy rule (48), to allow the net negatively charged virus to come close enough to the net negatively charged clay for protonation of the virus and then its adsorption by cation exchange. The greater adsorption of reovirus to low concentrations of M homoionic to Na, Ca, and Mg than to comparable quantities of K homoionic to the same cations was probably the result of the higher CEC of M.

The type of cation saturating the exchange complex of a clay affects the adsorption of proteins (27), amino acids, peptides (12; T. D. Dashman and G. Stotzky, Soil Biol. Biochem., in press), viruses (60), nucleosides (32), and nucleotides (39). A clay mineral containing a predominance of one cation may not display the same adsorptive behavior as the same clay homoionic to the same cation. For example, the adsorption of atrazine by a bentonite that contained M saturated predominantly by Na (R. F. Revson and Co.) was not as great as that of M homoionic to Na (Wyoming bentonite, Clay Mineral Repository, Department of Geology, University of Missouri, Colorado) (7). Browne et al. (7) suggested that a "screening effect" may have existed as a result of small amounts of Ca (7.1%) and Mg (2.7%) on the exchange complex of the bentonite from Revson.

(iii) **Varied concentrations of reovirus and constant concentration of clay.** Adsorption isotherms were constructed from experiments wherein the concentration of the clays, either saturated with a natural mixed complement of cations or homoionic to Na, was maintained constant and the concentration of reovirus was varied. The linear or constant partition (C)-type (21) adsorption isotherms obtained (Fig. 8) indicated that as the virus concentration in the system increased, there was a concomitant increase in available adsorption sites on the clays. Although the virus titers approached 10^6 TCID₅₀/0.025 ml, saturation of the clay (i.e., the formation of a plateau) did not occur. C-type adsorption isotherms were not obtained in experiments wherein the virus
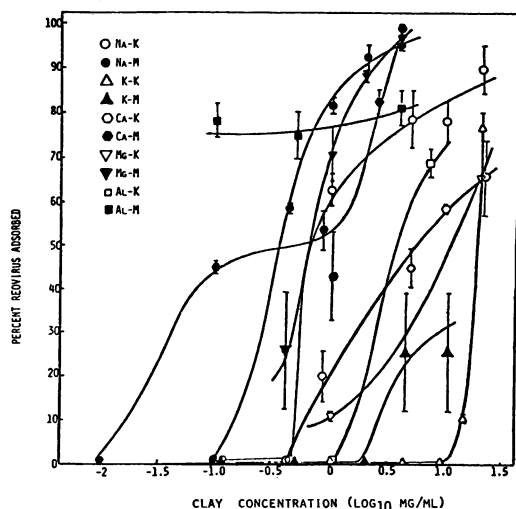
FIG. 7. Effect of cations saturating the exchange complex of M and K (i.e., homoionic clay minerals) on the percent adsorption of reovirus. Mean titer ± standard error of the mean.
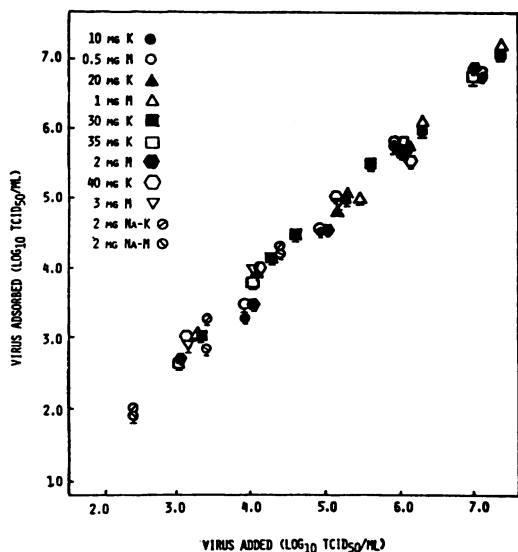
FIG. 8. Adsorption of reovirus by M or K as a function of increasing virus concentration. Adsorption isotherms were constructed from experiments wherein the concentration of the clays, containing either a mixed complement of cations or homoionic to Na, in distilled water was maintained constant, and the virus concentration was varied. The data represent 11 experiments, each with a different constant concentration of clay. Mean titer ± standard error of the mean.

concentration was maintained constant and the clay concentration was varied, as the adsorption isotherm displayed a plateau (Fig. 1 and 2). Larger amounts of clay (i.e., 2.5 to 45 mg of M and 30 to 500 mg of K per ml of synthetic estuarine and distilled water, respectively) were apparently necessary to remove close to 100% of the input virus population, probably as a result, in part, of the entrapment of the virus between the clay particles during centrifugation.

The C-type adsorption isotherm has been interpreted (21) as indicating the penetration of an adsorbate into the structure of an adsorbent. Giles et al. (21) cited the adsorption of di-, tri-, and tetrapeptides to M homoionic to Ca (23) as experimental evidence for the occurrence of C-type adsorption isotherms with clay minerals. The C-type adsorption isotherm obtained in the present study with both K (a nonexpanding clay mineral) and M (even though the virus did not penetrate the clay as shown by X-ray diffractometry; Lipson and Stotzky, in preparation) indicated that intercalation of the clay lattices is not necessary for the formation of the C-type isotherm. Harter and Stotzky (28) showed that adsorption of catalase by M homoionic to various cations did not result in expansion of the clay (electron microscopy showed that catalase was adsorbed and bound primarily on external

sites), even though the adsorption and binding isotherms were classified as C-type (58). Albert and Harter (3) and Harter (26) reported that C-type binding isotherms can be obtained when the pH_b is higher than the pI of the protein and that alteration of surface characteristics (e.g., type of cation saturating the exchange complex, decrease in pH_s) can change the shape of the isotherm.

The C-type adsorption isotherms obtained also indicated that a fixed proportion of reovirus was adsorbed, regardless of the concentration of virus added. This may have been the result of a heterogeneity in the net charge of the virus particles within a given population. Lance and Gerba (33) reported that the percentage of virus adsorbed to soil at various depths was approximately the same, regardless of virus concentration; they suggested that this was the result of differences in the net negative charge of individual viral particles within the population and that dilution of the virus did not change the percentage of charged particles and, consequently, the percentage adsorbed at each soil depth. Burge and Enkiri (9) showed that at least two populations of coliphage ϕX174 were distinguishable by the rate at which they were adsorbed by soil.

Experiments designed to investigate the influence of reovirus heterogeneity on adsorption showed that more than 90% of the virus remaining in the supernatant after centrifugation of the virus-clay complex was adsorbed by 1 mg of fresh K or M (Fig. 9). The removal from the supernatants of more than 90% of the nonadsorbed virus by fresh 1-mg samples of clay and the inability of 1 mg K and, to a lesser extent, M initially to adsorb large quantities of infectious
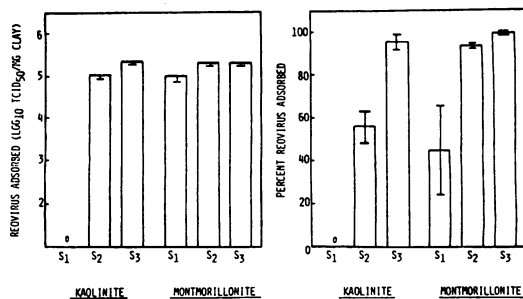

FIG. 9. Adsorption of nonadsorbed reovirus by the addition of fresh M or K. Nonadsorbed virus in supernatant 1 (S_1) was added to 1 mg of fresh M or K (i.e., virus-free clay), and infectious virus titers were determined in the supernatants. The supernatants from this system (S_2) were reacted with an additional 1 mg of fresh M or K, and the resultant supernatants (S_3) were treated as above. Control titer: $2.0 \times 10^5 \pm 0.55 \times 10^5$ TCID$_{50}$/ml of distilled water. Mean titer ± standard error of the mean.
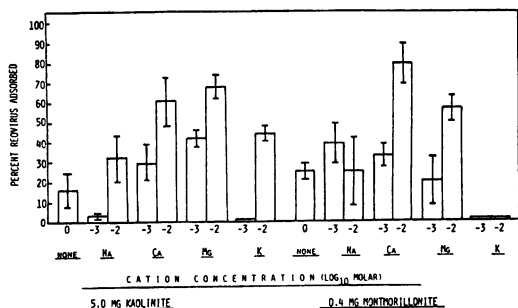
FIG. 10. Effect of cations added to distilled water on adsorption of reovirus by clay minerals containing a natural mixed complement of cations. Mean titer ± standard error of the mean.

virus (Fig. 1) may have resulted from the blockage of adsorption sites on the clay by the preferential adsorption of noninfectious virus particles. Noninfectious particles, identified as components within reovirus populations by genetic analyses (2) and reaction to proteolytic enzymes (1), are termed defective interfering and potentially infectious particles, respectively.

Ahmed and Fields (2) showed that defective interfering particles of reovirus type 3 are genetically heterogeneous, contain multiple mutations, and, most importantly, have a mutation on the S4 RNA segment. This segment encodes for the σ3 outer capsid polypeptide, which is necessary for the infectivity of reovirus type 3 (13).

Effect of cations on adsorption of reovirus by clay minerals. The addition of 10^{-3} or 10^{-2} M Mg or of 10^{-2} M Ca or K ions to distilled water enhanced the adsorption of reovirus to K, but significantly less virus was adsorbed in the presence of 10^{-3} M Na or K ions than in distilled water alone, and 10^{-2} M Na or 10^{-3} M Ca ions did not significantly affect the amount of adsorption. Adsorption to M was enhanced by 10^{-2} M Mg or Ca ions but not by 10^{-2} or 10^{-3} M Na or by 10^{-3} M Mg or Ca ions. Adsorption to M was significantly less in the presence of 10^{-2} or 10^{-3} M K ions than in distilled water alone (Fig. 10). These data explain the better adsorption of reovirus to the clays in synthetic estuarine than in distilled water, as the concentrations of cations added approximated those in the synthetic estuarine water. The cations probably suppressed the thickness of the diffuse double layer, thereby permitting the net negatively charged reovirus and clay particles to approach each other sufficiently for adsorption by physical forces (e.g., van der Waals forces, hydrogen bonding) and protonation to occur at the virus-clay interface. These concepts of colloid chemistry have been used to explain surface interactions between clays and microbial cells (47, 48)

and viruses (51, 53) and the adsorption of viruses to host cells (4, 17, 43) and to a cation-exchange resin (44). Adsorption of coliphage T2 to K (10), of encephalomyocarditis virus to M (51), and of poliovirus and coliphage T7 to M and K (38) was enhanced by the addition of divalent cations to the suspensions. Consequently, numerous investigators routinely employ Ca in their bentonite concentration procedures for the recovery of viruses from soil (50) and sewage (62).

Estuarine and freshwater ecosystems are characterized, in part, by fluctuations in salinity and by different concentrations and types of dissolved salts, respectively. These aquatic systems also reflect the inorganic and organic inputs from soils. The data presented in this study suggest mechanisms whereby reovirus, and possibly other enteric viruses, adsorb to naturally occurring particles (e.g., clay minerals) characteristic of these environments. The persistence of enteric viruses in aquatic and terrestrial environments has been related to their adsorption onto naturally occurring inorganic particulates such as clay minerals. The data presented in this study may be applicable to environmental virology, especially to techniques of virus concentration and isolation in environmental monitoring and pollution assessment studies.

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