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Effect of Proteins on Reovirus Adsorption to Clay Minerals

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Organic matter in sewage, soil, and aquatic systems may enhance or inhibit the infectivity of viruses associated with particulates (e.g., clay minerals, sediments). The purpose of this investigation was to identify the mechanisms whereby organic matter, in the form of defined proteins, affects the adsorption of reovirus to the clay minerals kaolinite and montmorillonite and its subsequent infectivity. Chymotrypsin and ovalbumin reduced the adsorption of reovirus to kaolinite and montmorillonite homoionic to sodium. Lysozyme did not reduce the adsorption of the virus to kaolinite, but it did reduce adsorption to montmorillonite. The proteins apparently competed with the reovirus for sites on the clay. As lysozyme does not adsorb to kaolinite by cation exchange, it did not inhibit the adsorption of reovirus to this clay. The amount of reovirus desorbed from lysozyme-coated montmorillonite was approximately 38% less (compared with the input population) than that from uncoated or chymotrypsin-coated montmorillonite after six washings with sterile distilled water. Chymotrypsin and lysozyme markedly decreased reovirus infectivity in distilled water, whereas infectivity of the virus was enhanced after recovery from an ovalbumin-distilled water-reovirus suspension (i.e., from the immiscible pelleted fraction plus supernatant). The results of these studies indicate that the persistence of reovirus in terrestrial and aquatic environments may vary with the type of organic matter and clay mineral with which the virus comes in contact.

Laboratory and field investigations have shown that the persistence of viruses in aquatic and terrestrial systems is enhanced when the viruses are associated with naturally occurring inorganic particulates such as clay minerals and sediments (2, 11, 17, 27, 30, 35). The prolonged infectivity of viruses in receiving or recreational waters and in soils has been ascribed to virus adsorption and binding to particulates in these environments (11, 20, 21, 25, 29, 31; Lipson and Stotzky, Water Res., in press). The detection and apparent persistence of the hepatitis A virus antigen, rotavirus, and other enteric viruses in well water (7a, 14) may also reflect, in part, an association between these viruses and particulates in the aquifer.

Organic matter is an integral part of aquatic and terrestrial systems, and its effect on interactions between particulates and microbes exerts a profound influence on the ecology of the microbiota in these environments (4, 10, 13, 34).

The effect of organic matter on the adsorption of viruses to particulates in soils and estuarine waters has been the subject of a number of studies (9), and conflicting results have been reported. For example, secondary sewage and humic acid had no effect on the adsorption of polio- or echovirus to estuarine sediments (17), nor did the accumulation of organic matter near the surface of soil columns affect adsorption of poliovirus to soil (19). However, bovine albumin, egg albumin, and domestic wastewater reduced the adsorption of coliphage T2 and egg albumin reduced the adsorption of poliovirus to kaolinite (K) (5). The suspension of soil in secondary sewage effluent reduced adsorption of coliphage f2 and poliovirus, suggesting that organic components in the wastewater may have competed for adsorption sites on the soil particulates (28). In contrast, adsorption of coliphages T1, T2, and f2 by several soil types increased with increasing organic carbon content (8).

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The mechanism(s) by which organic matter influences the adsorption of viruses to particulates is poorly defined, probably because most studies have used heterogeneous organics (e.g., sewage) or organics with similar biophysical properties (e.g., similar pI and molecular weights). Consequently, we studied the effect of specific organics, in the form of chymotrypsin, ovalbumin, and lysozyme (ranging in molecular weight from 17,500 to 54,000 and in pI from 4.6 to 11.2), on the adsorption of reovirus type 3 to the clay minerals K and montmorillonite (M) and on its subsequent infectivity.

MATERIALS AND METHODS

Virus and cell culture. The growth medium for the L-929 mouse fibroblast cells consisted of Hanks minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories), 1% L-glutamine, 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 used. Cell cultures and their supernatants were frozen and thawed three times, followed by purification of the virus by treatment with fluorocarbon, as described previously (21).

Clay minerals. The preparation of clay minerals homoionic to sodium was described previously (7, 21).

Titration of reovirus type 3. Reovirus type 3 was titrated on L-929 cells cultured in flat-bottom, clear plastic microtitration plates (Costar) (21, 36). Cytopathic effect was observed microscopically, and endpoints were expressed as 50% tissue culture infective dose ($TCID_{50}$), according to the procedure of Reed and Muench (26).

Preparation of clay-protein complexes. The proteins selected were globular and differed in pI and molecular weight (Table 1). Ovalbumin, α-chymotrypsin, and lysozyme were obtained from Sigma Chemical Co. (control no. A-5503), Mann Research Labs, Inc. (lot no. B3047), and Nutritional Biochemicals Corp. (control no. 5073), respectively. The clay-protein complexes were prepared as follows: clay homoionic to Na was added to distilled water to yield a final concentration of 20 mg/2 ml. A 20-mg portion of protein was

TABLE 1. Some properties of the proteins, homoionic clays, and clay-protein complexes

Protein-clay	pH of pI[a]	Mol wt[a,b]	pH in distilled water
Protein			
Ovalbumin	4.6–4.7	43,500–54,000	6.1
α-Chymotrypsin	8.1–8.6	17,500–24,500	3.7
Lysozyme	11.0–11.2	13,930–17,200	6.3
Homoionic clay			
M-Na	—[c]	—	6.4
K-Na	—	—	5.9
Clay-protein complex			
M-Na-ovalbumin	—	—	6.3
K-Na-ovalbumin	—	—	5.6
M-Na-chymotrypsin	—	—	6.8
K-Na-chymotrypsin	—	—	6.1
M-Na-lysozyme	—	—	5.9
K-Na-lysozyme	—	—	5.7

[a] Harter and Stotzky (12).
[b] Laskowski (22).
[c] —, Not determined.

added to the suspension, which was agitated every 5 min during a 1-h reaction period. The clay-protein suspensions were washed six times with distilled water (40,000 × *g* at 4°C) to remove loosely bound protein, and the pelleted clay-protein complexes from the last wash were used in the experiments (12). The clay-protein pellets were resuspended in distilled water and used either immediately or after temporary storage (i.e., <48 h at 4°C). Of the added proteins, approximately 45% (9 mg) lysozyme, 70% (14 mg) chymotrypsin, and 96% (19 mg) ovalbumin was bound to M (12), and approximately 2% (0.4 mg) lysozyme and 13% (2.6 mg) ovalbumin was bound to K (1).

Adsorption of reovirus by clay minerals homoionic to sodium and complexed with proteins. A 0.1-ml amount of stock virus was added to 0.9 ml of distilled water containing 1 mg of K or M homoionic to Na and complexed with chymotrypsin, ovalbumin, or lysozyme. The suspensions were agitated every 5 min during a 30-min reaction period at room temperature (approximately 23°C) and centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatants were titrated for infectious virus. The amount of adsorbed virus was calculated by subtraction of the number (titer) of viruses in the experimental (clay-protein complex or only clay) supernatants from the number of viruses in the distilled water control. The amounts of reovirus adsorbed by clays homoionic to Na and complexed with the proteins and by clays only homoionic to Na were compared (Fig. 1 and 2).

Effect of chymotrypsin, ovalbumin, and lysozyme on reovirus infectivity. A 0.9-ml portion of distilled water containing 10 mg of ovalbumin, chymotrypsin, or lysozyme was inoculated with 0.1 ml of stock virus. The protein-virus mixtures were reacted for 30 min at room temperature, with agitation every 5 min, and then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants and the resuspended immiscible chymotrypsin and ovalbumin fractions (10 mg of chymotrypsin or ovalbumin did not dissolve completely in 0.9 ml of water) were diluted 10-fold in distilled water and titrated for the presence of infectious virus. The control consisted of 0.1 ml of virus stock in 0.9 ml of distilled water.

Treatment of L-929 cells with lysozyme and chymotrypsin. A 1-mg amount of lysozyme or chymotrypsin was added to 1.0 ml of distilled water and agitated on a Genie Vortex mixer, and 0.025 ml of each protein solution was placed onto a confluent monolayer of L-292 cells. After 30 min at room temperature, the monolayers were washed once with maintenance medium and inoculated with reovirus. The control consisted of cell cultures washed once in maintenance medium but not pretreated with protein before inoculation of the virus. As the infectivity of reovirus was unaffected in distilled water containing solubilized ovalbumin, neither the effect of ovalbumin on L-929 cells in culture nor the infectivity of the virus adsorbed to M-ovalbumin complexes were studied.

Desorption of reovirus from M homoionic to sodium and complexed with chymotrypsin or lysozyme. A 0.9-ml amount of distilled water containing 1 mg of M homoionic to Na and complexed with chymotrypsin or lysozyme was inoculated with 0.1 ml of virus to yield final concentrations of $3.2 \times 10^5 \pm 0.43$ and $1.1 \times 10^6 \pm 0.16$ $TCID_{50}$/ml, respectively. The suspensions were agitated every 5 min during a 30-min reaction period at room temperature and centrifuged at 10,000 × *g* for 10 min, and the supernatants were titrated for infectious virus. The clay-protein-virus complexes were

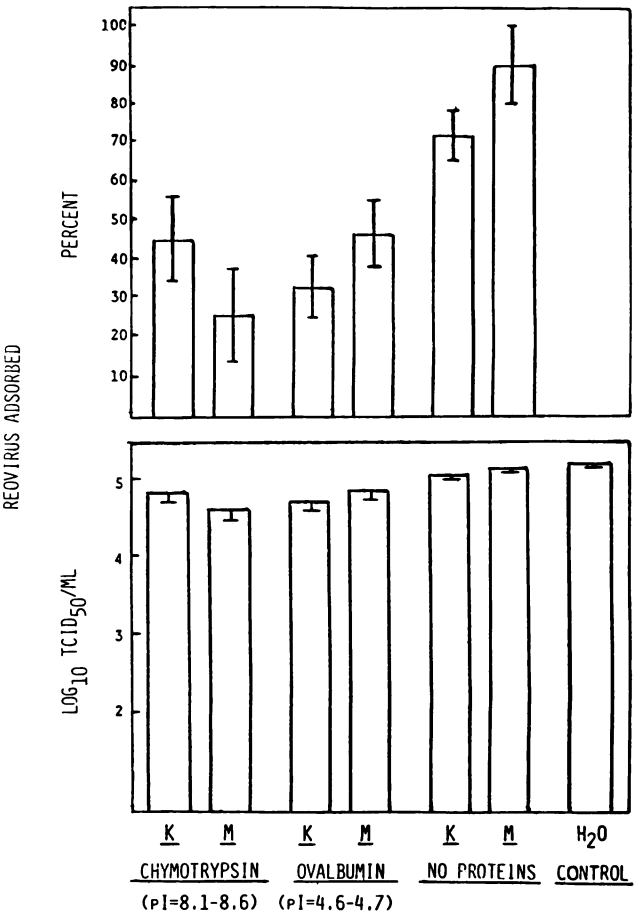

FIG. 1. Adsorption of reovirus by 1 mg of kaolinite (K) or montmorillonite (M) homoionic to sodium and complexed with chymotrypsin or ovalbumin. Control titer: $1.9 \times 10^5 \pm 0.18$ $TCID_{50}$/ml. Mean titer ± standard error of the mean.

sequentially washed six to seven times with 1-ml samples of sterile distilled water, followed by titration of each supernatant. The controls, treated identically as the experimentals, consisted of 1 mg of M homoionic to Na suspended in 0.9 ml of distilled water and inoculated with 0.1 ml of each of the virus stocks. Inasmuch as different viral concentrations were used in the presence of chymotrypsin and lysozyme, the amount (titer) of virus recovered from each clay-protein-virus system was calculated from their respective control.

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. When logarithmic plots were used, the vertical bars indicate the value of the standard error of the mean below the mean. Control and experiments were compared by Student's t test. $P < 0.05$ was assumed to be statistically significant. Computations were performed on a Tektronix 31 programmable calculator.

TABLE 2. Desorption of reovirus from montmorillonite (M) homoionic to sodium and complexed with chymotrypsin or lysozyme

Wash no.	Titer of supernatant ($TCID_{50}$/ml; $\bar{x}$ ± SEM)	
	M-chymotrypsin[a]	M-lysozyme[b]
A[c]	$2.2 \times 10^5 \pm 0.52$	$3.5 \times 10^5 \pm 2.1$
1	$3.8 \times 10^4 \pm 2.10$	$1.5 \times 10^5 \pm 0.22$
2	$2.0 \times 10^4 \pm 0.72$	$1.3 \times 10^5 \pm 0.01$
3	$7.0 \times 10^3 \pm 1.24$	$5.5 \times 10^4 \pm 2.80$
4	$5.6 \times 10^3 \pm 3.80$	—
6	—[d]	$2.0 \times 10^3 \pm 0.72$
7	$<10^2$	—

[a] Total virus recovered from M-chymotrypsin system: $2.9 \times 10^5 \pm 0.38$ $TCID_{50}$/ml. Control titer: $3.2 \times 10^5 \pm 0.43$ $TCID_{50}$/ml. Percent recovered: 90.6 ± 12.10.

[b] Total virus recovered from M-lysozyme system: $6.9 \times 10^5 \pm 1.1$ $TICD_{50}$/ml. Control titer: 1.1×10^6 0.16 $TCID_{50}$/ml. Percent recovered: 62.3 ± 9.58.

[c] Virus recovered in supernatant before start of washing sequence.

[d] —, Not determined.

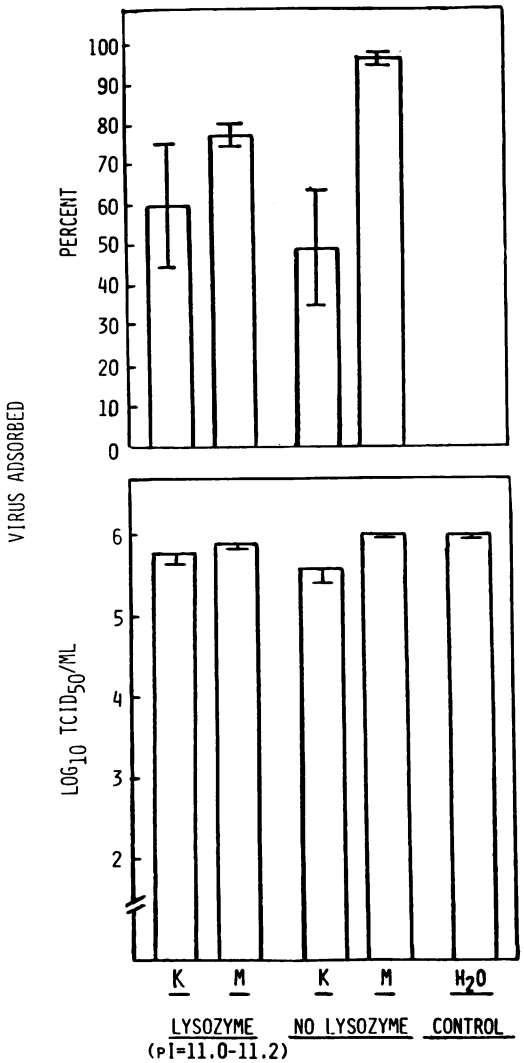

FIG. 2. Adsorption of reovirus by 1 mg of kaolinite (K) or montmorillonite (M) homoionic to sodium and complexed with lysozyme. Control titer: $1.1 \times 10^6 \pm 0.16$ $TCID_{50}$/ml. Mean titers ± standard error of the mean.

RESULTS

Adsorption of reovirus by clay minerals homoionic to sodium and complexed with chymotrypsin, ovalbumin, or lysozyme. The differences in titers between experimental (clay-protein complexes) and control (clay only) virus suspensions indicated that chymotrypsin and ovalbumin reduced the adsorption of reovirus to K and M homoionic to Na by approximately 26 and 66% and 39 and 45%, respectively (Fig. 1). Lysozyme did not reduce the adsorption of the virus to K, but it did reduce adsorption to M by approximately 20% (Fig. 2).

Desorption of reovirus from M homoionic to sodium and complexed with chymotrypsin and lysozyme. The complexation of chymotrypsin with M had no significant effect on the desorption of reovirus, but when lysozyme was complexed with M, the amount of virus desorbed after six washings was decreased by approximately 38% (Table 2). The preadsorption of chymotrypsin or lysozyme to M did not significantly affect the amount of virus desorbed after three washings, inasmuch as 72 ± 7.0% of the added reovirus was desorbed from protein-free clay homoionic to Na (Fig. 3). Repeated washings (i.e., greater than three) with distilled water was needed to show the decreased desorption of reovirus from the M-Na-lysozyme complex.

Effect of chymotrypsin, ovalbum, and lysozyme on reovirus infectivity. Chymotrypsin and lysozyme, in the absence of clays, decreased the infectivity of reovirus by approximately 85 to 99%, respectively, whereas ovalbumin enhanced the infectivity, in that approximately 150% of an input titer of 1.9×10^5 $TCID_{50}$/ml was recovered (i.e., the sum of virus infectivity in the ovalbumin pellet and supernatant) (Fig. 4). An insoluble protein fraction occurred during the preparation of a 1% solution of ovalbumin or chymotrypsin.

Effect of pretreatment of L-929 cells with chymotrypsin and lysozyme. Pretreatment of L-929 cells in monolayer culture with 1.0% chymotrypsin (0.013% protein per well) for 30 min had no significant adverse effect on virus infectivity. However, treatment of the cells with the same concentration of lysozyme decreased reovirus infectivity by 41.3 ± 8.75% (Table 3). The addition of 0.013% protein per well approximated the final protein concentration in the cell cultures in the studies of the infectivity of reovirus suspended in protein in the absence of clays.

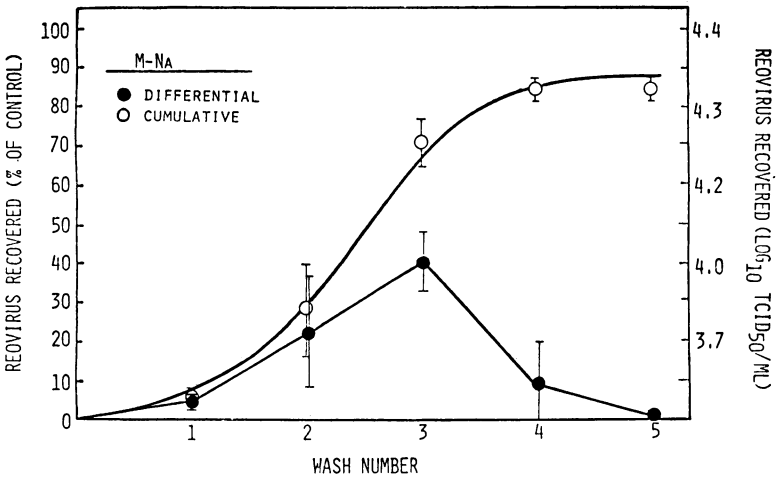

FIG. 3. Desorption of reovirus from montmorillonite (M) homoionic to sodium. Control titer: $4.6 \times 10^4 \pm 0.38$ ($\log_{10}$ 4.4 ± 0.07) $TCID_{50}$/ml. Mean titer ± standard error of the mean.

DISCUSSION

Data obtained from X-ray diffractometry and electron microscopy have indicated that lysozyme and chymotrypsin are adsorbed primarily onto the planar surfaces of M, whereas ovalbumin is adsorbed primarily to the edges, with some adsorption onto the planar surfaces (1, 12, 24). The decreased adsorption of reovirus to M complexed with lysozyme, chymotrypsin, and ovalbumin probably resulted from the blockage of negatively charged sites on the clay by each protein. The importance of negatively charged (i.e., cation-exchange) sites in the adsorption of reovirus to clay minerals has been reported (21).

Lysozyme and ovalbumin were probably bound to K by mechanisms other than cation exchange, as changing the bulk pH from 3.13 to 9.75 had little or no effect on adsorption of these proteins (1). Inasmuch as the area of K occupied by lysozyme was determined to be half the surface area of K (24), McLaren and co-workers (23, 24) suggested that lysozyme adsorbed only on one of the faces of K particles (probably the hydroxyl face of the aluminum octahedron) by hydrogen bonding. Although lysozyme was net positively charged at the pH of these studies, its adsorption to K probably occurred by a mechanism other than cation exchange (1). The inability of lysozyme to block reovirus adsorption to K concurred, in part, with the proposed mechanism that adsorption of reovirus was primarily to negatively charged sites on K as a result of protonation of the virus (21), as competition between the virus and lysozyme for negatively charged sites on K did not apparently occur. Maximum adsorption of chymotrypsin to K occurred below the pI of the protein (24), indicating that the protein adsorbed to negatively charged sites on the clay. Consequently, the decreased adsorption of reovirus to K complexed with chymotrypsin may have resulted from blockage of negatively charged sites.

The molecular weight of the proteins appeared to affect the amount (titer) of reovirus adsorbed to K. The large globular ovalbumin molecule (molecular weight, 43,500 to 54,000) probably inhibited reovirus adsorption to K by blocking negatively charged sites, although the protein was probably not adsorbed by cation exchange but by van der Waals forces or H bonding (1). The lysozyme molecule (molecular weight, 13,930 to 17,200) was apparently too small to block adsorption of the reovirus to K. Blocking or clogging of exchange sites or positions on clays by organics has been reported. For example, Hendricks (15) showed that organic molecules with a diameter of >8 nm^2 and held by van der Waals and coulombic forces were able to cover more than one exchange position on M and, therefore, reduce the net exchange capacity of the clay. A relation between the molecular weight of ovalbumin, chymotrypsin, and lysozyme and the adsorption of reovirus to M is unclear, inasmuch as the reduced adsorption of the virus by these proteins to M probably resulted from the blockage of negatively charged sites on the clay (1, 12, 24).

Free chymotrypsin reduced the infectivity of reovirus in distilled water by approximately 85%, whereas chymotrypsin complexed with M did not affect infectivity. Virus

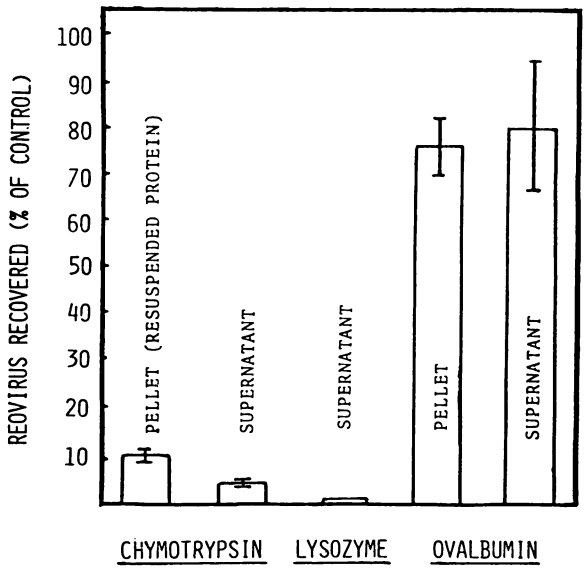

FIG. 4. Effect of 1% chymotrypsin, ovalbumin, or lysozyme on the infectivity of reovirus in distilled water. Chymotrypsin and ovalbumin control titer: $1.9 \times 10^5 \pm 0.18$ $TCID_{50}$/ml; lysozyme control titer: $1.1 \times 10^6 \pm 0.16$ $TCID_{50}$/ml. Mean titer ± standard error of the mean.

TABLE 3. Effect of pretreating L-929 cells in culture with 0.1%[a] chymotrypsin or lysozyme on the infectivity of reovirus

Protein	Infectivity titration ($TCID_{50}$/ml)	% Decrease in infectivity
Control	$4.7 \times 10^5 \pm 0.76$	
Chymotrypsin	$4.5 \times 10^5 \pm 0.30$	4.4 ± 0.43
Lysozyme	$2.8 \times 10^5 \pm 0.42$	41.3 ± 8.75

[a] 0.013% protein per well.

infectivity was decreased by free lysozyme by approximately 99% but by only approximately 38% when lysozyme was complexed with M. The lower inhibition of reovirus infectivity by lysozyme and chymotrypsin complexed with M was probably the result of the binding of the proteins to the clay, as the binding of some proteins to negatively charged surfaces (e.g., K, M, Celite) resulted in conformational changes in the proteins and, consequently, in altered biophysical activity. Evidence for this effect is suggested by the in vitro activation of the Hageman factor (serum glycoprotein factor XII) and of high-molecular-weight kininogen by K (6, 16) and by the enhanced activity of catalase when bound to M (33). Electrostatic attraction between the net positively charged proteins in distilled water in the absence of M and the net negatively charged reovirus probably also prevented contact of the virus with the host cells, thereby decreasing the infectivity titer.

The enhanced infectivity of the reovirus in the presence of ovalbumin may have been related to the low pI (4.6 to 4.7) of the protein. At neutral pH, both ovalbumin and reovirus have a net negative charge, and the electrostatic repulsive forces may have dispersed aggregates of the virus.

The pI of animal cells occurs at a pH of approximately 2.3 (3). Therefore, these cells had a net negative charge in Hanks minimal essential medium (pH 7.3), whereby lysozyme (pI = pH 11.0 to 11.2) and chymotrypsin (pI = pH 8.1 to 8.6) existed as net positively charged protein molecules. The adsorption of lysozyme to L-929 cells by electrostatic forces of attraction probably blocked some receptor sites necessary for virus adsorption. Chymotrypsin did not significantly inhibit virus infectivity, perhaps because the pI of chymotrypsin is only approximately 1 pH unit above that of the Hanks minimal essential medium and the amount of chymotrypsin adsorbed to the negatively charged cells was less than that of lysozyme. Ovalbumin, because of its net negative charge, did not apparently interact with the L-929 cells.

Conclusion. Chymotrypsin and ovalbumin reduced the adsorption of reovirus to K and M homoionic to Na, and lysozyme reduced adsorption to M but not to K. Each protein (except lysozyme bound to K) probably competed with the virus for adsorption sites on the clays. The reduction of reovirus adsorption to K and M was related to either the molecular weight of the protein or the adsorption of each protein to cation-exchange sites on the clays. Chymotrypsin and lysozyme markedly reduced the infectivity of reovirus in distilled water, whereas this effect was less pronounced when the proteins were bound to M, suggesting that the biophysical characteristics of the proteins that were responsible for reducing viral infectivity were altered at the clay-protein-virus interface.

The type and size of microbial populations differ in various soils and most aquatic ecosystems, and, therefore, variations in the type of proteinaceous cellular metabolites (e.g., enzymes, antibiotics) would be expected to occur in these environments. The infectivity of reovirus, and possibly of other human enteric viruses, may reflect their association with these and other proteins in the water column and at the clay-protein-virus interface. The data presented in this study support this suggestion.

ACKNOWLEDGMENTS

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