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Ionic Bonding, the Mechanism of Viral Uptake by Shellfish Mucus

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An investigation was conducted to determine the processes involved in the contamination of shellfish by viruses. Results of binding-release studies show that the process involves the attachment of viruses to mucus secreted, and then ingested, by shellfish during feeding. Analysis of the mucus-virus bond involved selective degradation of the mucus and use of chemical agents to block carboxyl and sulfate groups on the mucus. Results obtained indicate that the attachment of virus to mucus is primarily ionic and involves the binding of viral particles to sulfate radicals on the mucopolysaccharide moiety of shellfish mucus.

Bivalve molluscs obtain their food via a process termed filter feeding, which permits them to selectively ingest small particles of organic matter sieved from large volumes of seawater. This method of feeding depends upon entrapment of potential food particles in mucus sheaths or strands secreted continuously by the shellfish as they pump water. The chemical composition of this mucus is essentially similar in all species of bivalves (15). The stringlike masses of food material and mucus are directed by ciliary action to the mouth region of the shellfish, where sorting takes place. According to its nature, the accumulated material may be swept, via ciliary action, into the mouth or along rejection paths to the exterior, where it is eliminated as pseudofeces (5). To date, it has not been clearly resolved whether the entrapment of organic matter is due to a netlike structure of the mucus or to ionic phenomena (8).

Although they are not a nutrient source for shellfish, microbial entities, including pathogenic bacteria and viruses, become enmeshed in the mucus and are ingested (1, 2, 4). Among the viruses accumulated is the agent of infectious hepatitis, and a number of outbreaks of infectious hepatitis caused by eating contaminated shellfish have been reported (10). Studies have shown the uptake of viruses by bivalves to be dynamic with high titers of virus being accumulated in relatively short periods of time (2, 11). In one investigation Liu et al. (11) reported that, under experimental conditions, 70% of the poliovirus 1 present in contaminated seawater was accumulated by the mucus of Northern Quahaugs (*Mercenaria mercenaria*) in a 48-h period. Di Girolamo et al. (3) worked with West Coast oysters and reported rapid uptake of poliovirus 1 by these shellfish. These investigators reported an 80 to over 90% uptake of virus in a 24-h period depending upon species. In other studies, Di Girolamo (unpublished data) worked with other members of the enterovirus group (i.e., echovirus and coxsackie B virus), and observed that these were also accumulated on shellfish mucus, but not at the same levels as poliovirus 1.

Because of the rapid, apparently dynamic nature of viral uptake by shellfish, it is important that the mechanism involved by determined. Understanding the process of uptake is a necessary step toward developing effective means of eliminating viruses from shellfish. Therefore, we conducted an investigation to determine the mechanism by which one viral group, the enteroviruses, is acquired by shellfish from contaminated seawater. This paper presents the findings of that study.

MATERIALS AND METHODS

Shellfish mucus. Mucus (as pseudofeces) was obtained by placing six Pacific oysters (*Crassostrea gigas*), ten Olympia oysters (*Ostrea lurida*), or eight Northern Quahaugs (*M. mercenaria*) in each of three glass aquaria containing 3 liters of aerated, filtered seawater (salinity, 28‰; temperature, 13°C). To each aquarium was added 50 ml of a 1% hypochlorite solution. The hypochlorite served to irritate the shellfish causing them to produce abundant mucus as pseudofeces. The mucus contained considerable chlorine which prevented bacterial degradation of pseudofeces at the bottom of the aquaria. This mucus was harvested at 24-h intervals over a 10-day period. The mucus was washed free of chlorine with filtered seawater and then stored in sterile test tubes at 4°C. Storage at this temperature was considered necessary to prevent possible chemical or microbiological degradation.

Shellfish used for pseudofeces (mucus) production were depurated in filtered seawater for 48 h prior to use. To prevent mortalities, no shellfish was allowed to go without feeding for more than 12 days.

Tissue culture. Primary African green monkey kidney cell cultures were used throughout experiments. Cell suspensions were purchased from Baltimore Biological Laboratories. Hanks balanced salt solution containing 0.2% Casamino Acids and 10% calf serum was used for cell growth, and medium containing Earl balanced salt solution was used for cell maintenance.

Plaque assays. The basic method used was that of Hsiung and Melnick (7). Two tissue cultures were inoculated for each dilution of all samples tested. Plaque counting was done each third, fourth, and fifth day after inoculation.

Virus uptake by shellfish mucus. To assay for virus uptake, an approximate 3% suspension of mucus was prepared by weighing out 1 g of pseudofeces and diluting this with 30 ml of filtered seawater. The solution was homogenized for 2 min on a Lourdes homogenizer at 6,500 rpm. Five 3-ml portions of the homogenates were withdrawn, and equal volumes of the test virus (poliovirus 1) diluted in seawater were added. Virus concentrations ranged from 10^2 to 10^5 virus plaque-forming units (PFU)/ml. The portions were incubated at 5°C for 75 min; tubes were agitated at 5-min intervals to insure virus attachment. After incubation, the supernatants were aseptically decanted, and the sediments were suspended in 3 ml of nutrient broth. Serial decimal dilutions in nutrient broth were prepared, and samples were assayed as described above. Nutrient broth was used as a diluent because it has been shown to cause viral deaggregation (6) and, hence, it facilitated recovery of virus from sediments. In determining virus uptake, titers of virus recovered from sediments were compared to those of the control virus suspensions.

Difference in uptake of enteroviruses by shellfish mucus. To determine if any difference existed in the accumulation of enteroviruses, the uptake of four viruses by shellfish mucus was studied. The viruses chosen represented the four subgroups of human enteroviruses. The model viruses were poliovirus 1, coxsackie A-9, coxsackie B-4, and echovirus 1. All experiments were conducted as described above.

Timed binding studies. A study was conducted to determine how rapidly viruses would bind to mucus and whether this binding was a continuous process or if, in time, it reached a saturation level. Samples (3 ml each) of 3% mucus homogenates were pipetted into each of five 20-ml sterile test tubes. To each was added an equal volume of a virus suspension in nutrient broth. The five virus dilutions used contained 10^2, 10^4, 10^6, 10^7, and 10^8 virus PFU/ml, respectively. To avoid possible degradation of the mucus, samples were incubated at 5°C for 5, 15, 30, 45, 60, and 75 min. Supernatants were aseptically decanted, sediments were resuspended in nutrient broth, serial dilutions were prepared, and samples were assayed. Percentages of uptake were calculated as described previously.

Timed release studies. A study was conducted to determine how rapidly accumulated viruses could be released, under proper conditions, from shellfish mucus. Samples of mucus homogenate in seawater were prepared as described elsewhere, mixed with an equal volume of virus suspended in nutrient broth, and then incubated for 1 h at 5°C. The viral suspension contained approximately 5.0×10^4 virus PFU/ml. After incubation, the supernatant was decanted and replaced with filter-sterilized seawater; 1 ml of this was withdrawn and assayed for virus content, and this constituted the 0-h sample. The mucus was then reincubated and assayed at 30-min intervals for 18 h.

Effect of salinity on the binding of virus. Seawater, the milieu in which shellfish live, is essentially a complex salt solution containing many mono- and divalent cations. The following study was conducted to determine if variations in seawater salinity and, hence, concentration of cations, have an effect on the adsorption of virus by shellfish mucus. Homogenates of mucus (3%) were prepared as in previous studies, using however, high- or low-salinity seawater. The seawater had salt concentrations of 2, 7, 14, 28, 50, and 55‰. High-salinity samples were prepared by mixing appropriate amounts of a synthetic salt mixture (Aqua-Marin Salts, Aquatrol Co., Anaheim, Calif.) with seawater of 28‰ salinity. Low-salinity samples were prepared by diluting 28‰ seawater with sterile, deionized water. All salinities were determined with an H2B hydrometer. Portions of each homogenate (1 ml) were mixed with an equal volume of a poliovirus 1 suspension prepared by using water of the same salinity as the homogenate. Samples were incubated for 1 h at 5°C; then the supernatants were aseptically decanted, and the sediments were resuspended in nutrient broth. Serial decimal dilutions in nutrient broth were prepared, and the samples were assayed.

Effect of pH on the binding and release of viruses by shellfish mucus. Each group of enteroviruses has a definite pH range at which adsorption or binding to receptor sites on susceptible host cells takes place; above and below this range, virus expression is repressed (R. L. Crowell, Bacteriol. Proc., p. 180, 1968). Although nonliving, molluscan mucus is a chemical substance of complex organic nature (15). Therefore, a study was conducted to determine if the effect of pH on the binding of viruses by shellfish mucus could, in any way, be analogous to the adsorption of viruses to receptor sites on living cells. Five 3% mucus homogenates were prepared using seawater, and then 3 ml of each homogenate was placed in 20-ml test tubes. The pH of each homogenate was adjusted with either 0.1 N HCl or 0.1 N NaOH so that the homogenate samples had pH readings of 3, 6, 7.5, 9, and 10, respectively. To each tube of homogenate was added an equivalent amount of a poliovirus 1 suspension in seawater at the same pH as the homogenate. The homogenates in the test tubes were then incubated and assayed.

To determine the effect of an acid environment of virus release, five 3% mucus homogenates were prepared and mixed with an equivalent amount of

poliovirus in seawater and then incubated. After incubation, the seawater in four of these homogenates was aseptically decanted and replaced with seawater of pH 7.0, 6.0, 5.0, and 3.0, respectively. The fourth sample, of pH 8.0, served as a control. Samples were assayed after incubation for 1 h at 5°C.

Chemical digestion studies. To determine if there was a specific moiety in shellfish mucus to which viruses could bind, the protein, lipid, and mucopolysaccharid fractions of mucus were selectively degraded by chemical means. Four 1-g samples of mucus homogenate were used per study. One was digested with 0.25% trypsin for 1 h at 37°C, another was digested with a 50% solution of diethyl ether, the third was hydrolyzed with 0.1 N HCl for 12 h at 37°C, and the fourth was hydrolyzed with 0.01% testicular hyaluronidase (pH 5.0) for the same length of time. All treated mucus was washed free of residual chemicals in filtered seawater until a pH of 7.5 to 8.0 was obtained. Portions (3 ml each) were mixed with an equal volume of poliovirus 1 in seawater containing 10^4 virus PFU/ml. These were incubated, and then samples were withdrawn and assayed as described previously.

Effect of Astro-blau on virus binding. Astro-blau is a thalocyanin derivative that reacts specifically with the carboxyl and sulfate groups of acid and sulfated acid mucopolysaccharides at pH 5.0, forming a blue precipitate (12). Viruses are known to bind to sulfated acid mucopolysaccharides (16), and such mucopolysaccharides have been shown to be constituents of shellfish mucus (9). Consequently, binding of carboxyl and sulfate groups of this mucopolysaccharide moiety should substantially lower virus binding (adsorption) by shellfish mucus. The following experiment was conducted to test this hypothesis. Thirty milliliters of a mucus homogenate was mixed with an equal volume of an Astro-blau solution containing 0.2 mg of Astro-blau per ml. The mixture was held at 25°C for 1 h and then centrifuged at 2,000 rpm for 15 min. The supernatant was decanted and replaced with filter-sterilized seawater; this procedure was repeated until all unbound Astro-blau was removed. Three 3-ml samples of the homogenate were withdrawn and placed in sterile 20-ml test tubes to which were added equal volumes of a poliovirus 1 suspension in filtered seawater containing 10^4 virus PFU/ml. Samples were incubated and assayed as described in preceding studies.

Effect of CPC on virus uptake. Cetyl pyridinium chloride (1-hexadecyl pyridinium chloride [CPC]) has been shown to bind ionically to sulfate radicals (13). Hence, if viruses were binding to such groups, blocking these receptor sites with CPC should greatly repress the uptake of viral particles. To determine this, the following study was performed. To five 3-ml portions of mucus homogenate were added equal volumes of an aqueous solution containing 2 mg of CPC per ml. The samples were centrifuged at 1,200 rpm for 20 min, and then the supernatants were decanted and replaced with 3 ml of filter-sterilized seawater. To each sample was added 3 ml of a poliovirus suspension in seawater containing 10^4 virus PFU/ml. To test the effect of CPC on poliovirus, equal amounts of the stock CPC solution were added to each of the five 3-ml samples of the poliovirus suspension. Control samples consisted of virus-homogenate mixtures minus CPC. Samples were then incubated and assayed.

RESULTS

Virus uptake by shellfish mucus. The results of this study are shown in Table 1. Poliovirus was accumulated by the mucus at all viral titers tested. Uptake averaged approximately 65.5% of the total virus inoculated per milliliter.

The hypothesis that simple entrapment does not explain the binding of all particulate matter especially of viral particles, by shellfish mucus is supported by studies showing the binding of the four model enteric viruses (Table 2). A definite gradient was observed in the binding of these viruses by mucus; on the average, 68% of the poliovirus, 63% of the coxsackie A-9, 56% of the coxsackie B-4, and 38% of the echovirus inoculated per milliliter were accumulated by the mucus. These viruses were of the same size, and each serotype displayed differences in spontaneous aggregation among its various strains. Consequently, the observed differences in uptake must be due to some phenomenon other than entrapment.

Timed binding studies. The results of timed binding studies are shown in Fig. 1 and 2. Viruses were rapidly bound by shellfish mucus

TABLE 1. *Uptake of poliovirus by shellfish mucus.*

Amt of poliovirus added to mucus homogenate (PFU/ml)	Amt of poliovirus bound by shellfish mucus (PFU/g)	Virus uptake by shellfish mucus (%)
5.8×10^5	3.8×10^5	65
4.8×10^4	2.8×10^4	60
9.0×10^3	5.8×10^3	64
9.0×10^2	6.6×10^2	72

TABLE 2. *Accumulation of four representative enteric viruses by shellfish mucus*

Virus sample	Control virus titer	Amt of virus bound by mucus (PFU/g)	Virus uptake by shellfish mucus (%)
Poliovirus 1 (1sc-2ab)	1.8×10^4	1.2×10^4	68.0
Coxsackie A-9	1.6×10^4	1.0×10^4	53.0
Coxsackie B-4	6.3×10^4	3.5×10^4	53.0
Echo 1	1.5×10^4	5.7×10^3	38.0

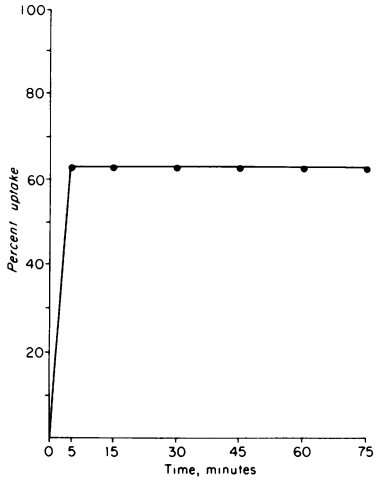

FIG. 1. *Uptake of virus by mucus during incubation at 5°C for 75 min.*

(Fig. 1), with binding reaching a maximum (4.4 × 10^4 virus PFU/g) after only 5 min of incubation. Incubating samples for longer periods did not increase binding. Under experimental conditions, the percentage of the virus bound appeared to be a function of the original virus titer (Fig. 2). The higher the original titer, the lower the percentage of virus bound (41% virus binding at a titer of 10^8 virus PFU/g as opposed to 87% binding at a titer of 10^2 virus PFU/g). However, there are a number of possible explanations for these results (e.g., aggregation, technical manipulation, etc). Consequently, this phenomenon is undergoing further study.

Timed release studies. The results of timed release studies are reported in Fig. 3. There was a rapid initial decline in virus titer, with only 10^4 virus PFU/g remaining after 90 min of elution. This period of rapid elution was followed by a longer period of very gradual release, so that 5.0 × 10^3 virus PFU/g were still recovered from the mucus after 18 h, at which time the experiment was terminated.

Salinity studies. The results of salinity studies are summarized in Fig. 4. The binding of virus appeared to be inversely proportional to the salt content of the seawater. A decrease in salinity from 28 to 12% produced a 20% increase in viral binding (1.2 × 10^4 to 1.6 × 10^4 virus PFU/g). Conversely, an increase in salinity to 45% produced a 15% reduction in poliovirus binding. Attempts to determine the effect of salinities above 45% were hampered by what appeared to be cytotoxic effects of these solutions on tissue monolayers.

pH studies. It appears from these studies (Fig. 5) that pH has a direct influence on the binding of viruses by shellfish mucus. Under our experimental conditions, maximum binding of virus (1.6 × 10^4 virus PFU/g) occurred at a pH of 7.5, with acid or alkaline solutions tending to reduce binding.

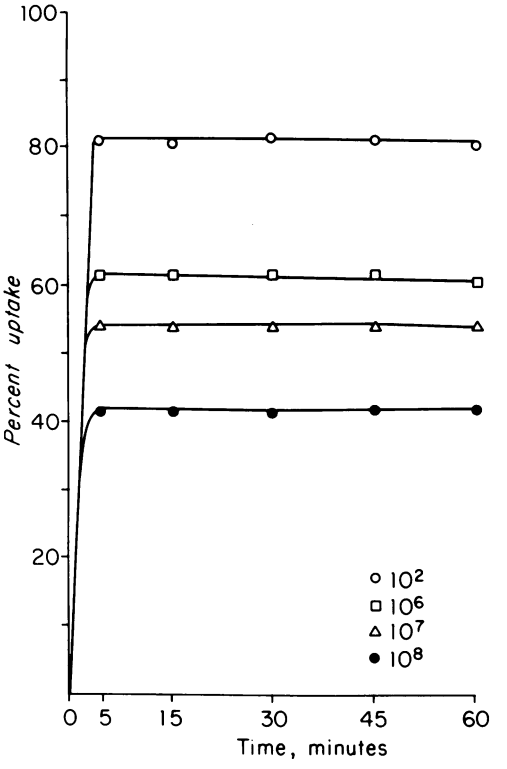

FIG. 2. *Effect of time on the binding of high and low titers of poliovirus by shellfish mucus.*

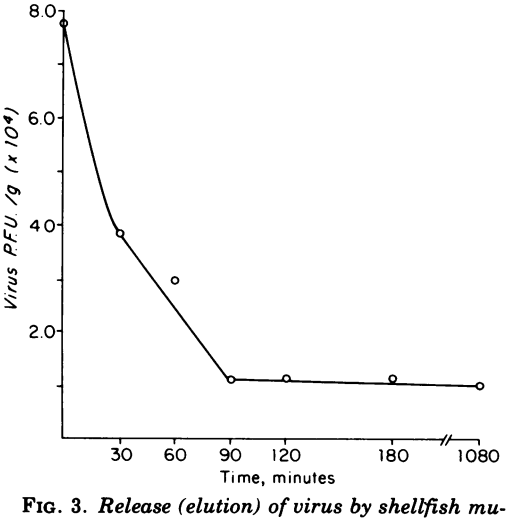

FIG. 3. *Release (elution) of virus by shellfish mucus.*

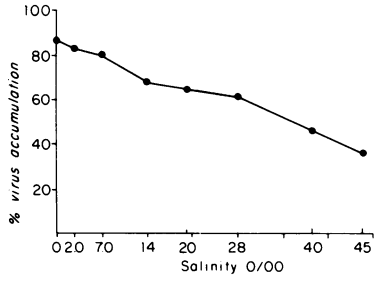

FIG. 4. *Effect of salinity on the binding of poliovirus by shellfish mucus.*

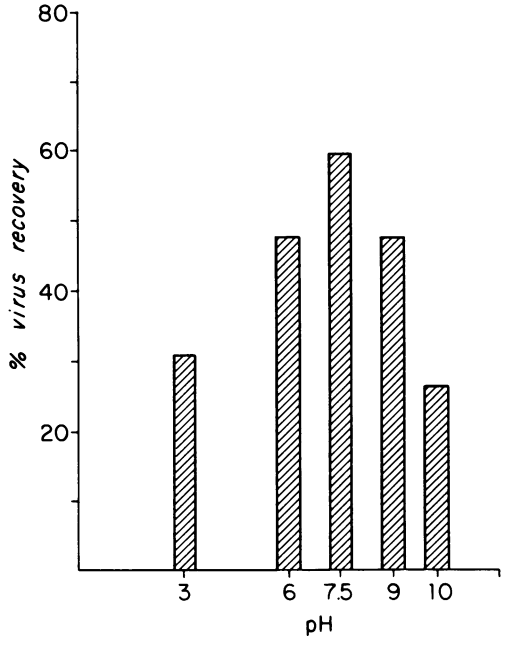

FIG. 5. *Effect of pH on the binding of poliovirus to shellfish mucus.*

The results of these pH studies also indicate that acidification of mucus triggers the release of bound virus in almost direct proportion to the pH change (Table 3). On the average, changing of pH of mucus from 7.0 to 6.0 produced a 10.5% release of virus; further acidification to pH 5.0 produced approximately a 26% release of virus.

Elution of virus was found to be more rapid in seawater to pH 5.0 than at 8.0 (Fig. 6). Better than 70% of the virus (3.0×10^4 virus PFU/g) was released from mucus after 30 min of elution at pH 5.0 compared to the same approximate release after 60 min of elution at pH 8.0.

Chemical digestion studies. The results of chemical digestion studies are summarized in Table 4. Treating the mucus with either trypsin or ethyl ether had no effect upon the binding of virus, suggesting that the compound responsible for viral attachment is neither a protein nor a lipid. However, a marked decrease in binding was noted when mucus was treated with either 0.1 N HCl or hyaluronidase. On the average, virus accumulation was reduced 50 to 60% in HCl-treated samples and 70 to 80% in enzyme-digested mucus.

Astro-blau studies. The results of Astro-blau studies are shown in Table 5. Under experimental conditions, reacting shellfish mucus with Astro-blau produced a definite reduction in virus binding. Accumulation dropped from 6.5×10^3 to 1.2×10^3 virus PFU/g, a reduction of better than 80% in actual binding.

CPC studies. The results of CPC studies are presented in Table 6. Reacting CPC with shell-

TABLE 3. *Effect of pH on the release of viruses from shellfish mucus*

Sample	pH of sample	Amt of virus bound by mucus at each pH level (PFU/g)	Virus bound by mucus (%)
Control virus suspension	7.0	1.2×10^4	0
Mucus homogenate	8.0	7.2×10^3	62.0
Mucus homogenate	7.0	7.4×10^3	53.0
Mucus homogenate	6.0	6.2×10^3	52.5
Mucus homogenate	5.0	5.6×10^3	47.0
Mucus homogenate	3.0	3.6×10^3	31.0

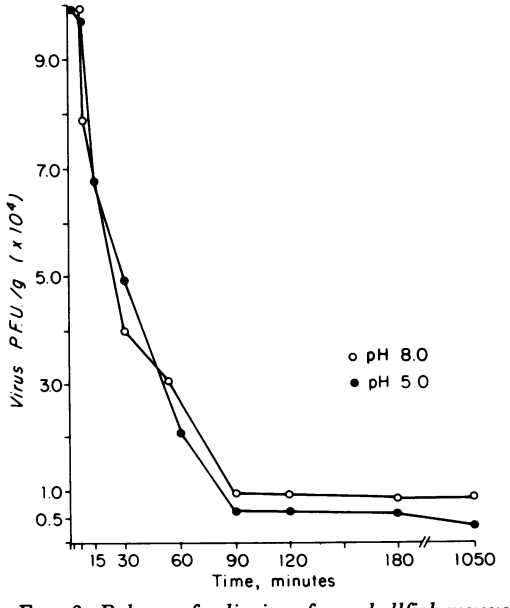

FIG. 6. *Release of poliovirus from shellfish mucus at pH 5.0 and pH 8.0.*

TABLE 4. *Effect of chemical digestion on the uptake of virus by shellfish mucus*

Sample	Treatment	Virus PFU/g or ml	Virus uptake (%)
Control virus	None	1.0×10^4	0
Control virus and shellfish mucus	Untreated	5.9×10^3	69
Shellfish mucus	Trypsin digestion	5.7×10^3	65
Shellfish mucus	Ethyl ether digestion	5.9×10^3	69
Shellfish mucus	Hydrolysis with 0.1 N HCl	3.7×10^3	35.2
Shellfish mucus	Digestion with 0.01% hyaluronidase	1.5×10^3	17.5

TABLE 5. *Effect of Astro-blau treatment on the binding of poliovirus to shellfish mucus*

Sample	Recovery of poliovirus from shellfish mucus (PFU/g)	Poliovirus recovery (%)
Control virus	1.0×10^4	0
Untreated mucus homogenate	6.5×10^3	65.0
Astro-blau-treated mucus	1.2×10^3	12.0

TABLE 6. *Effect of CPC treatment on the binding of poliovirus to mucus*

Sample	Recovery of poliovirus from mucus (PFU/g)	Recovery of poliovirus (%)
Control virus	1.0×10^4	0
Virus and untreated mucus	6.4×10^3	64.0
Virus and CPC-treated mucus	7.0×10^2	7.0

fish mucus produced an almost 1-log reduction in uptake from 6.5×10^3 to 7.0×10^2 virus PFU/g. This represents a reduction of about 90% in uptake.

DISCUSSION

The results of uptake studies indicate that viral uptake is dynamic, and that high titers of virus can be accumulated by shellfish mucus in a short period of time. Uptake studies with selected enteric viruses also suggest a possible gradient in virus accumulation. Since shellfish feed by trapping matter in mucus sheaths, it is obvious that entrapment in mucus is the principle way by which shellfish acquire viruses and become contaminated. These findings also suggest that viral uptake could be due to some mechanism other than simple entrapment.

This hypothesis, that viral binding and uptake is due to some means other than entrapment, is supported by the results of timed binding and release (elution) studies. Under experimental conditions, maximum uptake of viruses occurred within 5 min after exposure. However, timed release studies show that the binding reaction is reversible. The percentage of virus was greatest at lower, not higher, concentrations, suggesting adsorption to some specific site. It is also important to determine if these results were due to saturation of binding sites or to an equilibrium reaction. These possibilities are currently under investigation, and results will be reported at a later date.

Considered as polyelectrolyes, virus particles could be accumulated by shellfish mucus in at least five ways: (i) mechanical entrapment; (ii) direct chemical bonding; (iii) Van der Waal bonding; (iv) H^+ ion bonding; and (v) ionic bonding.

Since the results of timed release studies show that the reaction between virus and mucus can be reversed, mechanical entrapment and direct chemical bonding seem to be unlikely mechanisms of attachment, because both these forms of bonding reverse only with great difficulty. However, the fact that there is a relatively firm bond produced between virus and mucus, as indirected by uptake studies, makes Van der Waal bonding appear to be unlikely as the sole means of attachment. However, this type of bonding could be important if viruses are present in the environment in unusually high titers. Thus, results of these studies suggest that attachment of viruses to mucus is due to either ionic or H^+ ion bonding.

The weakening of the virus-mucus bond by increases in ionic concentrations or alteration of pH, as shown in salinity and pH experiments, makes H^+ ion seem unlikely as the sole or major means of viral attachment to mucus. Thus, it was observed that decreasing salinity of a mucus homogenate from 28 to 14‰ caused a 10% increase in viral accumulation. These findings show that cation concentration has a definite effect on virus adsorption by mucus and suggest that this effect is due to competition between cations and viral capsid coats for anions present on the mucus. These findings also indicate that seawater salinity will have an

effect upon uptake of viruses by feeding shellfish. Other workers have also published results considering the effect of salinity (and pH) on the uptake of viruses by shellfish (14).

The results of pH studies make it evident that virus uptake by shellfish mucus is influenced, to some extent, by H^+ ion concentration. Thus, increasing the H^+ ion concentration of mucus by decreasing the pH from 7.5 to 3.0 produced a 55% reduction in virus binding. From these studies it is also obvious that acidification of mucus causes the release of bound viruses in almost direct proportion to the change in pH. These results are significant since they suggest the possibility that the acid pH of the oyster digestive tract could trigger the release of some viral particles into the lumen of the stomach and gut.

Strong supportive evidence for the hypothesis that ionic bonding is involved in the attachment of viruses to shellfish mucus is presented by the results of chemical digestion and binding studies.

From the results of chemical digestion studies it appears that virus adsorption is probably associated with the acid mucopolysaccharide moiety of mucus and, specifically, with hyaluronic acid which, according to Thomas (17), exists as hyaluronasulfate in mucoid compounds. Under test conditions, virus uptake by mucus was reduced 50% after treatment with 0.1 N HCl and over 70% after enzymatic digestion with hyaluronidase.

The results of chemical binding studies show clearly that viruses bind specifically to certain anions present in the mucus. Blocking the sulfate and carboxyl groups of the acid mucopolysaccharide with Astro-blau produced approximately a 75% reduction in virus uptake. Uptake was reduced by 90% when the mucus was treated with CPC, which complexes specifically with sulfate radicals.

Therefore, the results of the experiments described in this investigation support the hypothesis that the principle mechanism of viral attachment to shellfish mucus is via ionic bonding. The most likely site of attachment appears to be sulfate radicals in the mucopolysaccharide fraction of the mucus. This finding is important because it offers a means by which to explain, and possibly quantitate, the mechanism of viral uptake and depuration by shellfish. These findings also suggest that the binding of viruses to mucus is similar to the first step in attachment of viruses to host cells, the reversible ionic bonding of the virus to the host cell membranes.

These studies are significant because they indicate that there are a number of avenues still to be explored in understanding the mechanisms of viral uptake and depuration by shellfish. It is important to have some idea as to the number of sulfate radicals available to bind viruses and to identify the compound with which these radicals are associated. Uptake of viruses by such means as Van der Waal bonding and binding to carboxyl groups should be explored. Clarification is also needed as to the possible nature of virus-mucus exchange in the digestive tract of shellfish and of the means by which viruses penetrate into the bodies of these animals.

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