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Virus Accumulation by the Rock Oyster *Crassostrea glomerata*

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The accumulation of virus by the New Zealand rock oyster *Crassostrea glomerata* has been studied in a static seawater system using radioactively labeled reovirus type III and Semliki Forest virus. The uptake of virus was found to be less rapid than for the bacterium *Escherichia coli* and to be unaffected by the presence of the marine alga *Dunaliella primolecta* in the seawater. Accumulation was dependent on virus concentration, with saturation achieved at 4×10^{10} reovirus particles per oyster, implying that an oyster possesses a large but finite number of sites for virus adsorption. When the rates of uptake of two viruses of similar size but differing surface properties were compared, the rate of accumulation of the lipoprotein-enveloped Semliki Forest virus was found to be less than that for the protein-enclosed reovirus. This observation, together with the finding that the oyster shell has a strong affinity for virus, suggests that surface properties, rather than size, are the principal factors governing the accumulation of viruses by filter-feeding marine bivalves.

The importance in the human diet of many common species of filter-feeding bivalves and their demonstrable role in the initiation of outbreaks of infectious disease have resulted in a number of studies being carried out on the processes involved in the accumulation of viruses by shellfish. Many of these studies have been carried out using the commercially important hard clam *Mercenaria mercenaria* (5), the Eastern oyster *Crassostrea virginica* (17), or the Northern quahaug (4). The technical difficulties involved in detecting or quantitatively measuring the virus in large volumes of water, or in the tissues of individual specimens, have hampered this work.

Some of these difficulties have been overcome in the work we report in this communication, by the use of highly purified radioactive virus labeled with [^{32}P]orthophosphate or [^{3}H]uridine. The use of radioactive virus enables the uptake of virus particles to be measured easily and the specific infectivity of virus to be measured by relating the number of particles present to the plaque-forming units in the same sample.

The edible New Zealand rock oyster *Crassostrea glomerata* is particularly suited to this type of study since it survives out of water over long periods of time and will readily filter water under the static conditions and relatively small water volumes necessary for a study of this type. Conventional double-isotope counting techniques have enabled the kinetics of accumulation of reovirus type III to be related to the rate of accumulation of both the bacterium *Escherichia coli* and the lipoprotein-enveloped togavirus Semliki Forest virus (SFV). The results obtained suggest that surface properties, rather than size, play an important role in determining the extent to which particular types of virus particles are accumulated by filter-feeding marine bivalves.

MATERIALS AND METHODS

Oyster samples and seawater. Oysters were obtained from local commercial sources within 48 h of harvest. Before use, the shell exterior was cleaned by vigorous scrubbing with a hard brush to remove mud and fouling organisms. Seawater was collected at the Leigh Marine Laboratory of the University of Auckland and was allowed to settle in large plastic drums. The water was then subjected to pressure filtration through a Gelman Preflow 400 prefilter (10-μm pore size) followed by a 0.45-μm Sartorius cellulose nitrate membrane filter. The filter-sterilized water was stored in sealed bottles at 4°C before use.

Virus, bacteria, and algae. Reovirus type III (Dearing strain) was propagated in mouse L cells and purified by zone sedimentation on sucrose gradients and isopycnic sedimentation on CsCl gradients as described previously (3, 21). Radioactive reovirus was prepared by including 10 μCi of [^{32}P]orthophosphate per ml in phosphate-deficient culture medium.

SFV was propagated in mouse L929 cells or chicken embryo fibroblasts and purified from the supernatant culture fluid by the method of Kennedy and Burke (16). The concentration of reovirus was determined using the relationship 9.1 absorbancy units at 260 nm = 1 mg/ml, and the particle number was determined from the relationship 4.6×10^{12} particles = 1 mg (8).

The concentration of SFV was determined by plaque assay, and a plaque-forming unit-particle ratio of 1:100 was assumed.

E. coli strain PB28 was obtained from P. L. Bergquist of this department and had the genotype F^- DNA_{ts} *thr leu lac thy* Sm^r B_1^-. The bacteria were labeled with [^{3}H]thymidine by propagation at 32°C in the medium of Adelberg and Burns (1). The washed pellet of bacteria was resuspended at 10^9 cells per ml and stored at −90°C in a buffered salt solution containing 30% (vol/vol) glycerol which is known to preserve the physical integrity of these cells (P. L. Bergquist, personal communication).

The marine green alga *Dunaliella primolecta* was provided by M. Barker, Zoology Department, University of Auckland, and was propagated in the medium of Guillard (11).

Radioactivity measurements. ^{32}P radioactivity present in seawater samples or oyster homogenates was measured by counting Cerenkov radiation in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. Other aqueous samples that contained ^{3}H or ^{125}I and/or ^{32}P radioactivity were diluted before counting with 10 volumes of a water-miscible scintillation fluid composed of equal volumes of a toluene-based scintillation fluid [300 mg of 2,5-diphenyloxazole (PPO) and 30 mg of 1,4-bis-(5-phenyloxazolyl)benzene per liter (POPOP)] and Triton X-100.

Preparation of ^{125}I-labeled bovine serum albumin. The lactoperoxidase procedure of Philips and Morrison (20) was used. The iodinated bovine serum albumin was extensively dialyzed against filtered seawater to remove unincorporated iodine and stored at 4°C before use.

Accumulation studies. A standard sample of three oysters was incubated, left valve downward, in 600 ml of seawater contained in a 4-liter polypropylene beaker. A 5.0-cm, Teflon-coated magnetic stirrer in the center of each beaker was operated at approximately 400 rpm to provide both aeration and circulation of the seawater. All experiments were performed at room temperature (19 ± 1°C) and oysters were preequilibrated in this system for 1 h before use. Filtering commenced within the first 10 to 15 min and continued in an essentially uninterrupted fashion for 30 to 40 h. Those oysters that did not commence filtering during the equilibration period were discarded.

After uptake of radioactive materials, the oyster tissue was removed from the shell and homogenized in seawater (VirTis "45," half-speed, 5 min), and the radioactivity present in homogenates was measured. Radioactivity present on the beaker walls and oyster shells was eluted with a measured volume of boiling 0.1% sodium dodecyl sulfate. Samples of the residual seawater were filtered through 2.5-cm Whatman GF/C glass fiber filters to collect feces and pseudofeces.

RESULTS

Preliminary experiments on virus accumulation. A series of preliminary experiments was carried out to establish some basic parameters for the experimental uptake system used in this study. The ability of the oysters to filter actively and remove suspended material from solution was monitored by the neutral red method of Cole and Hepper (6) and Badman (2). It is clear that the oysters are able to clear suspended material from the seawater rapidly (Fig. 1A).

However, factors other than the metabolic activity of the bivalve caused suspended material to be removed from solution: this became evident when virus was added to beakers containing only seawater (Fig. 1B). Progressive removal of virus from suspension occurred which was found to be independent of the type of container used. This removal resulted from irreversible adsorption of virus to the walls of the container but was reduced to a negligible level by the addition of bovine serum albumin to the seawater to a concentration 25 times that of the virus.

Similar losses of radioactivity by adsorption to the container walls were observed when ^{3}H-labeled *E. coli* was added to unfiltered seawater (Fig. 1C): these losses were eliminated when the seawater was filter sterilized before use.

Accumulation of reovirus. When radioactive reovirus was added to seawater that contained oysters, approximately half the virus was removed within the first 24 h (Fig. 2A).

To distinguish accumulation that resulted from the filtering activity of the oyster from passive adsorption to the oyster tissue or shell, a similar experiment was carried out using oysters killed by immersion in liquid nitrogen (Fig. 2B).

It is clear from Fig. 2B and Table 1 that substantial quantities of virus were removed from suspension by nonfiltering oysters as a result of adsorption of the virus both to the shell and, to a lesser extent, to the oyster tissue. The accumulation of virus as a result of active filtration therefore represented only approximately half the total removed from suspension, the most important non-accumulative losses resulting from adsorption to the shell.

Accumulation of reovirus under different experimental conditions. A series of experiments in which the uptake of reovirus was studied under differing experimental conditions revealed that uptake was not greatly affected by temperature between 15 and 25°C (data not shown) but was dependent on the virus concentration (Table 2). Saturation was achieved at approximately 8 μg of virus per oyster tissue equivalent (ca. 4×10^{10} particles). Above this value no further virus was associated with the oyster tissue, implying that each oyster possesses a limited number of adsorption sites for reovirus. In contrast, saturation was not reached for adsorption of virus onto the shell over the 100-fold range of virus concentration studied.

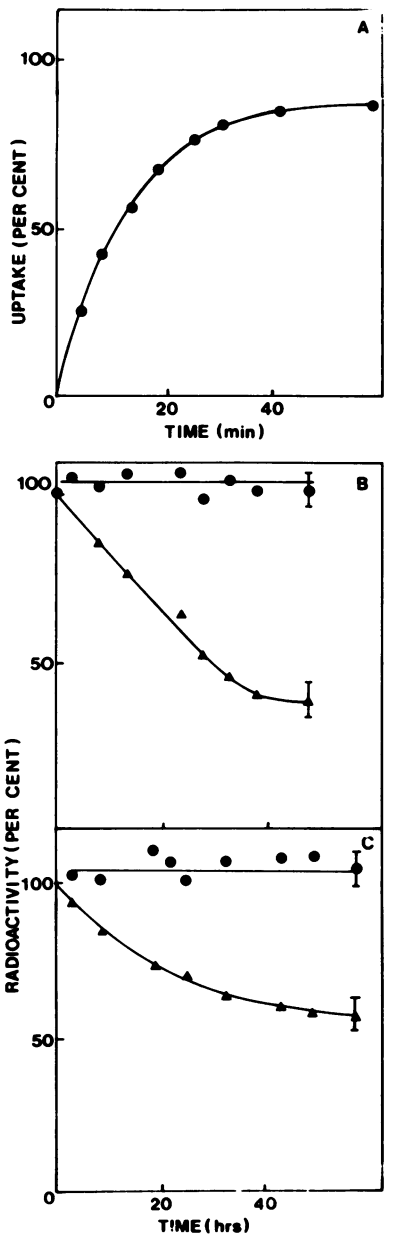

FIG. 1. *Preliminary experiments on the accumulation of radioactive microorganisms in the static seawater system. (A) Rate of clearance of neutral red from suspension by C. glomerata. The initial concentration of neutral red was 0.2 absorbancy unit at 300 nm/ml. (B) Effect of bovine serum albumin on adsorption of ^{32}P-labeled reovirus to beaker walls. ^{32}P-labeled virus (250 μg, 3 × 10^4 cpm) was added to each of two beakers that contained 600 ml of seawater. One beaker received a 25-fold excess of bovine serum albumin (●); the other did not (▲). (C) Effect of filtered and unfiltered seawater on the adsorption of ^{3}H-labeled E. coli to beaker walls. ^{3}H-labeled E. coli (600 μg, 8 × 10^5 cpm) was added to each of two beakers that contained 600 ml of seawater (▲) or seawater that had been subjected to ultrafiltration (●).*

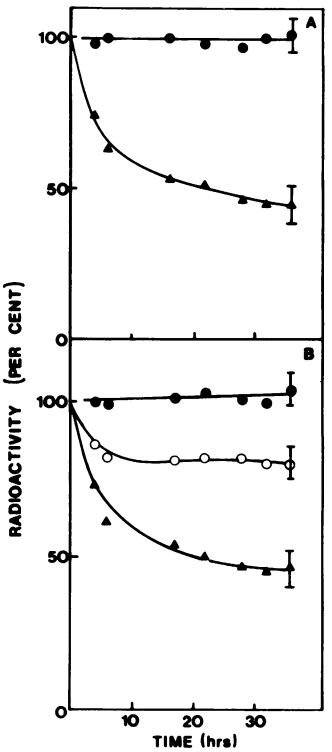

FIG. 2. *Accumulation of ^{32}P-labeled reovirus by oysters. (A) Removal of virus by three oysters (▲) compared with radioactivity monitored in seawater that lacked oysters (●) (control). The virus concentration was approximately 1 μg/ml (2,000 cpm/ml), and a 25-fold excess of bovine serum albumin was present. (B) Uptake by living oysters (▲) compared with those frozen and thawed (○) and a seawater control (●). The seawater that contained dead oysters was made 10^{-4} M sodium azide to inhibit microbial growth. A 20-μg amount of ^{32}P-labeled reovirus (3 × 10^4 cpm) was added to the seawater which contained a 25-fold excess of bovine serum albumin.*

The rate of virus uptake was not influenced by the presence in the surrounding seawater of the marine alga *D. primolecta* (Fig. 3), implying that virus uptake was independent of the presence of food material.

To determine whether the bovine serum albumin present in the seawater was accumulated by the oyster, oysters were supplied simultaneously with both ^{125}I-labeled albumin and ^{32}P-labeled reovirus. ^{125}I-labeled albumin was removed from solution by the oyster (Table 3), but the distribution of the albumin differed from that of the virus, with greater than 60% of the

TABLE 1. *Distribution of ^{32}P-labeled reovirus after incubation with live and dead oysters*

Component[a]	Live oysters		Dead oysters	
	% Radioactivity	Particles[b]	% Radioactivity	Particles
Oyster tissue	33.7	3.1×10^{10}	3.1	2.8×10^{9}
Shells	3.8	3.5×10^{9}	10.8	10^{10}
Pseudofeces and feces	3.8	3.5×10^{9}	0.7	6.4×10^{8}
Beaker walls	2.6	2.4×10^{9}	1.0	9.2×10^{8}

[a] At the conclusion of the experiment detailed in Fig. 2b, the distribution of radioactivity was analyzed.
[b] 20 μg (9.2×10^{10} particles) of virus was added to the seawater.

TABLE 2. *Effect of virus concentration on the accumulation of reovirus by oysters*

Component	Initial amt of virus present (μg)[a] in water			
	10[b]	100	500	1,000
Oyster tissue[b]	2.6	24.2	23.0	24.0
Shells[b]	0.67	8.1	39.5	106.0
Pseudofeces and feces[b]	0.69	67.0	32.0	61.0

[a] Four beakers each containing 600 ml of seawater and three oysters were seeded with ^{32}P-labeled reovirus (10 μg = 4×10^{5} cpm) and 0, 90, 490, and 990 μg of nonradioactive virus, respectively.
[b] After 40 h the distribution of radioactivity was measured and related to the amount of virus present calculated from the known specific radioactivity of the virus preparation. A 1-μg amount of virus equals 4.6×10^{9} particles.

albumin removed from solution accounted for by adsorption to the shell. This distribution differs markedly from that of the virus but does not exclude the possibility that the presence of bovine serum albumin may influence the subsequent uptake of virus particles.

Accumulation of reovirus and *E. coli*. To investigate the relative rates of accumulation of bacteria and virus, oysters were supplied with ^{32}P-labeled reovirus and ^{3}H-labeled *E. coli*, and the uptake of the two microorganisms was monitored simultaneously (Fig. 4).

As anticipated, bacteria were rapidly cleared from the seawater with a half-life of approximately 3 h. In contrast, approximately 9 to 12 h was required to reduce the virus concentration in the seawater to half its original value. At the end of the experiment the distribution of radioactivity was analyzed (Table 4), which revealed that clearance of *E. coli* from suspension was enhanced by the affinity of bacteria for the oyster shell.

Accumulation of reovirus and SFV. To determine whether the surface properties of virus particles play a role in their accumulation by the oyster, the uptake of ^{3}H-labeled SFV and ^{32}P-labeled reovirus was measured simultaneously. Reoviruses and group A togaviruses such as SFV have been shown to possess similar hydrated diameters of 85 and 70 nm, respectively, when examined by low-angle X-ray scattering (12; J. D. Harvey et al., manuscript in preparation), but the surface properties of these viruses differ. Whereas the outer surface of reo-

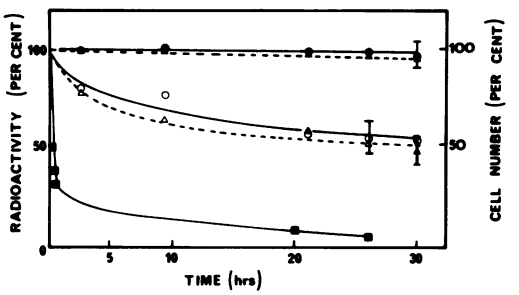

FIG. 3. *Accumulation of reovirus in the presence and absence of the marine alga D. primolecta. A 150-μg amount of ^{32}P-labeled reovirus (6×10^{11} particles; 2×10^{5} cpm) and alga (5×10^{9} cells) were added separately or together to 600-ml volumes of seawater that contained oysters. Samples were withdrawn at appropriate times, and the Cerenkov radiation was measured. The removal of algal cells from suspension was measured by counting cell numbers using a Coulter Counter (■). Virus uptake by oysters in the presence (broken lines) and absence (normal lines) of algae. (Solid symbols) Control; (open symbols) plus oysters.*

TABLE 3. *Distribution of ^{32}P-labeled reovirus and ^{125}I-labeled bovine serum albumin after incubation with oysters*

Component[a]	^{125}I (%)	BSA (μg)	^{32}P (%)	Virus (μg)[b]
Oyster flesh	2.6	26.0	21.0	12.6
Oyster liquor[c]	1.2	12.0	0.6	0.4
Shells	9.8	98.0	19.9	11.9
Pseudofeces and feces	1.7	17.0	2.6	1.5
Beaker walls	0.4	4.0	0.3	0.2

[a] Three oysters in 600 ml of seawater received 60 μg of ^{32}P-labeled reovirus (2×10^{5} cpm) and 1 mg of ^{125}I-labeled bovine serum albumin (BSA) (7×10^{5} cpm). After 40 h the distribution of the two isotopes was determined.
[b] A 1-μg amount of virus equals 4.6×10^{9} particles.
[c] Principally hemolymph.

virus is entirely proteinaceous (13), SFV is enclosed in a lipoprotein membrane typical of group A togaviruses (19). Figure 5 and Table 5 present results of an experiment in which oysters were presented with equal particle numbers of each virus and the uptake of both viruses was then monitored simultaneously. It is clear that the two viruses have been accumulated at different rates and that the subsequent distribution of radioactivity reflects differences in the fate of each type of virus particle.

Specific activity and integrity of reovirus after accumulation. The amount of virus present in tissue may be determined from the known specific radioactivity of the virus preparation. When this value is related to the infectivity present in extracts, information concerning the rate at which virus particles are inactivated is obtained. The results of such an analysis for reovirus are presented in Table 6, in which oysters that had accumulated virus for 48 h were subsequently dissected and homogenized, and the radioactivity and infectivity were measured. It is clear from Table 6 that the specific infectivity was reduced by approximately threefold over the 48-h period of incubation in seawater. Virus extracted from both the digestive diverticulum and the remaining tissue possessed a specific infectivity 10-fold lower than that of the virus in the seawater, implying that inactivation occurred after accumulation by the oyster but that for reovirus this was a relatively slow process.

To confirm that ^{32}P-labeled reovirus retained its physical integrity after incubation in seawater, virus incubated in seawater was subjected to isopycnic sedimentation on CsCl gradients. In-

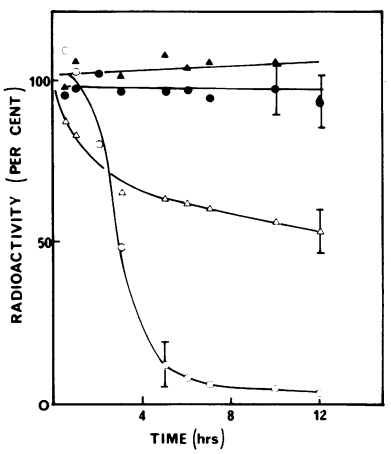

FIG. 4. *Accumulation of ^{3}H-labeled E. coli and ^{32}P-labeled reovirus by oysters. A 600-ml amount of seawater received E. coli (equivalent to 300 μg [wet weight] and 5 × 10^{5} cpm) and ^{32}P-labeled reovirus (15 μg and 10^{5} cpm). Symbols: (●) ^{3}H-labeled E. coli, control; (○) ^{3}H-labeled E. coli, oysters; (▲) ^{32}P-labeled reovirus, control; (△) ^{32}P-labeled reovirus, oysters.*

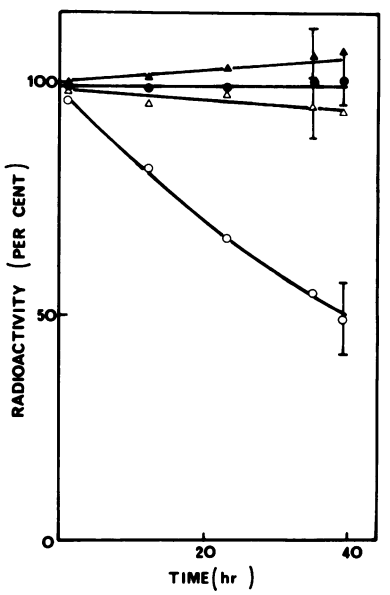

FIG. 5. *Accumulation of ^{32}P-labeled reovirus and ^{3}H-labeled SFV by oysters. A 600-ml amount of seawater received 2 × 10^{9} plaque-forming units of reovirus (10^{5} cpm) and 2.5 × 10^{9} plaque-forming units of SFV (10^{5} cpm). Symbols: (▲) ^{3}H-labeled SFV (control) and (△) in the presence of oysters; (●) ^{32}P-labeled reovirus (control) and (○) in the presence of oysters.*

TABLE 4. *Distribution of ^{32}P-labeled reovirus and ^{3}H-labeled E. coli after incubation with oysters*

Component[a]	^{3}H-labeled *E. coli*		^{32}P-labeled reovirus	
	% Radioactivity	Cells	% Radioactivity	Particles
Oyster flesh	46.0	8.3 × 10^{7}	19.9	1.4 × 10^{10}
Oyster liquor[b]	0.3	5.4 × 10^{5}	1.2	8.3 × 10^{8}
Shells	32.8	5.9 × 10^{7}	14.7	10^{10}
Pseudofeces and feces	10.8	1.9 × 10^{7}	6.7	4.6 × 10^{9}
Beaker walls	5.9	1.1 × 10^{7}	8.7	6.0 × 10^{9}

[a] At the conclusion of the experiment detailed in Fig. 4 the distribution of the two isotopes was determined. A 300-μg amount (1.8 × 10^{8} cells) of *E. coli* and 15 μg (6.9 × 10^{10} particles) of reovirus were added to the seawater.

[b] Principally hemolymph.

TABLE 5. *Distribution of ^{32}P-labeled reovirus and ^{3}H-labeled SFV after incubation with oysters*

Component[a]	% Radioactivity	
	^{3}H-labeled SFV	^{32}P-labeled reovirus
Oyster flesh	2.6	26.2
Oyster liquor[b]	1.1	0.5
Pseudofeces and feces	12.3	11.6
Shells	3.4	19.8
Beaker walls	3.1	9.9

[a] At the conclusion of the experiment detailed in Fig. 5 the distribution of radioactivity was analyzed. A total of 2.5×10^9 plaque-forming units SFV and 2×10^9 plaque-forming units of reovirus were added to the seawater.

[b] Principally hemolymph.

TABLE 6. *Specific infectivity of ^{32}P-labeled reovirus isolated from seawater and from oyster tissue*

Sample[a]	Incubation time (h)	Specific infectivity (PFU/10^6 cpm)[b]
Virus in 1× SSC		1.5×10^{10}
Virus in seawater	0	1.2×10^{10}
	2	1.3×10^{10}
	12	1.3×10^{10}
	24	1.3×10^{10}
	36	7.7×10^{9}
	48	4.8×10^{9}
Digestive diverticulum	48	5.8×10^{8}
Remaining tissue	48	4.3×10^{8}

[a] ^{32}P-labeled reovirus (670 μg, 1.6×10^6 cpm) was added to 600 ml of seawater that contained three oysters. SSC = 0.15 M NaCl plus 0.015 M sodium citrate.

[b] PFU, Plaque-forming units.

tact reovirus is known to possess a buoyant density of between $\rho = 1.358$ g/ml and $\rho = 1.385$ g/ml, depending on the amount of oligonucleotides present within the particle (8). After incubation in seawater, all the ^{32}P radioactivity was present in a discrete band at $\rho = 1.395$ g/ml, in contrast to the control virus which banded at $\rho = 1.375$ g/ml (data not shown). Thus, although the virus retained its physical integrity and most of its infectivity (Table 6), incubation in seawater resulted in an alteration in the buoyant density of the virus of 0.02 g/ml.

Uptake kinetics. The removal of microorganisms from seawater in these experiments may be assumed to conform to a first-order relationship of the form $dc/dt = \alpha C$, where C is the concentration of microorganisms, t is the time, and α is a rate of reaction constant. The half-life (the time taken to reach 50% of the initial concentration) may then be calculated from the slope of the semilogarithmic plot of the uptake data to yield the information presented in Table 7. This indicates that there were major differences in the uptake of the various microorganisms studied, with only the marine alga *Dunaliella* approaching the half-life value achieved for neutral red.

DISCUSSION

The use of radioactively labeled materials limited the volume of seawater that could be used in these uptake studies and required the use of high virus concentrations: experimental conditions therefore differed significantly from previous studies in which larger and more complex circulating aquaria were used (18). However, one advantage of the use of radioactively labeled and highly purified virus is that the subsequent fate of all virus particles may be followed. An unexpected finding that resulted from this approach was that sizable quantities of virus were found to be adsorbed to the oyster shell. This effect was presumably a result of charge-dependent surface interactions and was not related to the presence of other viable encrusting organisms, since the shells of oysters that had been frozen in liquid nitrogen exhibited the same adsorptive capacity as those of actively filtering animals. In contrast to the removal of virus resulting from adsorption to the shell, virus accumulation by the oyster itself was a process dependent on filtration.

Jørgensen and Goldberg (15) have found that a 1- to 2-μm diameter is the minimum particle size which can be filtered out of seawater by the gill filter of *C. virginica*. However, particles in the size range of viruses presumably may become adsorbed to the mucus sheet that covers the gills (7, 14) and then be transferred towards the mouth. Adsorption to the mucus sheet is the mechanism most likely to account for the uptake of reovirus, since virus suspensions used in this study were initially monodisperse and were not associated with larger particles that would be filtered by the gills. The poor accumulation of SFV when compared with reovirus may reflect either a lower affinity of the mucus sheet for lipoprotein-enclosed viruses or lower retention by the digestive system. The presence of radioactive SFV in both the pseudofeces and feces (Table 5) suggests that interaction of this virus

TABLE 7. *Half-life values for materials accumulated by C. glomerata*

Material	Half-life
Neutral red	12 min
Dunaliella	15 min
E. coli	3 h
Reovirus	9 h
SFV	>100 h

with the mucus sheet indeed takes place but that the virus is less efficiently retained within the digestive system.

The virus preparations used in this study were highly purified and were essentially monodisperse as judged by their sedimentation behavior on sucrose gradients. However, the possibility that either reovirus or SFV forms larger aggregates when diluted into saline water (10) cannot be excluded by this study. The differential uptake observed might therefore result from differing degrees of virus aggregation rather than different surface properties.

A clear dependence on virus concentration was observed for the accumulation of reovirus by oysters (Table 2). The maximum amount of virus associated with the tissue of an individual oyster was found to be approximately 8 μg or 4 $\times 10^{10}$ particles, suggesting that the oyster possesses a large but finite capacity to accumulate virus. This phenomenon could result from there being only a limited number of sites for virus adsorption on the mucus sheet covering the gills and a relatively slow rate of replacement of this layer by fresh mucus surfaces. Alternatively, the number of adsorption sites in the digestive diverticulum could be limited, which would account for the large amount of virus found to be associated with the feces and pseudofeces.

When the specific infectivity of virus was measured by relating the infectivity present in seawater and oyster extracts to the particle numbers present, reovirus was found to be essentially stable in seawater over a 48-h period, decreasing only threefold in specific infectivity over this period. However, virus accumulated by the oyster was 10-fold less infectious on a plaque-forming units-to-particle basis, demonstrating that some inactivation occurred after accumulation of reovirus by the oyster. Although virus inactivation was relatively slow and limited in extent, it suggests that both inactivation of the virus accumulated and physical rejection may be involved when the removal of infectious material from contaminated filter-feeding marine bivalves has been demonstrated experimentally.

ACKNOWLEDGMENTS

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