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Accumulation and Elimination of Coliphage S-13 by the Hard Clam, *Mercenaria mercenaria*

WALTER J. CANZONIER

Oyster Research Laboratory, New Jersey Agricultural Experiment Station, Rutgers—The State University, New Brunswick, New Jersey 08903

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Accumulation and elimination of viral particles by hard clams, *Mercenaria mercenaria*, were studied with the coliphage S-13 as a working model. *Escherichia coli* uptake and elimination were simultaneously monitored. Clams were exposed to low levels of S-13 (7 particles/ml) in running seawater for several days, achieving titers in tissues from 2 to more than 1,000 times the levels to which they had been exposed. Bacterial accumulation (previously established by other workers) was comparable. Upon exposure to virus-free running water, clams polluted to relatively low levels (100 plaque-forming units/ml) eliminated most of their bacterial contaminants in 24 to 48 hr. Viral contaminants, however, persisted for several days to weeks even under ideal conditions for clam activity, provided that the temperature remained below the inactivation threshold for the virus. Most of the accumulated virus appeared to be sequestered in the digestive gland. These sequestered particles are refractory to those mechanisms responsible for elimination of bacterial contaminants. This discrepancy points out the need for caution in evaluating the efficiency of shellfish depuration processes, especially if only a bacterial criterion is used as a monitoring system.

The indictment of shellfish as probable carriers of enteric viruses (3, 12, 15, 16, 18–20; O. C. Liu et al., Bacteriol. Proc., p. 151, 1968) has generated considerable interest in development of methods of cleansing oysters and clams of viral contaminants (depuration). Several laboratory studies have demonstrated the accumulation by clams and oysters of various viruses and their subsequent reduction upon the return of the shellfish to virus-free seawater (5, 10, 11, 13, 14; Liu et al., Bacteriol. Proc., p. 151, 1968). Some workers have also compared *Escherichia coli* with viruses in their accumulation and elimination by shellfish (6, 14).

The virus of principal public health significance is the etiological agent of infectious hepatitis. The isolation of this agent, however, is yet to be perfected and all work with shellfish has been confined to the use of model systems employing other viral entities. In these model systems shellfish are exposed from a few hours to several days to known concentrations of virus, in aquaria with standing, recirculated, or flow-through water supplies. Poliovirus and coxsackievirus (13), poliovirus (5, 10, 14), and bacteriophage (7) have been used in other laboratories. S. Y. Feng (Proc. Nat. Shellfish Ass. **57**:2, 1967), working in this laboratory with the hard clam, *Mercenaria mercenaria*, initiated studies with a phage of *Staphylococcus aureus* 80 and later introduced a smaller phage of *E. coli* C designated S-13. This work was continued by C. Fenton (S. Y. Feng and C. Fenton, *unpublished data*).

Feng and Fenton initially utilized high dosage of particles during the pollution phase which usually resulted in viral concentrations in excess of 2×10^3 particles per ml of clam tissue extract. At these high levels, clams eliminated a large percentage of the viral load within a short period of exposure to clean water (24 to 48 hr). This corresponded to an equivalent efficiency in simultaneous elimination of *E. coli*. However, a low titer of phage often persisted for several days in many of these experiments. The persistence of viral particles even after 48 hr of depuration raises the question of infectious potential, especially since the minimal infective dose for hepatitis and other enteric viruses is not well established. The exposure levels and subsequent levels of contamination in the experiments of Feng and Fenton were usually in excess of concentrations one might encounter under natural conditions. In the work reported here, therefore, the final contamination titers in clam tissue were held below 100 particles/ml by reducing the levels in the seawater to less than 10/ml.

A secondary objective of the present studies has been to determine the capacity of hard clams in accumulating and concentrating viruses when exposed continuously to very low levels in a running-seawater system. The concentrating capacity of hard clams and oysters has been established for bacteria (*E. coli*) and viruses when exposed to levels in excess of 40 particles or more per ml of seawater (2, 9, 11, 13, 14).

Previous workers at this laboratory and most at other laboratories had exposed clams to viruses by immersion in standing water with high viral titers. In the present work, to simulate more probable natural pollution situations and to achieve lower but more uniform contamination levels in the clam at the initiation of depuration, the presentation of phage (dosing) was at low levels for prolonged periods.

Feng and Fenton used pools of whole clams in their assay procedure, and no effort was made to concentrate or refine the homogenized tissue. In this present work, sensitivity at lower phage titers was enhanced by using only an extract of the digestive glands of individual clams.

MATERIALS AND METHODS

Clams. Northern hard clams (*Mercenaria mercenaria;* 100 to 150 g) were obtained from local commercial sources and conditioned in the aquarium system prior to initiation of the experiment. In any experiment, clams were selected to be approximately the same size.

Phage model. The bacteriophage used was designated S-13 (1), the host cell being *E. coli* C. This is one of the smaller viruses, approximately 20 nm in diameter, about the size of the poliovirus. Phage and host cells were obtained from E. Simon and E. S. Tessman, Purdue Univ. High phage titers (10^7 to 10^9/ml) were produced in 250-ml nutrient broth (Difco) cultures of *E. coli*, the cells were removed by centrifugation, and the supernatant fluid was stored over chloroform at 4 C until dispensed.

Assay. Extracts of clam tissue were prepared by homogenizing in a blendor the digestive gland of individual clams in an equal weight of 0.8% nutrient broth (Difco) for 1 min at high speed. Ten milliliters of the brei was placed in 13-ml tubes to which 2 ml of chloroform was added; the tubes were capped and shaken and then centrifuged at 25,000 × *g* at 4 C for 1 hr. A 1-ml sample of supernatant fluid (including gelatinous layer), or an appropriate dilution in nutrient broth, was spread uniformly over a base layer of plate count agar (Difco) with 0.0025% $CaCl_2$ added. After 10 min, this was overlaid with about 5 ml of 1.5% agar containing 10^7 to 10^8 host cells per ml and 0.05 M tris(hydroxymethyl)aminomethane buffer adjusted to *p*H 7.6. Plates were incubated 4 to 6 hr at 37 C.

Each sample consisted of 10 to 20 clams, and 5 or 10 ml of extract was plated for each clam. It was estimated that the lower limit of recoverability was accordingly 0.2 or 0.1 plaque-forming unit (PFU) per ml of clam tissue extract.

Bacterial system. A strain of fecal *E. coli* B was grown in nutrient broth at 45 C and assayed by a pour plate technique, developed by V. Cabelli (2), employing a modified MacConkey agar. The clam digestive glands were ground in a blendor as for phage, but whole brei or dilutions thereof were plated. Six to 10 clams, processed individually, were used for each sample.

Experimental aquaria and seawater system. Seawater was pumped continuously from the Shrewsbury River, Monmouth County, N.J. Salinities range in this river from 17 to 26%. During all experiments reported, they were 20% or greater.

The piping and tank system was constructed of polyvinylchloride, polyethylene, and expoxy-coated wood. The clam treatment tanks measured 61 by 244 cm with a flow of 150 to 200 liters per hr per tank.

Water temperature was controlled when necessary by passage through a Teflon heat exchanger. All water was sterilized by passage through a thin layer (1 to 2 mm) beneath 15 G. E. Germicidal Lamps (30-w; UV). This UV-treatment system is essentially as described for the unit used in the Public Health Service Laboratory at Purdy, Wash. (8), with the exception that a smooth rather than riffled surface was used.

During the pollution phase, phage and bacterial suspensions were refrigerated at 4 C and dispensed by a peristaltic pump into a small mixing chamber receiving the inflowing water before its entry into the aquarium. This chamber also counterbalanced a spring-loaded switch controlling the peristaltic pump, thus preventing excessive dosing in the event of a temporary reduction in water flow.

During the course of the experiment, aquaria were drained and flushed daily to remove accumulated detritus, mucus, and feces.

Environmental data. Various physical parameters including temperature, salinity, turbidity, and chlorophyll levels of incoming water were determined daily or at more frequent intervals. As a direct index of suitability of the conditions for clam activity, shell movements of 12 to 24 clams were recorded continuously throughout these studies. In addition, a population of 100 clams was held under observation in the aquarium system, and the number with valves open and siphons extended was recorded three times daily. All experiments were conducted during periods of relatively high activity.

RESULTS

Accumulation of phage. Two hundred clams were placed in running water and dosed with approximately five phage particles/ml. Samples of 20 clams were removed at 16, 40, 160, and 184 hr. Samples of seawater were removed from the aquarium at frequent intervals (24 hr or less) throughout the experiment to monitor phage levels. Exposure levels never exceeded seven per ml and had a mean of five per ml. At 212 hr, the

TABLE 1. *Uptake of bacteriophage S-13 by hard clams*[a]

Date	Exposure time (days)	Exposure level[b]	Mean titer[c]	Accumulation factor[d]
20 January	10	0.05	57	1,140
28 January	15	4–7	75	14
16 April	18	3–5	90	22
29 April	2	1	54	54
7 May	8	9	92	10
27 May	9	4–6	107	23
19 June	7	3–7	70	14
26 June	1	70	1,754	25
3 August	6	6	14	2.3
9 September	7	0.4–2.2	7	3.2
18 September	7	4	24	6
23 September	7	0.4–1.2	5	5
8 October	7	0.8–8.0	83	10
27 October	7	2	395	220
14 May	1	3,000	1,816	0.7
5 June	1	475	151	0.3

[a] All in flow-through seawater except last two in aerated standing water.

[b] Concentration of S-13 in seawater in PFU/ml.

[c] Concentration of S-13 in digestive glands of 10 or 20 clams in PFU/ml.

[d] Concentration in clams/concentration in seawater.

dosing was increased by a factor of 14 (70 per ml), and the clams were again sampled at 231 hr.

The means for 20 clams in each sample through 184 hr ranged between 52 and 86 per ml. The mean for all samples was 70 per ml. This titer is approximately 10 times the level to which they were exposed.

After 19 hr at the higher dosage, the sample mean was 1,754 per ml, somewhat more than 25 times the dosage level.

Capacity of clams to accumulate phage in their tissues considerably in excess of the levels to which they were exposed was demonstrated in the pollution phase of 14 additional experiments (Table 1). In these experiments, the accumulation factor (ratio of clam titer to seawater titer) for the means of each sample ranged between 2 and 1,100. Individuals often had titers in excess of 100 and occasionally as high as 1,500 times the dosage levels.

In a few cases, negligible accumulation was attributable to generally low activity as indicated by other observations. For example, in the last two experiments listed in Table 1 the clams were exposed in standing water. Under such conditions, activity is severely inhibited and many clams fail to open for long periods.

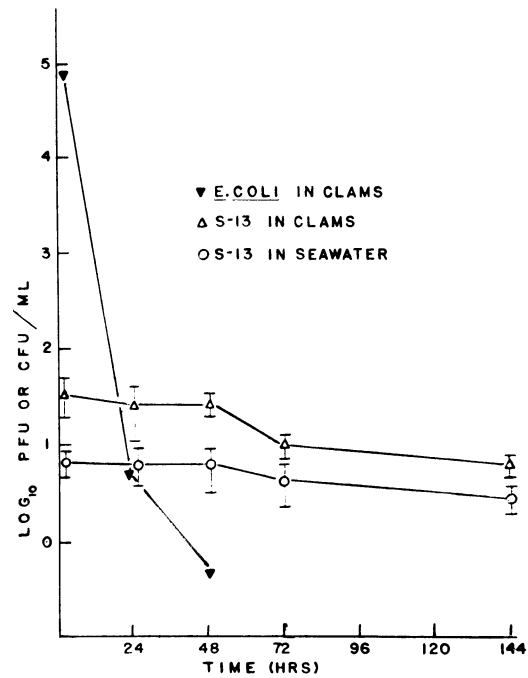

FIG. 1. *Elimination of phage S-13 and E. coli in clams and inactivation of S-13 in seawater at 16 C (October 1968). Confidence intervals of 95% indicated for samples representing 20 individual clams or 30 ml of seawater.*

Depuration experiments. These experiments were conducted throughout the year and under broadly varying conditions. Four experiments are considered representative and illustrate features of retention and elimination of viral particles in hard clams.

(i) This experiment (October 1968), conducted at 16 C, illustrates clearly that bacteria and phage are handled quite differently by the hard clam (Fig. 1). The mean viral titer for 10 clams in the initial sample was 33 PFU per ml. The initial *E. coli* level was 10^5 colony-forming units (CFU) per ml. At the time of initial sampling, three flasks, each containing seawater with phage at 6 PFU/ml, were immersed in the aquarium as a control for phage inactivation at the temperature of the depuration phase. *E. coli* elimination proceeded rather rapidly; within 24 hr the level had dropped to 10 CFU/ml and by 48 hr to less than 1 CFU/ml, approaching the lower limit of reliable assay by the method used.

In contrast, reduction in phage proceeded very slowly and in 144 hr (6 days) reached a level less than one log below the initial titer. Phage in seawater exhibited an identical pattern of reduction.

(ii) A similar experiment (September 1968),

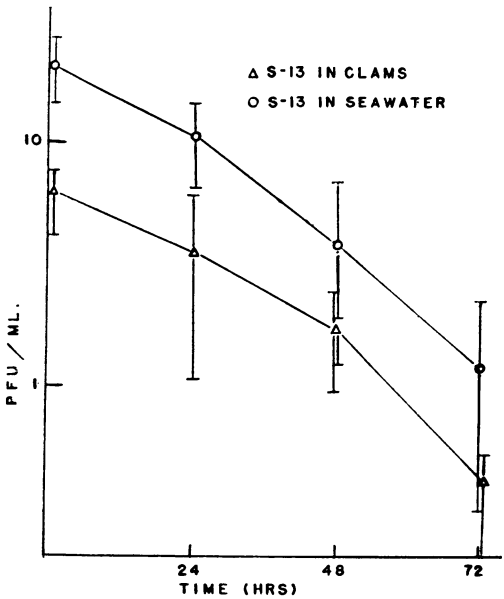

FIG. 2. *Elimination of phage S-13 in clams and inactivation of S-13 in seawater at 24 C (September 1968). Confidence intervals of 95% for samples representing 20 individual clams or 10 ml of seawater.*

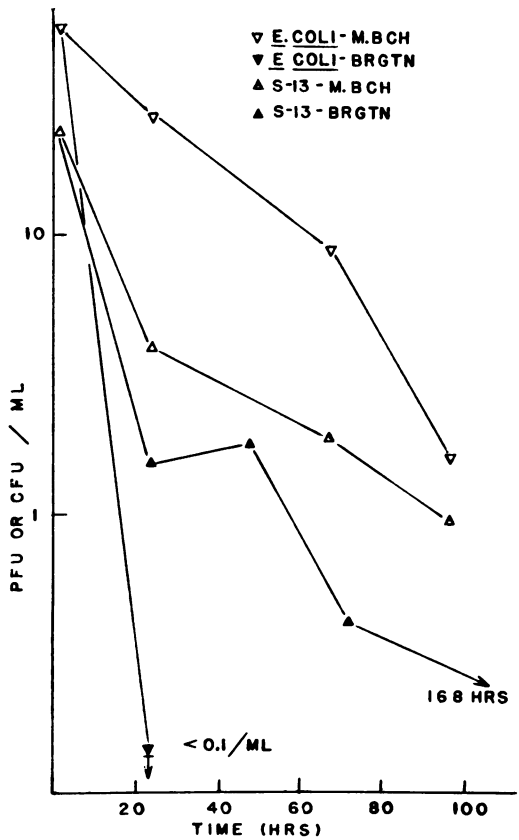

FIG. 3. *Comparison of elimination of phage S-13 and E. coli by clams depurated at Monmouth Beach and Brigantine. A reduction in seawater temperature occurred at both sites during the period of exposure (September 1969).*

conducted at 24 C, illustrates the effect of higher temperature on the rate of phage reduction. Again the rate of change in phage titer in clams paralleled that in seawater although the slope for both is considerably steeper than in the previous experiment and the limits of reliable assay were reached in 72 hr (Fig. 2). Additional studies of the kinetics of inactivation of S-13 and P-80 phage have indicated that temperature is indeed the primary factor in phage inactivation in a seawater medium and that there can be considerable differences between two viral types in response to this factor.

(iii) Depuration at the Monmouth Beach Laboratory was compared with that at a temporary facility located at Brigantine in southern New Jersey. Water quality at that site, as judged by clam activity, was superior to that obtainable in the Shrewsbury River. One experiment (Fig. 3) again illustrates the discrepency in elimination of viruses and bacteria. Elimination of *E. coli* at Brigantine was rapid, dropping to less than 0.1 CFU/ml in 24 hr, but was quite unsatisfactory at the Monmouth Beach Laboratory. Reduction of S-13 at both sites was better than that obtained for *E. coli* at Monmouth Beach, but even at Brigantine some phage were detectable after 168 hr. This experiment illustrates the lack of correlation between elimination of phage and bacteria. The slightly better elimination of phage at Brigantine was apparently a function of water temperature, which attained a peak of 29 C at this site during the period of observation while remaining less than 25 C at Monmouth Beach.

Thirty-three experiments with varying conditions of temperature and clam activity have been conducted in addition to those illustrated. The data strongly suggest that a portion of the phage is sequestered at low levels in the clam tissues and this sequestered phage is not diminished appreciably by activity of the clam. The reduction in phage titer in every case could be accounted for by temperature-dependent inactivation in situ.

(iv) To test this hypothesis, an experiment was designed to compare reduction in phage titer in active and inactive clams at temperatures above and below 16 C. Above this temperature, rate of S-13 inactivation in seawater is greatly accelerated (*unpublished observation*). A large group of clams were exposed for 8 days to low levels of phage at

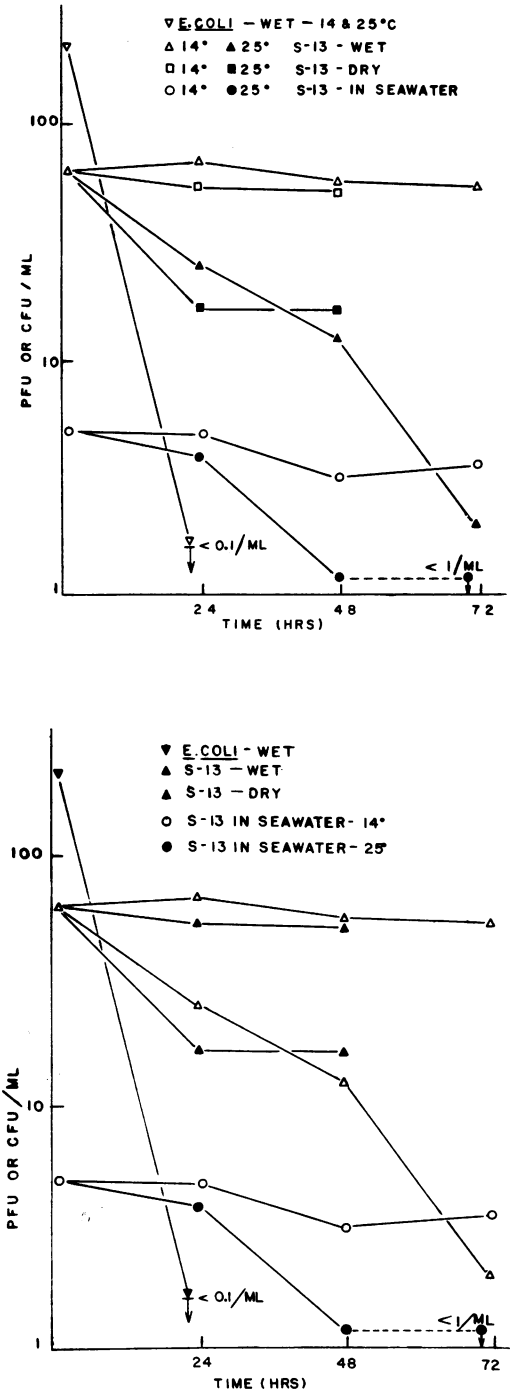

FIG. 4. *Comparison of phage S-13 elimination by clams exposed to running seawater and confined in plastic bags at two temperatures. Elimination of E. coli in running seawater was essentially the same at both temperatures and is represented by a single series of observations (November 1969).*

14 C. All clams were also exposed to *E. coli* for the last 24 hr of the phage pollution phase. For the depuration phase, four lots were treated as follows: (i) running seawater at 14 C, (ii) running seawater at 25 C, (iii) dry storage in plastic bags immersed in the 14 C aquarium, and (iv) dry storage in plastic bags immersed in the 25 C aquarium.

Phage in flasks of seawater were also immersed in the aquaria at 14 and 25 C, as an inactivation control during the depuration phase.

Clams in lots (i) and (ii) were sampled at 24, 48, and 72 hr. There were insufficient clams to sample groups (iii) and (iv) at 72 hr (Fig. 4). The *E. coli* cells, initially 200 CFU/ml, were reduced to less than 0.1 CFU/ml at 24 hr and were undetected at 48 hr. This indicated high clam activity. No difference in *E. coli* elimination was detectable between warm and cool treatment.

Clams at 14 C (group i) retained essentially constant phage titers for at least 72 hr. There was no difference between wet and dry treatment in the first 48 hr. Phage titers in seawater at 14 C also remained constant.

At 25 C, the titer in both clam groups (ii and iv) and in seawater was reduced at a rate similar to that described in the 24 C experiment above (Fig. 2).

Retention of S-13 has been observed in clams in the laboratory for as long as 34 days during periods of good clam activity. Field experiments, in conjunction with relaying of clam from condemned to approved shellfish waters, show even greater periods of phage retention. Marked clams polluted with S-13 at 76 PFU/ml were mixed with transplants. After a period of 2 months (10 October to 12 December) on the bottom in an area considered good for clamming, 50 to 90% of the virus was retained. Water temperatures during 50 days of this period ranged between 8 and 16 C, sufficient for clam activity, although always below the inactivation threshold level for S-13. During a subsequent period of 3 months at temperatures lower than 8 C, the phage persisted in both wet stored and refrigerated samples.

DISCUSSION

The experimental evidence reported here illustrates several aspects of viral accumulation and elimination by hard clams. In comparison with earlier work, the use of low viral titers allows a more reasonable projection for the prediction of the kinetics of viral uptake and elimination in naturally polluted clams. Indeed, the pattern of elimination when initial titers are less than 100 PFU/ml is strikingly different from that in cases of clams polluted to levels in excess of 1,000 PFU/ml. In the latter, elimination of a major

portion of the viral contaminants is quite rapid and parallels the elimination of such bacterial contaminants as *E. coli*.

The accumulation of bacterial and viral particles by clams and other shellfish has been reported for several species. In some cases, viral accumulation has not exceeded the exposure levels and was often several orders of magnitude less. Mitchell et al. (14) have obtained concentrations ranging from 10 to 27 times the exposure levels in oysters with poliovirus, and accumulation factors of 1 to 180 have been reported for poliovirus in Olympia oysters and Manila clams (6). In the current studies, accumulation factors in excess of 1,500 have been obtained for individuals; for pooled samples, however, the factor usually fell between 6 and 50 times the exposure levels, with a maximum level of 1,100. It should be noted that results of others are probably based on whole clam samples, whereas the determinations reported here utilized digestive gland only. Correction factors of 0.15 or 0.3 may be applied to accumulation factors in Table 1 to render them comparable with accumulations determined on the basis of assays of total clam or drained meat, respectively. These correction factors are based on determinations of viral distribution in various tissues (*unpublished data*).

A significant feature in all accumulation studies in which levels above environmental titers have been achieved is the use of a flow-through system. Accumulation of both bacteria and viral particles is generally poor in standing water systems (5, 11, 13; Feng and Fenton, *unpublished data*). This reflects unfavorable conditions for continued physiological activity (e.g., ventilation and feeding). Polluted clams may be those individuals that have opened temporarily to sample the aquarium water. The variation in accumulation in this series (Table 1) is perhaps due to clam activity and the quantity and quality of particulate material in the seawater. The significance of this second factor was not investigated in this series but, as Hoff and Becker (6) point out in their study of poliovirus uptake, the presence of particles to which virus may adhere greatly enhances uptake. It should be noted that other workers used relatively high exposure levels, ranging from 40 to 1,000 PFU/ml of seawater. The experiments reported here generally utilized pollution levels of 1 to 8 PFU/ml of seawater in the pollution phase. This difference in the exposure levels plus the high stability and recoverability of S-13 must be considered in comparing accumulation reported here with that in studies with poliovirus. Extension of accumulation studies to poliovirus and other enteroviruses requires development of improved recovery procedures at low titers and determination of the inactivation kinetics of the viruses under the conditions of the experimental system.

The accumulation of viruses to high levels in certain tissues emphasizes the potential health hazard of shellfish exposed to even the lowest levels of viral pollutants.

The pattern of viral elimination reported here is somewhat different from that obtained by most other workers. Their conclusions, at least implicit, have been that viral particles are readily purged by the direct physiological activity of the contaminated shellfish. The present study strongly suggests that under certain conditions, i.e., low titers accumulated over an extended period, retention of some of the virus can be quite prolonged and independent of clam activity as measured by bacterial elimination. Indeed, reduction of viral titers in clams contaminated at low levels can be accounted for solely as a result of inactivation in situ under the influence of temperature and other physical factors prevailing during depuration. The results of Seraichekas et al. (21), also with low initial titers, suggest a prolonged retention of small numbers of poliovirus in a few of the shellfish sampled, even after 72 hr of depuration. At higher initial titers, these workers found poliovirus to be rapidly reduced and handled essentially by the same mechanism as the *E. coli* contaminants. Simultaneous observations on viral inactivation under the condition of depuration were not reported. It should be noted that in the experiments of Seraichekas et al. the pollution phase was at 15 C and depuration at 20 C. The significance of residual contamination is difficult to interpret without some knowledge of the persistence of the viral particle of interest. This is especially true in the case of prolonged depuration and low initial titers. Considerable variation in the uptake and elimination of two phages (S-13 and a large phage of *Staphylococcus aureus*) indicated the potential influence of viral stability in the kinetics of these two phenomena. The phage of *S. aureus* was not readily accumulated, and residual particles were rapidly eliminated from the clam. This phage was quickly inactivated under the conditions prevailing during the experiments as determined by seawater controls (S. Y. Feng and W. J. Canzonier, *unpublished data*).

It has been assumed in the design of control procedures recommended for depuration processes that elimination of particulate biological material is a function of the physiological activity of the shellfish. This is certainly well documented for some bacterial contaminants and appears to hold true for a large portion of the viral particles harbored by heavily polluted shellfish. The cur-

rent work and some results of other workers indicate that accumulation of viral particles may involve two related but distinct processes. The first process, and this may apply to all particles entering the mantle cavity, is the collection and entrapment of particles (food, bacteria, viruses) and the subsequent entry of many of these particles into the digestive tract. Smaller particles may be adsorbed onto larger particles or entrapped in mucus. Most of these particles will be eliminated in the feces and pseudofeces when a heavily polluted clam is placed in clean water under conditions suitable for ventilating and feeding. In this phase, viral and bacterial elimination should proceed at the same rate and this has indeed been well demonstrated (11, 14) by others and in this laboratory. The conditions necessary for adequate clam activity to effect removal of bacteria and a large portion of the viral contaminants can be defined. The effectiveness of elimination of these contaminants can also be monitored by appropriate bacterial assays.

It appears, however, that small numbers of viral particles can, during prolonged exposure, be sequestered in clam tissues. This second process may occur even with low environmental levels of virus. The majority of such sequestered particles may be recovered from the digestive gland and associated tissues and a smaller fraction from the hemolymph (10, 11; *unpublished data*). These particles apparently are not easily dislodged and their elimination is quite independent of clam activity. In the case of heavily contaminated clams, the majority of viral particles are rapidly eliminated, creating the impression of extremely effective depuration. In the case of clams exposed to low levels for extended periods, the elimination pattern is quite different since the majority of particles have been effectively sequestered and not labile to rapid release. This has been confirmed by observations of clams exposed at different levels under various conditions and subsequent studies of elimination. All experiments listed in Table 1 were followed by elimination studies. In cases where the titer was quite high, there was initially a rapid decline to a lower level, but in all cases the phage persisted for periods commensurate with with phage inactivation observed in seawater at the temperature of the experi nents.

The degree of coliform contamination is the criterion in accepting shellfish for depuration and in evaluating the subsequent effectiveness of the depuration process (4). This seems to assume that bacterial elimination is a reliable index to viral elimination. The results described here show that viruses can persist in shellfish depurated to acceptable coliform levels. The stability of particular viral pathogens under the conditions of depuration and handling is of considerable significance if the small numbers that remain sequestered in tissues are potentially an infective dose. The stability of viruses under such conditions is yet to be established. On the basis of the epidemiological evidence of shellfish-associated hepatitis (3, 12, 20) and of the persistence of the etiological agent of this disease in water (17), this virus could be expected to remain in clams indefinitely under conditions prevailing in depuration and storage.

If depuration of hard clams is to be accepted as a commercial practice, additional controls will be necessary to assure a safe product. Such controls might include a monitoring system more critical than the coliform standard now recommended. Additional processing, such as inclusion of a postdepuration period of warm storage (25 C or higher) to inactivate residual viruses, might be considered. The feasibility of such a procedure would be limited by the inactivation thresholds of the viruses of concern and the tolerance of the clams to the temperatures necessary.

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