that the enzyme activity paralleled its tumor inhibitory capacity. Further fractionation of the asparaginase preparation by DEAE column chromatography indicates that the activity is associated with the third peak. Comparative *in vivo* assay of all the peaks obtained once more demonstrates that only in the fractions showing enzyme activity was there any tumor inhibitory effect.

Broome has reported that in tissue culture, the 6C3HED cells dependence on L-asparagine is closely related to their sensitivity to guinea pig serum inhibition(11). In light of these observations, it is possible that the mechanism of regression *in vivo* is due to the enzyme converting all available asparaginase to aspartic acid which the tumor cannot utilize in the host. The possibility that the asparaginase enzyme or globulin affects the cells by direct attachment to the tumor cells was investigated. We were unable to detect asparaginase enzyme activity on tumor cells treated with the enzyme. Nor was there any loss of enzyme activity of GPS after such treatment with tumor cells. In addition the fluorescent antibody method was negative for visible attachment of guinea pig globulin components on viable tumor cells.

Summary. The guinea pig serum tumor inhibitory activity was effective for the ascites form of the Gardner lymphosarcoma. The active protein appears to be an enzyme L-asparaginase. Inhibition of the enzyme activity in normal pooled guinea pig serum in all cases caused a loss of the *in vivo* tumor inactivating capacity of the serum. Comparison of enzyme activity of fractionated serum correlates well with tumor destroying ability.

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Fate of Poliovirus in Northern Quahaugs.* (30841)

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Earliest documented outbreaks of infectious hepatitis associated with consuming raw oysters were reported in Sweden in 1956(1,2). Following this, two similar incidences occurred in the United States(3,4) and 4 outbreaks involving approximately 900 cases were traced to consumption of raw quahaugs or hard clams, *M. mercenaria*(5-7). Although the number of such cases is very small as compared with total incidence, it is important in that definite vectors be identified and measures taken to control this mode of transmission.

Studies on interaction of viruses and oysters have been conducted in recent years(8-10). It was shown that several species of shellfish were capable of picking up a significant amount of virus very rapidly and retaining it for as long as 28 days in dry storage.

* Contribution No. 14 from this Center.

Metcalf and Stiles(8) also isolated strains of Enterovirus from Eastern oysters harvested from Little and Great Bay, New Hampshire. Only a study of hard clams by Atwood, Cherry and Klein(11) on uptake of a B-5 Coxsackie virus showed a similar result. The present study was concerned with the fate of viral contaminants in shellfish using northern quahaugs and an attenuated poliovirus strain as working model. Three aspects of this subject have been investigated in a preliminary fashion: (a) patterns of uptake of virus by shellfish from their environmental water; (b) quantitative distribution of virus in various parts of body; and (c) mechanisms of viral elimination by shellfish.

Materials and methods. Virus. LSc 2ab strain of type I poliovirus was used throughout experiments. Whole virus stock was propagated in primary African green monkey kidney (MK) tissue culture (TC) cells and kindly supplied by Lederle Research Laboratories, American Cyanamid Chemical Co., Inc. as vaccine rejects. Early safety testing on these batches of virus showed equivocal results, but later testing demonstrated no detectable viral contaminants. The virus pool contained approximately $10^{8.0}$ PFU/ml and was stored at −20°C until used. Its survival time in filtered seawater from Narragansett Bay, R. I. was studied. At 22°C, the highest temperature used for all experiments, 50% inactivation of the virus occurred after 4 days, and 90% inactivation after 9 days.

Tissue culture. Primary African green MK cells were used throughout experiments. Cell suspensions, purchased from Flow Laboratories, were used to prepare monolayer cultures in 3 oz. prescription bottles. Each bottle was seeded with approximately 3,000,000 cells. Hanks' balanced salt solution (HBSS) containing 0.5% lactalbumin hydrolysate and 2% calf serum was used for growth of the cells and Earle's balanced salt solution with same per cent of hydrolysate and calf serum used for maintenance(12). Appropriate concentrations of antibiotics were incorporated in all media, including penicillin G, streptomycin and/or amphotericin B.

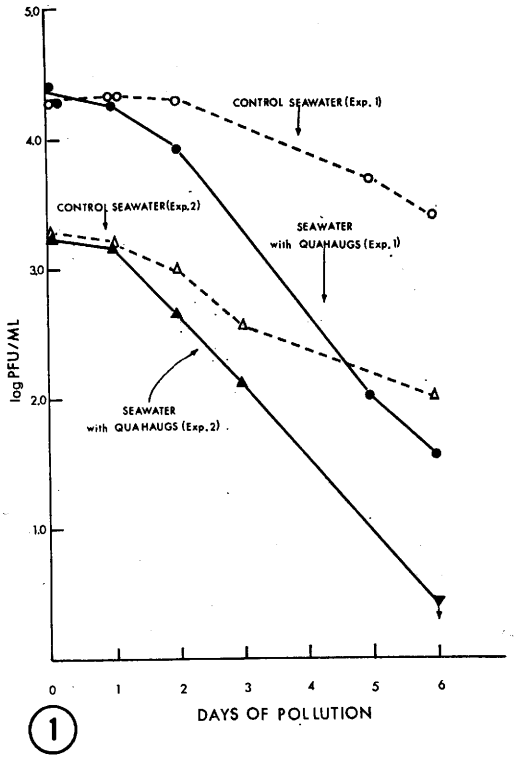
Plaque assay. General procedures used were described originally by Hsiung and Melnick (13). The bottles were ready for use between 5 and 7 days of incubation at 37°C after seeded with MK cells. Medium in bottles was decanted and 0.2-0.5 ml of appropriate diluted samples were added into each of duplicate bottles. Incubation for 2 hours at 37°C was allowed for viral absorption. Cell sheet was then overlaid with 10 ml of growth medium containing 3% Noble agar and 0.0017% neutral red. After agar became solidified, bottles were returned to 37°C incubator. Plaques were counted between 3rd and 6th days of incubation. Score obtained on last day is considered as final count. In text, total PFU's contained in 1 ml or gram of each sample were presented.

Quahaug or hard clam. The Northern Quahaug, *Mercenaria mercenaria,* used for experiments, was procured from local fishermen and had been harvested by tonging from nearby growing areas approved for human consumption. Usually 3-4 hours elapsed between time of harvesting and arrival at laboratory. They were stored in large basins in wet lab with a constant supply of fresh seawater pumped in from Narragansett Bay, R. I. until used. The shellfish were of a size comparable to "cherry stone" clams in restaurants, each weighing 100 ± 10 g with shell. The combined volume of mantle fluids and a portion of lymph from one shellfish ranged from 14 to 22 ml; digestive diverticulum, including stomach, weighed between 1.0 and 3.0 g; gill and mantle membrane 4-6 g and the remaining body 4.5-10.0 g. Contaminated quahaugs were stored at −20°C, if not dissected and homogenized immediately. Frozen shellfish were found much easier to handle than fresh ones. Each weighed organ was washed 3 times in HBSS, 25, 10, and 10 ml, respectively, then homogenized at 14,000 rpm for 3 minutes with sufficient HBSS added to constitute a 10% homogenate (w/v). Homogenates were clarified by low speed centrifugation before being assayed by plaquing.

Results. Uptake of virus. Several experiments were carried out to explore preliminarily the mode of viral uptake by quahaugs by following up virus titer of their surrounding seawater. The procedures used for a typical experiment are as follows: Each of two 24-

liter pyrex aquaria was filled with 4 liters of fresh seawater from Narragansett Bay, R. I. Four ml of stock virus and 6 active quahaugs were added into one aquarium and the same amount of virus, without shellfish, into the other. The aquaria were placed in an 18°C water bath and water in aquarium aerated with pumped-in air. Samples of 10 ml seawater were removed periodically. At each sampling, 1 liter of fresh seawater was added to each aquarium in order to keep shellfish active. During the entire period, all quahaugs were pumping and sufficient feces produced, indicating that animals under study were most likely in an active state. In another experiment, identical procedures were used, except only 1/10th amount of virus was added into each tank. All water samples were assayed for viral contents by plaquing in duplicate MK bottles. Results are illustrated in Fig. 1. Reduction in virus titers in control tanks was a combined result of dilution by adding seawater during samplings and natural inactivation under the particular condition. That in tanks containing quahaugs was a combined result of these two, plus amount presumably taken up by shellfish. As shown, virus titer of water samples from tanks containing quahaugs decreased more rapidly than those from control tanks. Difference in titer between the 2 tanks was detectable by 48 hours and was greater than 30-fold by 6 days. Initial concentration of virus in water did not seem to play a role for the pattern and rates of viral reduction. Quahaugs in these tanks were assayed for viral contents at end of experiments and found to contain significant amounts of virus in various systems. This finding indicates that viral uptake by shellfish under these conditions was probably a continuous process.

Accumulation and localization of virus in quahaugs. To elucidate the pattern of viral accumulation in various organs of shellfish, an experiment was conducted as follows: Each of two 24-liter aquaria contained 8 quahaugs and 4 liters of fresh seawater contam-



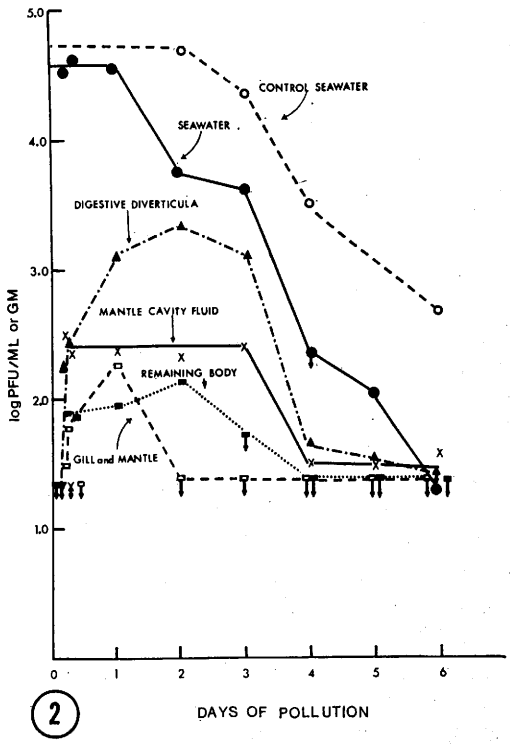


FIG. 1. Uptake of Poliovirus by Quahaugs in Static Aquaria.

FIG. 2. Accumulation and Localization of Poliovirus I in Quahaugs.

inated with poliovirus type I at $10^{4.50}$ PFU per ml. One additional tank containing only seawater with same viral content served as control. All aquaria were placed in an 18°C water bath and the seawater aerated. Water samples from all tanks and one shellfish from each tank were harvested and stored at −20°C at varying time intervals, during which one liter of fresh seawater was added. Immediately prior to processing of the shellfish, each was thawed with valves open in a petri dish at room temperature and the drained fluids collected. The fluid sample contained total amount of mantle cavity fluid and a portion of hemolymph from shellfish. Following this, digestive diverticula containing stomach was dissected out, then gills and mantle membranes, and last, the remaining body. All parts were washed 3 times with sterile HBSS and prepared for 10% homogenates. All preparations were assayed for viral content. Results are illustrated in Fig. 2.

As shown, differences between average viral titers of water samples from 2 tanks containing quahaugs and control water titers were relatively smaller and more erratic in this experiment than those in previous experiments. This was probably due to a gradual removal of shellfish from tanks for sampling. Maximum uptake in shellfish fluids was reached within 4 hours of exposure and persisted for 3 days, followed by a sharp drop in titer corresponding with decrease in titer of surrounding water. Maximum uptake in digestive diverticulum portions was reached in 24 hours and persisted until end of 72 hours. Their content also receded rapidly thereafter, following the steep drop of water titer. Both gills and remaining bodies contained little virus and no virus was detectable from these parts of body after 48 hours of exposure. Of particular interest is the finding that digestive diverticula picked up highest quantity of virus in first 3 days of exposure. This is in agreement with that described for the Eastern oysters contaminated with either poliovirus or Coxsackie virus by Metcalf and Stiles(8).

Since drained fluids and digestive diverticulum portions contained most of viral contaminants, total contents in these parts of quahaugs were computed using above data. Viral content in each whole shellfish was the sum of viral contents in 4 portions of the animal plus those found in the first washing with HBSS. Average values of fluids and diverticula of 2 quahaugs from each sampling and average sums of 2 whole shellfish are presented in Fig. 3. As shown, during first 3 days of pollution, drained fluids and digestive diverticula contained comparable amounts of virus. During fourth to sixth day, viral content in drained fluids became comparatively greater than that in digestive diverticula in spite of sharp decline of total content in whole animals.

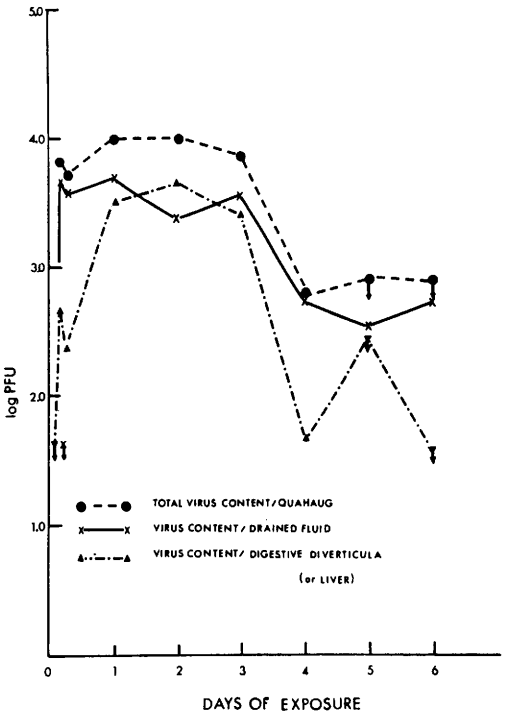


FIG. 3. Total Viral Contents in Drained Fluids, Diverticula with Stomachs, and the Whole Quahaug.

Viral turnover in digestive system. To determine whether virus is passing through digestive system continuously or merely retaining in this system after a plateau is reached during entire contamination period, the following experiment was carried out. Two 12-liter pyrex tanks were set up in usual manner, each containing 2 quahaugs and 2 liters of fresh seawater with $10^{4.50}$ PFU's of poliovirus per ml. Quahaugs, total amount of

TABLE I. Turnover of Poliovirus in Digestive System of Quahaugs.

Parts of quahaug	24 hr PFU	24 hr %	72 hr PFU	72 hr %
Lymph (total)	5.17×10^3	26.0	1.93×10^3	5.4
Body & gill (total)	1.35×10^3	6.8	5.00×10^2	1.4
Digestive diverticula & stomach (total)	7.50×10^2	3.8	1.18×10^3	3.3
Feces (total)	1.26×10^4	63.4	3.20×10^4	89.9
Total	1.99×10^4	100.0	3.56×10^4	100.0
Seawater titer (per ml)	4.25×10^4	—	2.00×10^2	—

fecal matter, and 10 ml seawater were harvested from one tank at the end of 24 hours, and those from the other at 72 hours. Shellfish were dissected and 10% homogenates were prepared. Feces was washed once with 10 ml HBSS and then homogenized. All samples were assayed for viral content. Total viral content in duplicate organ samples and in feces accumulated in each tank were computed and are summarized in Table I. The sum of viral contents in various organs and feces represents total number of virus particles which had been picked up by shellfish from their environment for entire period up to time of harvesting. Of this, 63% was found in feces cumulated for 24 hours and 90% in feces cumulated for 72 hours. This seems to indicate that virus was continuously picked up by shellfish, passed through digestive system and was finally eliminated in feces. The actual viral content in feces might have been higher than was shown here, because a portion of virus may have been inactivated during process of elimination and from aging at the bottom of tank. The kinetics of possible viral inactivation in feces was not examined in present study.

Status of virus in digestive diverticula. To explore the status of viral particles harbored in digestive diverticula, a typical experiment was conducted as follows: Four quahaugs were exposed to seawater containing $10^{4.50}$ PFU's of virus per ml for 48 hours in usual manner. Digestive diverticula and stomachs were dissected out and pooled. The tissue pool was minced and divided into 3 equal aliquots. First aliquot, homogenized in usual manner, was considered as a control of total viral content in the tissue pool. Second aliquot was treated with 0.25% trypsin at 37°C for 2 hours and centrifuged at a low speed. Supernate was collected and sediment homogenized. Third aliquot was further minced to very fine condition, suspended in HBSS and spun at a low speed. Only supernate was saved. All preparations were assayed for viral content with results summarized in Table II.

As shown, trypsinization released approximately 80% of virus, whereas mere mincing released 50%. This finding indicates that virus in digestive diverticula had not penetrated any types of cells, nor adsorbed chemically onto surface of any type of cells to a significant extent.

Status of virus in drained fluids. To determine which part of drained fluids from contaminated quahaugs contains virus and whether phagocytosis of virus has occurred in circulatory system, one experiment was conducted as follows: From 4 quahaugs which had just been exposed to seawater with $10^{4.50}$ PFU's of poliovirus per ml for 48 hours at

TABLE II. Poliovirus in Minced Digestive Diverticula and Stomachs.

Aliquot	Preparation	Material analyzed	Total PFU/g	% of total
#1	Homogenized as is	Total Homogenate	2.3×10^3	100.0
#2	Trypsinized and low speed centrifuged	Supernate	2.8×10^3	80.5
		Sediment (homogenized)	6.8×10^2	19.5
#3	Minced, extra fine, suspended in HBSS & low speed centrifuged	Supernate	1.1×10^3	47.9

TABLE III. Distribution of Poliovirus in Mantle Cavity Fluids and Drained Hemolymph.

Fluids	Volume (ml)	Preparation	PFU/ml	Total PFU	%
Mantle cavity	47	Supernate	*$<2.5 \times 10^0$	$<1.2 \times 10^2$	—
		Wash	$<2.5 \times 10^0$	" "	—
		Sediment	" "	" "	—
Hemolymph	25	Supernate	1.6×10^2	3.9×10^3	62.3
		Wash	$.7 \times 10^2$	1.8×10^3	28.8
		Sediment	2.3×10^1	5.6×10^2	8.9
		Total		6.3×10^3	100.0

* Non-detectable in undiluted preparation.

18°C, mantle cavity and body fluids were collected by opening the valves and letting them run into a sterile petri dish for 2-3 minutes at room temperature. Following this, each quahaug was transferred to another petri dish and more fluid allowed to drain out for 60 minutes. Both the early and late fluids were pooled and measured for volume. Total volume of early fluids was 47 ml and later fluids 25 ml.

The early fluids were light straw-color and transparent; the later samples were thick and milky, containing many blood cells and phagocytes. Both fluids were centrifuged at a low speed and supernates were collected. Sediments were washed once with HBSS and then homogenized. All preparations were assayed for virus content with results given in Table III. It is interesting to note that no virus was detectable in early fluids, whereas considerable amounts of virus were found in later fluids. Of this, 62% was in supernate, 29% in wash fluids, and 9% associated with cells. This finding indicates that great majority of virus found in shellfish liquor was probably in hemolymph and not in mantle cavity fluids, and that viruses were not phagocytized to a significant degree by any type of cells in circulatory system.

Discussion. The present study has clarified several aspects concerning basic mechanism of viral pollution in shellfish. First, viral uptake by various parts of shellfish body was extremely rapid. Within a few hours, maximum contamination was attained. The level of contamination was maintained so long as sufficient viruses were present in surrounding seawater. As virus reduced in water, viral content in shellfish receded correspondingly. This finding is in general agreement with that observed for oysters(8,9) and quahaugs(11) except that Coxsackie B-3 virus was retained at a significantly high level in digestive diverticula of eastern oysters when virus titer of their surrounding water had dropped to a much lower level(8). Whether or not this was due to different species of shellfish being used for experiments remains to be determined.

Secondly, most of viral contaminant was found distributed equally in digestive diverticula (including stomach) and hemolymph. Other parts of body contained little virus throughout observation periods. From a physiological viewpoint, this finding appears understandable. The feeding mechanism of shellfish involves propelling and screening a large quantity of seawater which, for quahaugs, varies from 0.05 to 0.15 liters/hr/g of meat(14). Viral particles in pumped seawater were probably ingested in conjunction with food particles such as algae, then passed through digestive tract, with some retention in the diverticula, but finally were eliminated in feces. This route has been known for some time as the major source of bacterial contamination in shellfish(15).

Recovery of large amounts of virus from, and a continuous increase in viral content of feces as exposure prolonged, indicate that a continuous turnover of virus was occurring in this system. The finding of a high viral plateau maintaining in digestive diverticula for days does not necessarily contradict this assumption, because intake of virus by shellfish might have balanced out by its output during this period. One may also explain the plateau phenomena by supposing that a certain amount of virus is retained in this system without further uptake when a thresh-

old is reached. Data in Table I seem to exclude such possibility.

By the same reasoning, virus turnover in hemolymph of quahaugs may have behaved similarly during plateau period. The present study, however, did not reveal whatsoever how virus was being taken into, and how it was eliminated from the circulatory system. Since there is complete lack of knowledge on how hemolymph is formed and what its fate is in quahaugs, virus turnover in this system remains obscure until more basic information becomes available.

Lastly, it is our major concern as to whether virus in the digestive system has penetrated certain types of cells or chemically adsorbed onto cellular surface. Data in Table II show that virus was not intracellular, and also imply that virus was probably not chemically bound to any type of cells. Thus, it may be assumed from present evidence that virus found in digestive system was only transient. Since food elements are eliminated in feces very rapidly, virus in this system would probably be eliminated in a similar manner. This finding offers a theoretical foundation that virus contained in digestive system can be cleansed readily if contaminated shellfish were placed in clean seawater under appropriate conditions.

In addition to contamination of digestive system, one would be equally concerned with viral contaminants in circulatory system. It is felt that high viral content in hemolymph is at least in part due to the high levels of viral pollution in seawater being used in these experiments. Under natural conditions, contamination level of virus in estuarine water is probably minimal, because multiple environmental factors would dilute as well as inactivate viruses derived originally from sewage. In certain experiments which will be reported elsewhere, when quahaugs were contaminated in aquaria with constant supply of running seawater, viral uptake into their hemolymph was indeed very low as compared with that taken into digestive diverticula. This seems to bear out the assumption that hemolymph of shellfish contaminated in nature would not contain large amounts of virus. In other words, the viral contamination of circulatory system should be an insignificant problem when one deals with naturally polluted shellfish.

Summary. The fate of virus contaminants in shellfish was investigated by using the Northern Quahaug and a strain of type I poliovirus as working models. It was shown that process of viral contamination in quahaugs was a dynamic one. Highest level of contamination was attained within a matter of hours followed by plateauing so long as viral level was maintained in surrounding seawater. Among all organs examined, digestive diverticulum, including stomach and hemolymph were found to harbor majority of contaminants. Further study indicated that viruses contained in these systems most likely did not penetrate into or adsorb chemically onto any type of cells. Implications of these findings have been discussed.

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