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Viral Depuration of the Northern Quahaug[1]

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A study was conducted to evaluate critically the feasibility of using the self-cleansing mechanism as a practical means to obtain virus-free shellfish. Two systems supplied with fresh running seawater, three strains of human enterovirus and the Northern quahaug, were used as working models. Preliminary experiments in the experimental system under arbitrarily selected conditions showed that depuration of poliovirus-polluted quahaugs could be achieved by the method used for the Eastern oyster. The factors affecting viral depuration studied so far included: (i) initial concentration of shellfish pollution; (ii) temperature of seawater; and (iii) salinity of seawater. It was shown that purification of the lightly polluted shellfish was achieved sooner than of the heavily polluted ones. The efficiency of viral depuration was roughly a function of the water temperature within the range tested (5 to 20 C). Reduction of salinity to 50 to 60% of the original level stopped this process completely, but 25% reduction in salinity did not affect significantly the rate of depuration. Preliminary study in the pilot system showed that viral depuration in the large tank appeared to be equally as efficient as that in the small experimental tanks under the particular conditions.

In the past decade, several outbreaks of infectious hepatitis (12, 15, 18, 19, 20, 21) have been traced to the consumption of raw shellfish. The total number of cases reported in these outbreaks amounted to approximately 1,700. Although it is difficult to account for all sporadic cases associated with shellfish, there is indication that such cases do occur from time to time (4). Further, secondary cases resulting from contact with shellfish-associated patients may be sizable on a yearly basis. Clinically, this disease has a protracted course, is extremely debilitating to the patients, and may even result in occasional deaths. Thus, there is a definite need for research in this area with the aim of developing means of minimizing or eradicating this mode of transmission. Effort has been made in this area in our laboratory with the following objectives: (i) to gain more understanding on the basic mechanisms as to how shellfish are able to transmit the infectious hepatitis virus to man; (ii) to evaluate whether or not the depuration process may be used as a practical means in obtaining clean shellfish. The present communication describes the experimental results concerning the latter objective. The pattern of viral accumulation and elimination by the Eastern oyster, *Crassostrea virginica*, has been reported previously (21). Thus, the present study has been devoted to the Northern quahaug, *Mercenaria mercenaria*. Since the human hepatitis virus was not available for this study, three strains of human enterovirus were used as the working models.

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MATERIALS AND METHODS

Viruses. The LSc 2ab strain of type 1 and the Leon 12ab strain of type 3 of poliovirus, and the POW strain of B-4 coxsackievirus, were used in the experiments. The stock polioviruses were grown in primary African green monkey kidney (MK) tissue cultures and were kindly supplied by the Lederle Research Laboratories, American Cyanamide Co., Inc. The virus pools contained approximately $10^{8.0}$ plaque-forming units (PFU)/ml and were stored at −20 C before use. The coxsackievirus seed was obtained from S. Kibrick, Boston University School of Medicine. The stock virus was propagated in our laboratory in 3-oz (90-ml) prescription bottles containing monolayers of African green MK cells. After the cells showed 4+ cytopathic effect (CPE), the bottles were frozen and thawed three times. The fluids were pooled and centrifuged at 2,000 rev/min for 30 min, and the sedimented cell debris was discarded. The virus pool was stored at −20 C until it was used. The virus content was also approximately $10^{8.0}$ PFU/ml.

Tissue culture. Primary African green MK cells were used throughout the experiments. The monkeys, weighing 2 to 3 kg each, were purchased from the Hurtlast and Thoresen Farm, Neptune, N.J. The procedures used for preparation of monolayer tissue

cultures in 3-oz prescription bottles were essentially those described by Hsiung (7).

Shellfish. The Northern quahaug was used throughout the study. The shellfish were purchased from local fishermen, and were of the size served in restaurants as "cherrystones"; each weighed 100 ± 10 g with shell, and measured 2.5 by 3.0 inches (6.4 by 7.6 cm). Upon arrival in the laboratory, they were stored in large tanks in the wet laboratory, supplied with constant incoming fresh seawater from Narragansett Bay, Rhode Island. The period of storage was generally more than 1 week, offering ample time for the shellfish to be adapted to the conditions of this laboratory. This also served as a process of purification of contaminants they had acquired in nature. The volume of shell liquor from each shellfish of this size amounted to 11 to 22 ml. Each digestive diverticulum with stomach weighed from 0.7 to 2.0 g. The combination of the gill and mantle membranes from each weighed 4 to 6 g, and the remaining body, 4.5 to 10.0 g. The shellfish harvested during experiments, if not immediately dissected or homogenized, or both, were stored at −20 C until time for processing.

Preparation of homogenates. In all experiments, each sample contained 5 or 10 quahaugs unless otherwise indicated. First, the shells were scrubbed with soap and water, and were rinsed thoroughly with tap water. The valves were opened by using a sterile clam knife to sever the adductor muscles. The mantle cavity fluids from quahaugs in each group were collected into a sterile beaker. Approximately 30 sec were allowed for draining the fluids from each shellfish, and this process was facilitated by lifting the mantle cavity membranes from the shell surface. Both the volume and weight of the fluid samples were measured. The meat was then shucked, and the shucked meat from the shellfish of each group was pooled and weighed. The meat was homogenized at the median speed of a Waring Blendor for 3 min after adding an equal weight of phosphate-buffered water at *p*H 7.2 or Nutrient Broth. The 50% (w/w) homogenates and the fluid samples were then centrifuged at 2,000 rev/min for 20 min. The supernatant fractions were stored at −20 C. On the day of assay, the samples were quickly thawed in a water bath at 37 C and clarified again at 2,000 rev/min for 20 min. The supernatant fluids were assayed for viral content by plaquing.

Plaque Assay. The procedures were essentially those described by Hsiung and Melnick (8). Throughout the text, the total number of PFU contained in each milliliter or gram of various specimens is presented.

Depuration system and seawater supply. The contact surfaces of the entire system were made of either polyvinyl chloride material or epoxy-based compounds. The seawater was continuously pumped in from the deep part of Narragansett Bay, Rhode Island. Duplicate intakes and pumps were provided in case of mechanical difficulties. The water was pumped into an 800-gal balance tank in the wet laboratory from which the water was distributed to various experimental systems by gravity. The flow rate to each system was controlled by polyvinyl valves. The entire system from the balance tank down to disposal of the waste water is depicted in Fig. 1. The water first passed through a heat exchanger, by which its temperature was maintained at a desired level, 15 to 20 C. After passing through a Purdy type of ultraviolet treatment box (9) for sterilization, the water flowed into a constant head box. From this box, the water was distributed into four mixing boxes, 6 by 9 by 10 inches (15.2 by 22.8 by 25.4 cm) each. In general, virus at appropriate dilutions was pumped into these boxes by a diaphragm pump and mixed thoroughly with the water by flowing through several baffles in the boxes. From each mixing box, the water flowed through a plastic tubing into a tank of 1 by 1 by 2 ft (30 by 30 by 60 cm), where shellfish were kept. The flow rate from the constant head box to the mixing box was usually adjusted to 2 liters per min. With the total capacity of each shellfish tank being 54 liters, the average retaining time of water in each tank was approximately 27 min.

The number of shellfish in each tank in each experiment has never exceeded 120 quahaugs for accumulation phase, whereas the number has not exceeded 50 for depuration phase. During the course of depuration after each sample of 5 or 10 quahaugs was removed, an equivalent number of clean shellfish was added into the tank, thereby keeping the number of shellfish in the tank constant for the entire experiment.

The pilot depuration tank was made of wood, and its interior was painted with epoxy material. The internal dimension was 2.9 by 4 by 6.6 ft (88.4 by 121.9 by 201.2 cm). After passing through a Purdy-type ultraviolet box, the water flowed into this tank from numerous side holes on two polyvinyl pipes on the bottom extending from the front to the middle of the tank. The flow rate into the tank ranged from 32 to 64 liters per min. The total capacity of the tank was approximately 1,700 liters. The tank could hold two layers of eight baskets of shellfish. Each basket held 1.5 bushels of shellfish. During the depuration experiment, these baskets were filled with normal quahaugs of various sizes. Study was carried out on the shellfish which were previously polluted in the experimental tanks and planted among the "dummy" shellfish. In general, the experimental shellfish were planted in unfavorable positions among the dummies, e.g., in the middle of basket, in the lowest level of shellfish in a basket, etc.

RESULTS

Preliminary experiments on viral depuration. Mitchell et al. (17) showed that the Eastern oysters eliminated the poliovirus and *Escherichia coli* very rapidly when the depuration process was carried out in a flow-through system. As a preliminary test of whether this finding holds true with quahaugs, the following experiment was conducted. One hundred quahuags were polluted by appropriate concentrations of the type 1 poliovirus and a strain of *E. coli* in a running water system for 48 hr. The shellfish were then rinsed thoroughly with tap water and transferred into a

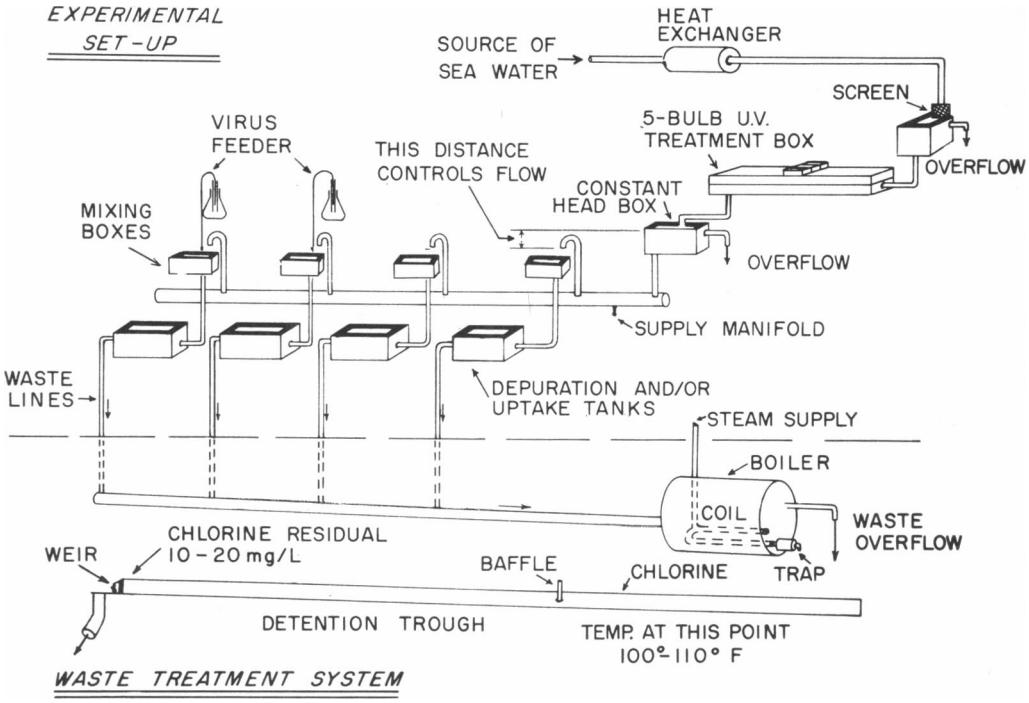

FIG. 1. *Diagram of experimental depuration system and seawater supply.*

clean tank for depuration. At varying times, a sample of 10 shellfish and a sample of seawater in the tank were obtained. The shellfish were processed and the shell liquor and 50% meat homogenates were prepared, as described. All preparations and the seawater samples were assayed for both viral and bacterial contents. The results of viral assays are illustrated in Fig. 2.

As shown, the seawaters contained very little or no virus when the shellfish were depurating in a clean tank. The virus was eliminated rapidly from both the shell liquor and meat of the shellfish under the particular experimental conditions. After 72 to 96 hr of treatment, no virus was detectable by the technique used, whereas small amounts of bacteria persisted in the shellfish for more than 96 hr. These data in general confirmed those observed for the oysters by Mitchell et al. (17).

Factors affecting viral depuration. From the preliminary experiments described above and those reported by Mitchell et al. (17), it appears clear that depuration of virus-polluted shellfish was feasible under proper conditions. The major way in which our experiments and those of Mitchell differed from those described previously (5, 16; S.Y. Feng, *personal communication*) was in the use of running-water system. In view of the encouraging results obtained in arbitrarily chosen

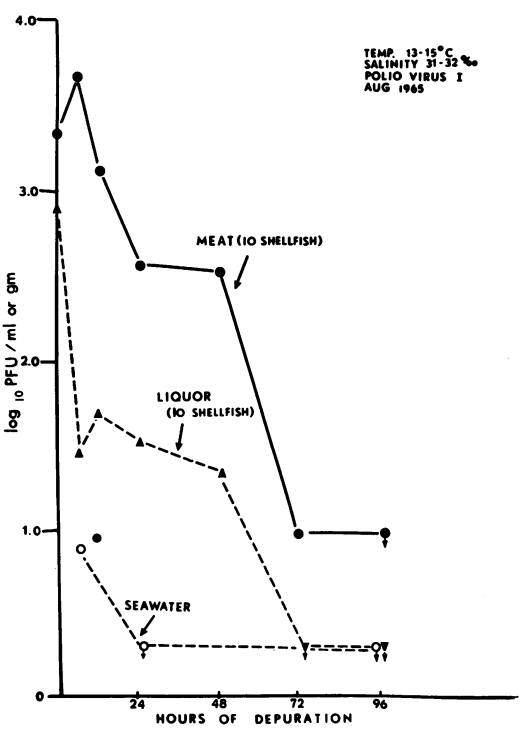

FIG. 2. *Preliminary experimental results of depuration of quahaugs polluted with poliovirus type 1.*

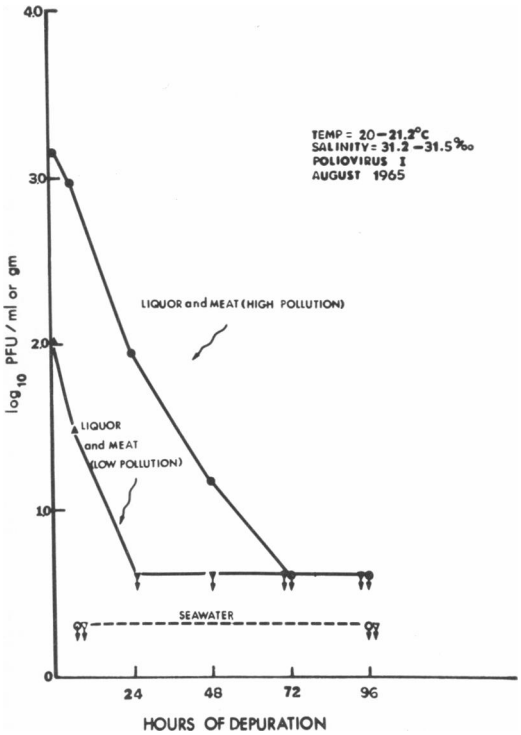

FIG. 3. *Effect of initial viral concentration in shellfish on viral depuration (poliovirus type 1).*

conditions, further effort was made to evaluate thoroughly various factors which may possibly affect the efficiency of depuration. From these experiments, it is hoped, first, that the factors which can facilitate this process may be singled out, and, second, that by using a combination of the favorable factors the depuration process may be developed into a practical means for commercial use. The factors studied thus far are discussed separately below.

Effect of initial viral concentration in shellfish. At the outset, one may suspect that depuration for the lightly polluted shellfish may require less time than for the heavily polluted ones. To document this assumption, the following experiment was conducted. Two groups of 50 quahaugs each were polluted with a high and a low level of the type 1 poliovirus in seawater. After 48 hr, the polluted shellfish were rinsed and transferred into a clean tank for each group. Groups of five quahaugs were harvested from the two depuration tanks periodically up to 96 hr. The shell liquor and meat from the shellfish of each group were pooled and homogenized. All homogenates and seawater were assayed for viral content, with the results shown in Fig. 3. As shown, at the time of starting depuration, the lightly polluted shellfish contained 50 PFU/ml of the homogenate, and the heavily polluted ones contained approximately 1,000 PFU/ml. The time required to depurate the former group to nondetectable level was 24 hr and that for the latter group was 72 hr. This finding definitely bears out the original suspicion that the time of depuration is proportional to the degree of pollution of the shellfish.

The same experimental design was repeated with the type 3 poliovirus. Both the shell liquor and meat samples from each group were collected and prepared separately. The assay results are summarized in Fig. 4. As shown, the lightly polluted shellfish were cleansed at 24 hr, whereas the heavily polluted ones were cleansed at 72 hr. These data confirmed those observed in the previous experiment with the type 1 poliovirus.

Effect of seawater temperature. The effect of water temperature on viral depuration was studied in a series of experiments during the winter of 1965–66. The range of temperatures tested was from 5 to 20 C. The viruses used in these experiments were poliovirus types 1 and 3 and the coxsackievirus type B-4. The general procedures for each of these experiments were as follows. One hundred quahaugs were polluted for 24 hr in running seawater of 15 C, containing approximately 50 to 100 PFU per ml. At the end of pol-

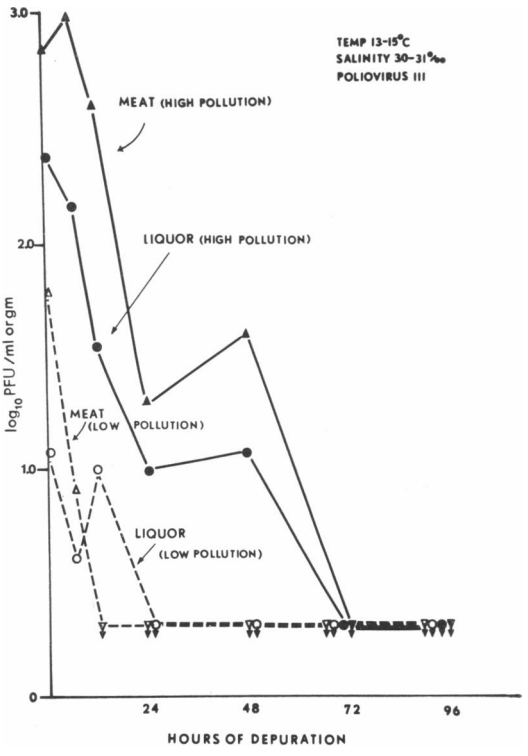

FIG. 4. *Effect of initial viral concentration in shellfish on viral depuration (poliovirus type 3).*

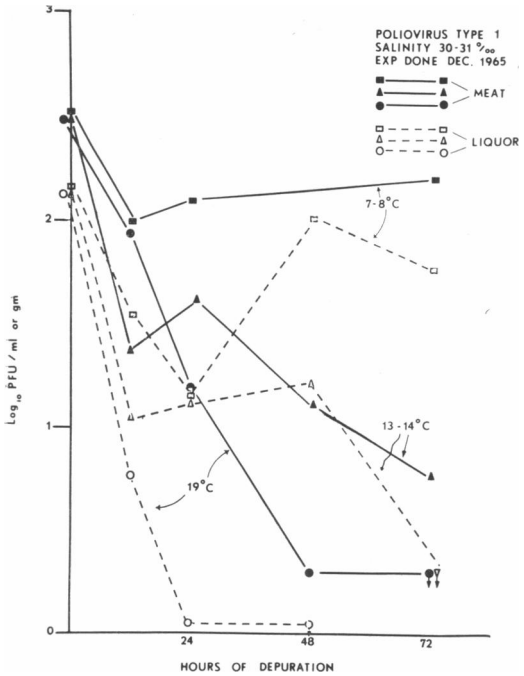

FIG. 5. *Effect of seawater temperature on depuration of poliovirus type 1-polluted quahaugs.*

lution, all shellfish were scrubbed and rinsed with tap water. Two groups of five shellfish were harvested at this time and considered as the zero-hour sample of depuration. The remaining quahaugs were divided equally into three groups, each of which was transferred into a clean tank supplied with seawater at different temperatures. During the next 72 to 96 hr, samples of shellfish and seawater were removed periodically. The shell liquors and meat from the shellfish in each group were pooled and processed in the usual manner. The viral assay for all samples in each experiment was generally done at one time. The results from three experiments are illustrated in Fig. 5–7. Except for the zero-hour sample, all seawater samples were found to contain no detectable virus; thus, these values are not included in the figures.

In Fig. 5 are summarized the results of depuration of the type 1 poliovirus-polluted quahaugs. The three groups of shellfish were treated in the tanks containing seawater at 7 to 8 C, 13 to 14 C, and 19 C. The solid lines represent the results of meat samples, whereas the broken lines represent those of liquor samples. At 19 C, the virus in shell liquors was reduced to nondetectable level after 24 hr of treatment, whereas the virus in meat samples was reduced to the same level at 72 hr. In 13 to 14 C water, the viral content of liquor reached nondetectable level by 72 hr, and that of meat was reduced considerably but has never reached the nondetectable level. In 7 to 8 C water, there was little depuration occurring in either liquor or meat for the entire experimental period.

In Fig. 6 are summarized the depuration results of the type 3 polluted shellfish. The three groups of shellfish were treated with seawater at 5 to 6 C, 14 C, and 18 to 20 C. It may be noted that at 18 to 20 C the viral content of both shell liquors and meat reached nondetectable level within 24 hr. The same level was also reached at lower temperatures, but at a slower pace. The difference in time of reaching the nondetectable level, especially in meat samples, between these two experiments may be attributable in part to the difference in initial level of pollution of these shellfish.

In Fig. 7, the results of depuration of the coxsackievirus-polluted shellfish are summarized. Only the assay results of the meat samples from two groups of shellfish are shown, because a mechanical failure of the third tank occurred in this experiment. The effect of temperature on the efficiency of depuration is clearly shown, confirming, in general, the observations with the polioviruses.

By using only the approximate time required to depurate the polluted quahaugs to contain the

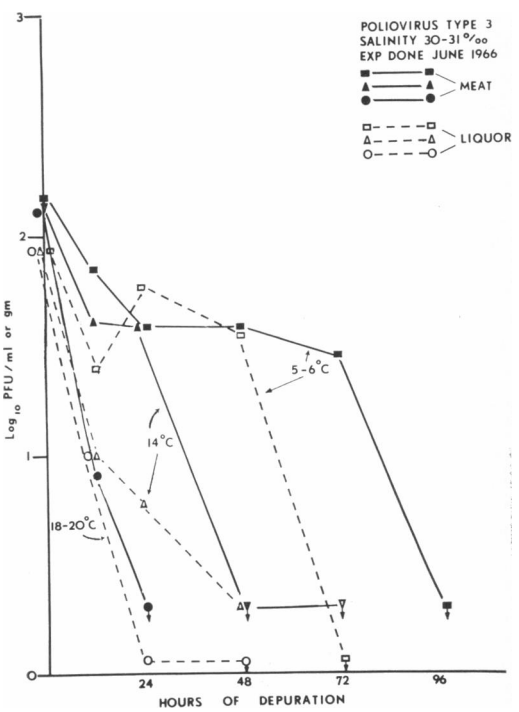

FIG. 6. *Effect of seawater temperature on depuration of poliovirus type 3 polluted quahaugs.*

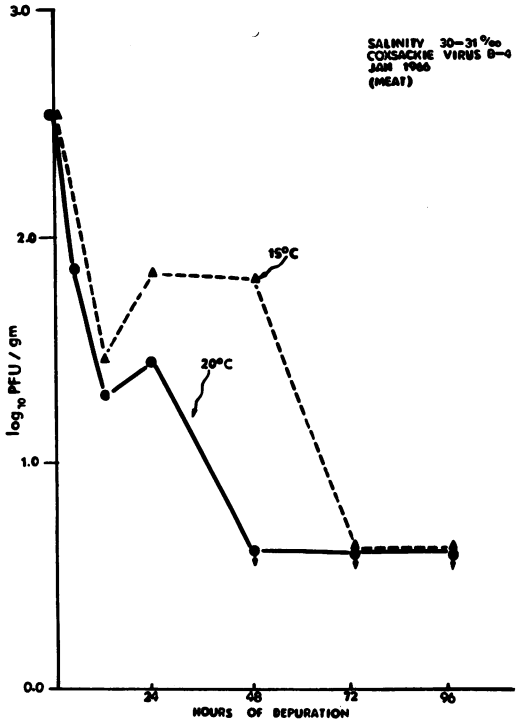

FIG. 7. *Effect of seawater temperature on depuration of coxsackievirus B-4 polluted quahaugs (meat specimens).*

nondetectable level of virus, the data from four such experiments are combined and tabulated in Table 1. It is clear from these results that the efficiency of viral depuration is roughly a function of the seawater temperature within the range tested. The highest temperature used, 20 C, has given the best results. Whether or not this will hold true for quahaugs harvested in other locales or for other species of shellfish remains to be determined. Although temperatures above 20 C have not been tested, on the basis of current knowledge of shellfish physiology one suspects that the efficiency of depuration may be reduced at a temperature not too much above 20 C.

Effect of salinity. The effect of varying salinities of the seawater on viral depuration was studied in several experiments while the water temperature was kept at a constant range. In one experiment, 100 quahaugs were polluted with the type 1 poliovirus in the usual manner for 24 hr. After sampling the duplicate samples of shellfish at the end of pollution, the shellfish were divided equally into three groups and were transferred into clean tanks. The seawater flowing through these tanks was at 18 to 20 C and with the following salinities: 17 to 21, 23 to 28, and 31‰. The quahaugs and seawater were sampled periodically up to 96 hr. The specimens were processed and assayed in the usual manner (Fig. 8 and 9).

In Fig. 8 the data of shell liquor assays are shown, and in Fig. 9 those of meat are shown. Depuration proceeded rapidly in the water with salinities of 31‰ and 23 to 28‰, whereas little depuration was obtained with those treated in seawater with salinities of 17 to 21‰. Although a slightly better depuration was shown for the shellfish depurated in 31‰ salinity than in 23 to 28‰ in this particular experiment, this difference was not observed in other experiments when the salinity of seawater only reduced approximately 25% of the original. It has been repeatedly observed, however, that a reduction of salinity to 50 to 60% of the original seawater completely stopped the process.

Depuration in pilot system. A preliminary study of depuration in the pilot tank was initiated to see what difficulty may be encountered during the process of scaling up. A total of 120 quahaugs were polluted with the type 1 poliovirus and a strain of group A *E. coli* in the experimental system. At the end of 24 hr, three groups of eight quahaugs were harvested and considered as zero-hour samples. The remaining 96 quahaugs were planted among the dummy quahaugs in eight baskets. At varying intervals, triplicate samples of eight quahaugs each were harvested. In general, a sample consisted of one shellfish from each of the baskets. Both liquor and meat from shellfish of each group were pooled and homogenized. The homogenates were assayed for bacterial content immediately and for viral content later. During each sampling, a seawater sample was also obtained and titrated. The results of viral assays from two such experiments are summarized in Fig. 10. The bacterial results will be described by Cabelli et al. elsewhere. As shown, the great majority of samples were cleansed to contain a nondetectable level of virus at 48 hr. There was no detectable virus in all samples at 72 hr. In general, the trend of viral depuration in the large tank ap-

TABLE 1. *Effect of seawater temperature on depuration of enteric viruses (four experiments)*

Prepn	Values	Time required to depurate at			
		20 C	15 C	10 C	5 C
		hr	*hr*	*hr*	*hr*
Liquor	Average	48	60	60	>72
	Range	24–72	48–72	48–72	>72
Meat	Average	48	78	84	>96
	Range	24–72	48–96	72–96	>96

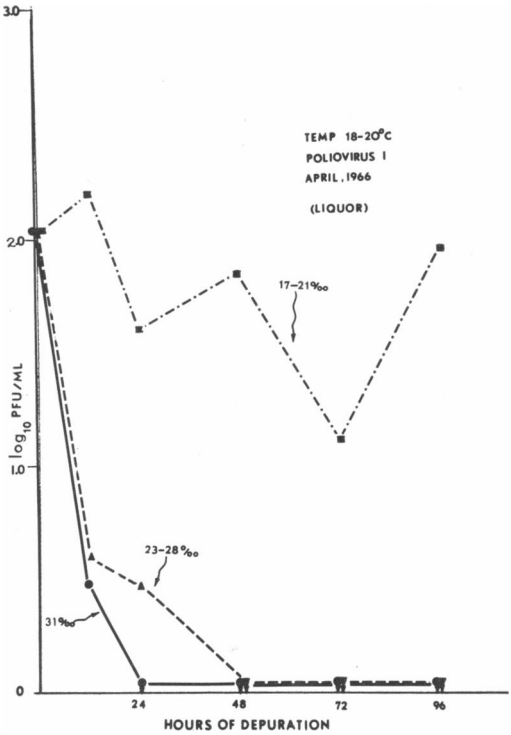

FIG. 8. *Effect of seawater salinity on viral depuration of quahaugs (liquor specimens).*

peared similar to those observed in the experimental system. One of the six samples did not depurate well at 48 hr, indicating variation among samples which might occur from time to time.

DISCUSSION

The present study attempted a critical evaluation of whether or not viral depuration of shellfish may be attained experimentally. If so, what are the best conditions under which this process can be conducted? For studies carried out to date, the working models selected included three strains of the human enterovirus and the Northern quahaug. The problem virus, infectious hepatitis virus, however, was unfortunately not available for this study. It is felt that information obtained with the viruses studied may not be completely without merit for the following reasons. (i) As reported by Metcalf and Stiles (16), strains of coxsackievirus and echovirus were recovered from Eastern oysters from the New Hampshire area. Although only hepatitis outbreaks have been associated with consumption of raw shellfish, the potential hazard of the transmission by shellfish of other types of virus cannot be overlooked. Therefore, cleansing of shellfish of numerous types of enteric virus may be needed in the future, in addition to that for infectious hepatitis virus. (ii) Studies with the model viruses would offer information as guidelines for improvement of depuration conditions, both in terms of biology and of engineering, since the success of viral depuration is probably hinged on shellfish biology. (iii) Biologically, the human enteroviruses simulate in many aspects the hepatitis virus. It is hoped that the information obtained from our study on these viruses is applicable to handling the hepatitis virus-polluted shellfish. We, however, are keenly aware of the possibility, remote as it may be, that the hepatitis virus may behave differently from other types of virus. This problem can only be resolved by testing shellfish which actually are polluted with the hepatitis virus per se.

Not long before the current study was initiated, several laboratories yielded experimental data which had cast some shadow on this approach. From their data, these investigators seemed to imply that, although depuration of bacterially contaminated shellfish had been successful (1, 22), depuration of virus-polluted ones was not likely because of the nature of the contaminants. C. B. Kelly (*personal communication*) first pointed out that the failure of these investigators to depurate shellfish was chiefly due to the experimental systems used. The previous studies were uniformly

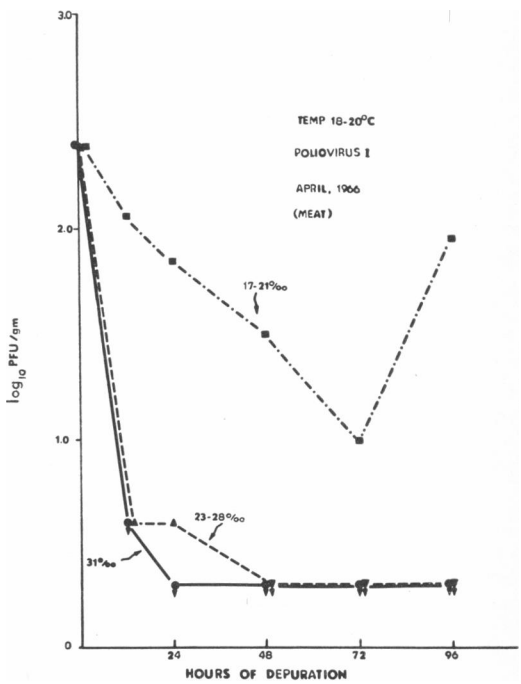

FIG. 9. *Effect of seawater salinity on viral depuration of quahaugs (meat specimens).*

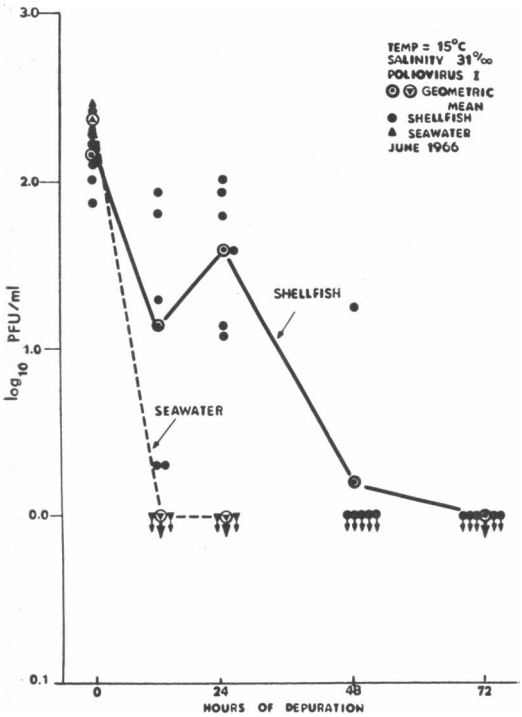

FIG. 10. *Depuration of poliovirus type 1 polluted quahaugs in pilot size tanks (results of two experiments).*

done in systems which contained only stationary water. Although the water was exchanged periodically, in retrospect such a measure was obviously unable to provide the shellfish with adequate condition to function. This reasoning was substantiated first by the work of Mitchell et al. (17), and later by ourselves (13) when running seawater was supplied to the depuration tanks. The two major species of shellfish for raw consumption in this country, the Eastern oyster and the Northern quahaug, were shown to be able to unload their viral contaminants rapidly when they were treated in these systems. The success of these experiments was also partially attributed to the use of the Purdy type of ultraviolet system (9), by which the seawater was completely sterilized before being used to treat the shellfish.

Although under arbitrary conditions, the preliminary results of viral depuration offered sufficient encouragement for the study to continue. Several factors which may affect viral depuration have been evaluated. As shown in Fig. 5–7, the water temperature seems to play a major role in affecting the process of purification. In many experiments, purification of the shellfish from poliovirus contamination was achieved within 24 to 48 hr at 19 to 20 C. This type of result certainly sheds more light on the practicability of the process. Whether or not this observation will hold true for other species of shellfish and the same species of shellfish harvested at other geographical locations remains to be determined.

Although salinity experiments did not reveal a particular range in which shellfish will depurate more efficiently, they provide a definite guideline in that the salinity of seawater used for depuration should not be lower than 50 to 60% of the original levels to which the shellfish were conditioned. Excessive reduction of the salinity is definitely detrimental to the process. As shown recently by C. N. Shuster, Jr., and F. C. Garb (*personal communication*), reduction of salinity to such an extent would also stop the shellfish from functioning.

The first factor studied was the degree of shellfish pollution in relation to the time required for depuration. Since there is little information available concerning what amounts of virus were present in the shellfish which produced human diseases, conclusion from these results would be only conjectural. Metcalf and Stiles (16) have isolated enteric viruses from oysters obtained in New Hampshire areas which were known to be polluted with raw sewage. The viruses present in these specimens apparently were not numerous in view of the technique used to isolate the viruses. According to T. G. Metcalf (Symp. Natl. Conf. Depuration, 1st), by plaquing out some of these specimens, the highest counts were not over 5 PFU per 10 oysters. During the period when Metcalf and Stiles did their sampling, no outbreak of any type of virus diseases was noted. Therefore, one still wonders how much virus would be found in shellfish if they were examined during the hepatitis outbreaks. Since no simple technique is available for isolating the infectious hepatitis virus at this time, to assay viral content of shellfish for this virus is impossible. Although attempts were made to determine what constitutes a minimal infectious dose for the infectious hepatitis virus in human volunteers (2, 11), the data obtained so far appear unrealistic. From these data, one would be reluctant to guess how many viral particles were in these shellfish which produced the illness. Thus, in reality, we have no idea regarding what levels of viral contaminants we are trying to depurate. The problem of the virus concentration in polluted shellfish in the field situation remains to be solved by further investigation. It is felt, however, that the levels of pollution used in the model systems in various laboratories are probably much higher than those which are actually occurring in nature. At least, the experiments conducted in this study have demonstrated that shellfish polluted with low levels of

virus could be purified to a certain level faster than those polluted with high levels. From this guideline, one would be encouraged to deduce that the naturally polluted shellfish would be purified even faster and easier than those containing the lowest concentration of virus tested in the laboratory.

Lastly, the data obtained for depuration studies in both experimental and pilot scales have been encouraging. For the past four decades, the fact that no obvious hepatitis outbreaks were associated with shellfish in some European countries may be due to the practice of depuration (6). All of the evidence indicates that this process may be useful commercially.

At this time, it should be stressed, however, that many problems remain to be solved before adoption of this process in our country. To achieve this step, the many conditions which may influence this process should be explored and studied. Pitfalls in scaling up to a commercial size should be uncovered, and definite measures should be designed to avoid these. After more is learned from using the model systems, other species of shellfish and the same species of shellfish grown in other locations should be thoroughly investigated. Only from such comprehensive studies can a set of generalized conditions and the many exceptions to these be clearly defined.

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