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Viral Depuration by Assaying Individual Shellfish[1]

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A study was carried out to further evaluate the practicability of viral depuration by assaying individual shellfish. The Northern quahaug and a strain of the type 1 attenuated poliovirus were used as the working model. Two types of depuration systems were employed: the small experimental tanks and a pilot-size tank with a capacity of approximately 24 bushels (836 liters) of shellfish. Volumes of the individual shellfish samples were found uniform throughout the experiments when a prior selection for the weight of the shellfish was made. There was also no significant difference in volumes of the individual samples during the course of depuration (24 to 96 hr). Under controlled hydrographic conditions, however, the uptake of virus in individual shellfish varied considerably. In general, the individual variability reached 10- to 100-fold. This wide variation would explain the variability of viral contents obtained in pooled samples during depuration as reported previously. During a later phase of depuration, although a great majority of shellfish were free of the virus, a few still harbored minimal amounts of contaminants. The presence of virus in some of the shellfish after various periods of depuration would, theoretically, be obscured by the pooling of the sampled shellfish. Further examination of the negative samples by assaying larger quantities than those routinely used revealed that a few still contained virus. To simulate naturally polluted shellfish as closely as technically possible, shellfish were polluted with minimal amounts of virus. The shellfish were cleansed more rapidly by the depuration process than were those polluted with more virus. Since the naturally polluted shellfish were shown to contain less virus than those studied in the laboratory, it is anticipated that the former type of shellfish may be cleansed more readily by this process within a reasonable period of time. Justification for a field trial of depuration in this country is presented.

Viral pollution of various species of shellfish has been well documented by epidemiological evidence (1, 2, 8, 11-14) as well as by laboratory studies (3, 4, 6, 9). Consideration has been given to developing the self-cleansing mechanism of shellfish as a practical, stopgap measure for this problem before the complete abatement of water pollution is achieved. In a previous communication (7), the effect of several parameters on depuration of virus-polluted Northern quahaugs, *Mercenaria mercenaria*, was presented. The time of depuration required to attain a desired quality is related to the initial level of viral contamination. The rate of viral depuration is also roughly a function of the temperature of the seawater used within the range tested, i.e., 5 to 20 C. The water salinity used for depuration should not be lower than 75% of the original level to which the shellfish has been acclimatized. Under these favorable conditions, the shellfish artificially contaminated with poliovirus and coxsackievirus were cleansed within 48 to 72 hr of depuration treatment. Another report (T. G. Metcalf, *in preparation*) describes a study in which the Eastern oysters, *Crassostrea virginica*, were similarly cleansed of their viral contaminants under controlled conditions. In the aforementioned reports, each of the samples examined consisted of a pool of at least 5 shellfish and, in most instances, 20 shellfish. The pitfall of using pools of shellfish is that the presence of virus in undepurated animals may be obscured by the majority of cleansed animals serving as a diluent of the sample. Although the uncleansed shellfish may be very few in number and may harbor minimal amounts of virus, they would still serve as carriers of virus that may result in human illness. For a more critical assay of the potential hazard of depurated shellfish, the current study was initiated to investigate the following aspects relevant to the future practice of commercial depuration: variability of the volumes of individual shellfish samples and the effect, if any,

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of depuration upon the volume; variability of viral accumulation in shellfish under controlled environmental conditions; variability of viral contents in shellfish and the percentage of animals being cleansed after various intervals of depuration; and the depurability of shellfish polluted with low levels of virus, simulating the natural situation as closely as the present virological technique permits.

MATERIALS AND METHODS

Shellfish. Northern quahaugs were used throughout the experiments. The size and condition of the shellfish and the manner in which they were handled in the laboratory were previously described (7).

Virus. The LSc 2ab strain of type 1 poliovirus was used in all experiments. The stock virus, which was grown in primary African green monkey kidney (MK) cells, was supplied by the Lederle Research Laboratories, American Cyanamide Co., Inc. The virus pool contained approximately $10^{7.5}$ plaque-forming units (PFU/ml) and was stored at −20 C before being used.

Tissue culture. Primary African green MK cells were used for assaying all samples. The methods for preparing the cultures have been described (7).

Depuration system and seawater supply. The experimental system and seawater supply in the wet laboratory have been described (7).

The pilot depuration system was as described previously (7), with the following modifications. The depuration tank held three layers of wire baskets, and each layer contained eight baskets. In each basket, about 1 bushel (35 liters) of quahaugs was placed. The depth of shellfish in each basket did not exceed three layers of shellfish of various sizes.

Pollution of shellfish. All quahaugs studied were polluted with the type 1 poliovirus in the experimental system. The method of pollution was as previously described (7). The seawater flowing through each experimental aquarium was kept at 2 liters/min. The virus diluted in distilled water was pumped into the mixing box at a rate of 1 ml/min. The desired degree of shellfish pollution was controlled by varying the dilution of the virus. Prior to the beginning of viral pollution, the shellfish were conditioned for at least 24 hr in the aquarium, where the seawater temperature was maintained at 15 C and the salinity at 30 to 31‰. Under the same conditions, the shellfish were polluted for 20 to 72 hr. After pollution, the quahaugs were rinsed with tap water and transferred into a clean aquarium or into the pilot tank for depuration. During the depuration phase, the hydrographic conditions were identical to those described above, except that the temperature of the seawater was raised to approximately 20 C.

Preparation of homogenates. Immediately after the shellfish were sampled, they were stored at −20 C until further processing. At this time, the shells were washed with soap and water. The entire contents of each shellfish, including mantle cavity fluids and meat, were placed in a metal homogenizing cup and blended at median speed for 120 sec (Lourdes). The homogenates were clarified by low-speed centrifugation. From each sample, 10-ml samples were put into three to four test tubes and stored at −20 C. Immediately before assaying, the samples were thawed rapidly in a water bath at 37 C and subjected to another cycle of low-speed centrifugation for clarification. The samples were diluted in appropriate amounts of the nutrient broth (Difco).

Plaque assay. The procedures were essentially those described by Hsuing and Henderson (4).

RESULTS

Variability of the volumes and effect of depuration on the volumes of shellfish samples. Approximately 20 experiments were carried out to evaluate viral depuration under varying conditions by assaying individual shellfish samples. The sample was prepared by pooling and homogenizing the total content of both shell liquor and meat from each quahaug as previously described. The volumes of all samples throughout these experiments were measured. This information serves as the base line for later evaluation of the individual variation of viral uptake and elimination by the shellfish. The results from one typical experiment carried out in the experimental tank system are summarized in Table 1. As shown, the average volume of 20 samples from the polluted shellfish before depuration was 40.1 ml, ranging from 35 to 44 ml. The shellfish with shells originally weighed from 90 to 110 g; 1 ml of sample weighed approximately 0.8 g. Thus, the combination of meat and liquor of one sample constituted roughly 30% of the whole shellfish. After various periods of depuration, the average volumes of the samples ranged from 37.9 to 39.1 ml. As shown by the 95% limits of the average volumes, there appeared to be no significant difference in the volumes of shellfish samples before and after depuration of 24 to 72 hr.

Viral accumulation examined by individual assays. For all practical purposes, the process of viral pollution of shellfish was carried out in the experimental tanks and lasted 20 to 24 hr for most experiments. After this period of pollution, a number of shellfish were sampled, processed, and assayed for viral contents. These values are considered the final degree of contamination and

TABLE 1. *Variability of volumes and effect of depuration on volumes of individual quahaug samples*

Hr of depuration	No. of samples	Avg volume	Range	95% Limits
		ml	*ml*	*ml*
0	20	40.1	35–44	35.2–45.0
24	20	39.1	32–44	31.4–46.8
48	20	37.9	32–44	30.6–45.2
72	20	39.1	34–44	32.7–45.5

were used as the base line for evaluating the efficacy of depuration. Logically, individual variations in initial contamination would be reflected in the values obtained later from the depurated animals at various intervals. This effect would probably be observed more clearly in the early depuration samples than in the later ones. From repeated experiments, the viral accumulation in shellfish persistently showed a wide range of variability. Thus, it was felt that this variation might be reduced by prolonged pollution. In one attempt, the shellfish were polluted for 72 hr. The results from three experiments are illustrated in Table 2. As shown, a greater variability of viral accumulation was observed in shellfish in experiment G62, whereas less variation was observed in experiments G68 and G86. The prolonged period of pollution may have played a role in reducing the degree of variation in viral uptakes as indicated by the data of experiment G86, where the pollution was carried on for 72 hr. It appears, however, that there must be other factors which may affect the uniformity of viral uptakes by the quahaugs. These may include the physiological condition of the shellfish, hydrographical conditions, overcrowding of the shellfish in aquaria, or others. At any rate, although viral pollution was carried out under similar conditions at different times, the range of individual uptakes could be as small as 10-fold, e.g., 15 to 150 PFU/ml in experiment G68 and <20 to 460 PFU/ml in experiment G86, and as large as 100-fold, e.g., 2 to 244 PFU/ml in experiment G62. When the 20 quahaugs from each experiment were pooled as in the previous reports (7, 10), the values of the pools for the three experiments were 55, 92, and 160 PFU/ml, respectively. The range of PFU values of the pooled samples was far less widespread than those shown by the individual assays. The former values would definitely obscure the actual occurrence of viral uptakes in these animals.

Effect of viral depuration examined by routine individual assays. Routinely, each sample was assayed by plaquing 0.25 or 0.50 ml in each of two 3-oz (85 g) MK bottles. When no plaque was observed in these two bottles, the value was scored as <2 or <1 PFU/ml for that sample. All experiments conducted were initially tested in this manner. The results from a typical experiment (G62) are summarized in Fig. 1. As shown, the average value of the zero-hour samples was 92 PFU/ml. If these quahaugs were pooled and assayed, the pool, theoretically, should give this value. At the 24th hr of depuration, the average value was 5 PFU/ml or less. At this time, five shellfish, or 25%, were shown to contain no detectable virus, whereas, four shellfish, or 20%, still contained 10 PFU/ml or more. At the end of 48 hr, if the pool were assayed, the value would be <2 PFU/ml. Yet, six shellfish, or 31%, still contained 2 PFU/ml when they were examined individually. Theoretically, only at the end of 72 hr would both the individual assay and the assay of the pool demonstrate no detectable virus when the routine examination procedures were used.

From the results of four such experiments in which initial levels of contamination were moderate—the average values of 20 samples before depuration were from 54 to 159 PFU/ml—the percentage of shellfish attaining the levels of <2 PFU and ≤ 2 PFU/ml at various time intervals of depuration were computed (Table 3). At 24 hr

TABLE 2. *Individual variability and effect of pollution time on viral accumulation*

Expt no.	Avg	Range	Standard deviation
24-hr pollution			
Expt G62	92.0[a]	2–244	85.6
Expt G68	54.9	15–150	33.8
72-hr pollution			
Expt G86	159.5	<20–460	100.14

[a] PFU/ml of sample.

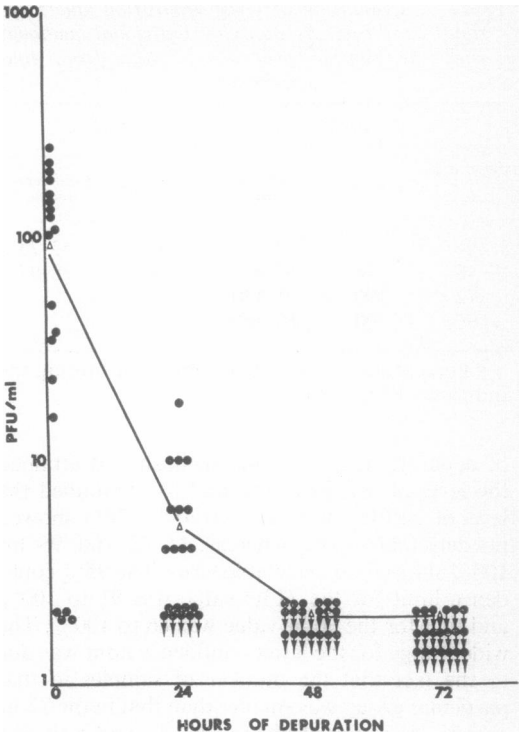

FIG. 1. *Depuration of shellfish in the experimental system (experiment G62) assayed by the routine procedures.*

TABLE 3. *Evaluation of viral depuration under optimal conditions by assaying individual quahaugs with the routine procedures (data from four experiments)*

Hr of depuration	PFU/ml (<2)		PFU/ml (≤2)	
	Per cent[a]	Confidence limits	Per cent[a]	Confidence limits
24	45	34–56	64	53–75
48	78	67–87	94	88–97
72	100	91–100		
96	100	86–100		

[a] Percentage of individual samples attaining the indicated PFU value.

of depuration, 45% of the shellfish had attained the level of <2 PFU/ml, and 64% attained the level of 2 PFU/ml, or less. At 48 hr, 78% showed no detectable virus, whereas, at 72 and 96 hr, 100% showed no detectable virus. The 95% confidence limit for the 72-hr value was 91 to 100% and that for the 96-hr value was 86 to 100%. The wider range for the latter confidence limit was due to the fact that the number of samples in that particular group was smaller than that in the 72-hr groups. As reported previously (7), under similar conditions only 48 hr was usually required to cleanse the virus in both liquor and meat. By using individual assay, however, complete cleansing required 72 hr. This difference seems attributable to the fact that few shellfish still harboring minimal numbers of virus at the end of 48 hr of treatment were discovered by assaying the individual shellfish samples.

Effect of assaying larger amounts of samples. The foregoing data clearly indicate that individual assays revealed that a few shellfish were not cleansed by the depuration process within the time studied. One would still wonder whether the negative samples might have harbored even fewer viruses which could not be detected by the routine test procedure. In several experiments, the depurated samples were reexamined by plaquing out larger volumes. The results from two typical experiments are illustrated in Fig. 2 and 3. By testing 1 ml of each sample, 6 to 20 samples were found to contain virus at 48 hr, whereas, by testing 2.5 ml of the same samples, 11 showed presence of virus (Fig. 2). At 72 hr, the routine test showed that none of the samples contained virus; the test of the larger volume revealed 5 samples still containing virus. In Fig. 3, the results from an experiment using the pilot tank system are illustrated. At 48 hr, one sample was shown to contain virus by routine testing, whereas, when a larger volume of each sample was tested, 11 samples were shown to have virus. At 72 hr, there was still one sample showing a detectable amount of virus by the latter test.

The average PFU/ml of each group and the percentage of shellfish showing no detectable virus by the particular tests were computed from the data of the two experiments shown in Fig. 2 and 3

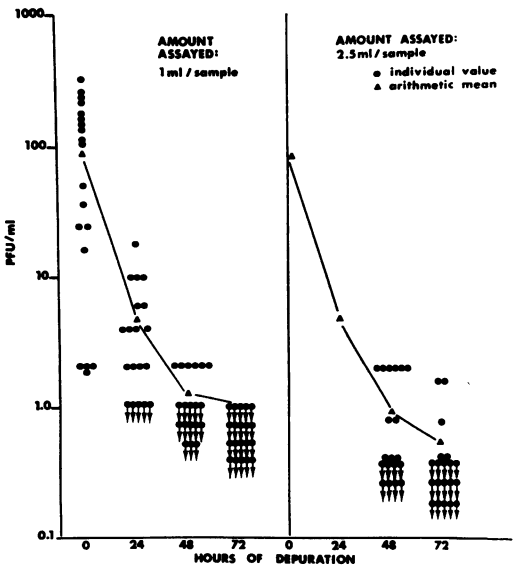

FIG. 2. *Effect of assaying different quantities of the individual samples on evaluation of viral depuration in the experimental system.*

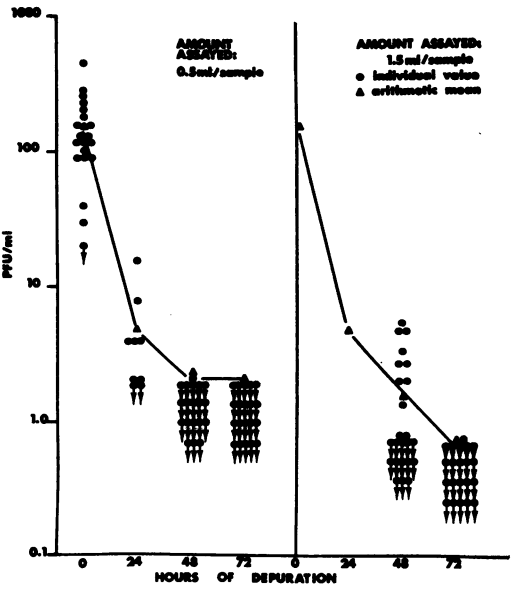

FIG. 3. *Effect of assaying different quantities of the individual samples on evaluation of viral depuration in the pilot tank.*

TABLE 4. *Effect of assaying different volumes of shellfish samples on residual virus after various periods of depuration*

Expt no.	Amt assayed	Length of depuration (hr)							
		0		24		48		72	
		PFU/ml[a]	% No virus[b]	PFU/ml	% No virus	PFU/ml	% No virus	PFU/ml	% No virus
	ml								
G62	1.0	87.40	0.0	4.75	20.0	<1.32	68.4	<1.00	100.0
	2.5					<0.95	42.1	<0.54	75.0
Difference						0.37	26.3	0.46	25.0
G76	0.5	437.90	0.0	14.17	25.0	<2.00	83.0		
	2.5					<1.68	25.0		
Difference						0.32	58.0		
G86	0.5	159.50	5.0	4.89	22.2	<2.00	94.7	<2.00	100.0
	2.5					<1.60	54.2	<0.67	95.2
Difference						0.40	40.5	1.33	4.8

[a] Average PFU/ml of the individual samples harvested at that time.
[b] Percentage of samples showing no detectable virus in the amount tested.
[c] Carried out in pilot depuration tank.

and an additional experiment of a similar type carried out in the experimental depuration system (Table 4). Further testing with larger volumes revealed definite differences in both parameters. At 48 hr of depuration, the differences in the average PFU values shown by the two tests were small, and the differences in the percentage of shellfish still harboring virus were considerable. The difference of the latter parameter at 48 hr ranged from 26.3 to 58.0%. At 72 hr, the differences became less apparent. This finding seems to indicate that prolonged depuration is beneficial in eliminating the small numbers of residual virus in the contaminated shellfish.

Depuration of shellfish polluted with low levels of virus. The field survey results reported by Metcalf and Stiles (9; T. G. Metcalf, *in preparation*) indicate that the viral pollution of shellfish under natural conditions was definitely minimal when the shellfish were obtained from prohibited and conditionally approved areas. Metcalf (*in preparation*) stated that no more than 5 PFU were found in each of the samples examined in his laboratory. Each sample in his study contained 10 Eastern oysters. Thus, even by assuming that all of the virus found in one sample came from a single shellfish, the total viral content in each shellfish would not exceed 5 PFU. For practical consideration, then, demonstration of the efficacy of the depuration process to cleanse shellfish polluted with very low levels of virus becomes essential. One experiment was carried out to illustrate this point. The virus level in shellfish used in this experiment was so low that an accurate assay for the samples can reasonably be achieved. A group of 50 quahaugs were polluted to contain an average of approximately 20 PFU/ml of the samples. At various time intervals,

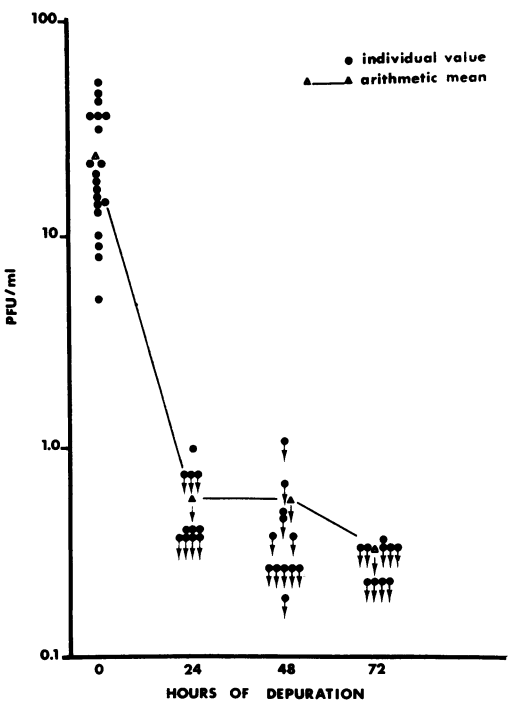

FIG. 4. *Depuration of quahaugs polluted with low levels of poliovirus (experimental system, experiment C91).*

groups of shellfish were sampled and examined repeatedly by plaquing out larger volumes of each sample than were routinely used. The results are illustrated in Fig. 4. At 24 hr of depuration, 4 out of 11 shellfish were found to contain virus. At 48 hr, no shellfish was found to contain virus. The slight variation in all the "less than" values was a result of the different number of tissue culture bottles used for testing each sample. Although all samples were tested with an equal number of tissue culture bottles initially, some of these failed to yield valid information because of bacterial contamination or toxicity (or both) of the tested material to the tissue culture cells. At 72 hr of depuration, all but one showed no virus. This result also confirms the findings of the previous experiments in which the variations were even more apparent when higher initial pollutions in shellfish were used. Essentially, this experiment demonstrates that less time is required to depurate the shellfish that are polluted with low levels of virus.

DISCUSSION

The present study has further evaluated the efficiency of depuration of virus-polluted shellfish by careful examination of each individual animal during the entire course of the experiments. The data obtained have answered a number of questions relating to viral depuration of shellfish and have offered some insight into the problem of infectious hepatitis virus transmitted by shellfish. In previous reports (7, 10) containing data of viral elimination in the Eastern oysters and the Northern quahaugs, definite variations in viral content in the samples containing a pool of 5 to 20 shellfish were shown. Judging from the data of the present study, variation of the pooled samples during various stages of depuration is, at least in part, due to the variability of viral uptake by the individual animals. Under identical conditions, the viral uptake of individual shellfish varied up to 100-fold (Table 2). This variation would definitely be reflected in the early phase of depuration, even though the elimination rates might have been more uniform. It is interesting to note, however, that the large variation in viral accumulation in individual shellfish was not observed when the experiments were carried out in aquaria containing only stationary seawater (5). The lesser variation may possibly be attributed to the following factors. Since shellfish under this condition were functioning at their minimal capacity, the degree of uptake was probably very low, so that less variation resulted. While this type of experiment was being conducted, the shellfish were placed far apart from one another so that they could function at all times, even though the activity was of low magnitude.

One of the major concerns for viral depuration study has been whether the small number of depurated shellfish harboring virus would be masked when the pools of shellfish were tested. The majority of shellfish containing no virus definitely would dilute those still harboring small numbers of virus. The data presented appear to bear out this assumption, in spite of the fact that actually larger amounts of the samples were tested than those assayed for the equivalent pooled samples. The individual assay has increased the sensitivity of the testing system. Furthermore, it has offered more understanding as to how depuration actually works. It is suspected that the viral depuration process depends heavily upon the physiological activity of the shellfish, although the present study has not documented this hypothesis. There is little doubt that the great majority of shellfish are capable of cleansing themselves when they are subjected to an ideal and clean environment. On the other hand, a very small number appeared not to be functioning well. Thus, a few shellfish still harbored virus after 48 to 72 hr of depuration. This seems to be the inherent drawback of the depuration process that has to be faced in the future if the process is to be adopted for commercial practice. This difficulty, hopefully, may be overcome by prolongation of the treatment.

Serious attempts were made in the present study to determine the residual amounts of virus after various intervals of depuration. More extensive testing did reveal the small numbers of virus which failed to depurate (Fig. 2–4). Regardless of the initial levels of contamination in shellfish, viral depuration proceeded rapidly in the first 48 hr. After 48 hr of treatment, most shellfish were free of more than 90% of their virus. The small numbers of shellfish containing a residue of virus might result from physiological inactivity of the shellfish during depuration after they had reached a high contamination level or had shown very low contamination levels throughout the entire experiment.

Since the naturally polluted shellfish were shown to contain fewer virus than those which have been studied in the laboratory, it is anticipated that the small amounts of virus would be cleansed more readily by this process under optimal conditions within a reasonable period of time. From the information obtained in the field and in the laboratory to date, it may be deduced that depurated shellfish from prohibited and conditionally approved areas would most likely attain a quality virologically equivalent to, if not

better than, those harvested from approved areas but not depurated.

Undoubtedly, the major public health problem for consumption of raw shellfish is the possibility of transmitting the infectious hepatitis virus. This potential hazard has been well documented (1, 2, 8, 11–14) in the past 10 years. Unfortunately, this virus is not available for laboratory study at this time. Direct evidence to show that depuration of hepatitis virus may be achieved, as has that of other human enteric viruses, is not forthcoming in the near future. If one is convinced that elimination of any foreign biological particles by shellfish chiefly relies upon physiological activity, depuration of hepatitis virus would seem to be likely. Since no better measure for coping with this problem is foreseeable, the depuration process should be tried as a stopgap measure to obtain shellfish of better quality. Thus, the health hazard associated with consumption of raw shellfish may be reduced to a minimal level. To implement this process for commercial practice throughout the country at this time may appear to be premature because of the extra expenditure imposed upon the industry and because of the lack of experience in mass processing. In order to critically attest whether this process is indeed helpful to this problem, and to gain practical experience with this process, at least several demonstration plants should be established at strategic locations along the coastal regions. The products from these plants may be distributed to consumers who are willing to try this type of commodity. Epidemiological follow-up may be carried out on those who have consumed the depurated products. This approach would seem to bypass the difficulty encountered in the laboratory, where the hepatitis virus cannot be studied readily. Also, experience obtained from the limited and controlled field study would lay the groundwork for the future implementation of this process on a larger scale. It should be stressed, however, that the demonstration plants should be carefully monitored by public health workers during this period, so that the human failure factor can be definitely ruled out.

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