biliary pigment is derived from sources other than hemoglobin produced by the breakdown of the erythrocytes in the blood stream. This is believed to be due to a normal existence of a certain degree of what has been called ineffective erythropoiesis. It is conceivable that this ineffective erythropoiesis can be increased in the polycythemia of the high altitude.

Actually the finding described by Merino (2) of an increment in the plasma bilirubin and in the excretion of fecal urobilinogen during the first and second weeks of exposure to a sea level environment in subjects brought down from high altitudes can be explained on this basis rather than on the basis of an abnormal red cell destruction. Reynafarje's findings(15) of a normal red cell survival by the radioactive chromium method in a group of subjects residents at the high altitude after descent to sea level seem to support this point of view.

We believe that the study of the degree of this ineffective erythropoiesis in the polycythemia of the high altitude can explain the increment of plasma bilirubin seen in natives residing at high altitude.

Summary. In 19 healthy natives living at high altitude, the 24-hour urine excretion of glucuronic acid before and after ingestion of 4 g of salicylamide was measured. No significant difference was found in the amount of salicylamide glucuronide excreted by the subjects of this group when compared with the amounts excreted by a control group.

Our results are compared with the work of other authors and the probable mechanisms of this indirect hyperbilirubinemia are discussed.

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Viral Pollution of Shellfish. 1. Some Basic Facts of Uptake.* (31520)

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Several hepatitis outbreaks have been implicated with consumption of raw shellfish (1-4). This finding prompted controlled studies in various laboratories. Hedstrom and Lycke(5) showed that the European oysters, *Ostrea edulis,* were capable of picking up significant amounts of poliovirus from their environmental seawater. Metcalf and Stiles(6) found the Eastern oysters, *Crassostrea virginica,* equally efficient in doing so. It is felt that since these workers used only stationary seawater aquaria, viral uptake by shellfish in their experiments did not exceed those present originally in seawater. By using an aquarium supplied with seawater running through, Mitchell *et al*(7) demonstrated that Eastern oysters were able to concentrate

* Contribution No. 18 from this Center.

poliovirus to 10-60 times that present in surrounding water. Our previous communication (8) on the Northern quahaugs, *Mercenaria mercenaria,* in a stationary water system essentially confirmed Metcalf and Stiles. In a later report(9), however, it was shown that quahaugs could also pick up greater amounts of virus when polluted in a running-water system. All available evidence points up to the possibility that shellfish may serve as potential vectors in transmitting human enteric viruses, but it is felt that some links are missing between available experimental data and those occurring in natural conditions. The present study concerns basic phenomena involved in viral pollution of shellfish. A strain of attenuated poliovirus and the Northern quahaugs were used as working models. Three particular aspects have been investigated: (a) Relation between levels of viral pollution of surrounding water and degree of shellfish contamination. (b) Patterns of viral pollution of shellfish in simulated natural environment. (c) Basic mechanism by which shellfish pick up virus from environment.

Materials and methods. Virus. LSc 2 ab strain of type I poliovirus was used throughout experiments. Stock virus used was grown in African Green Monkey kidney (MK) tissue culture (TC) and kindly supplied by Lederle Research Laboratories, American Cyanamid Co., Inc. Properties of this virus have been described(8).

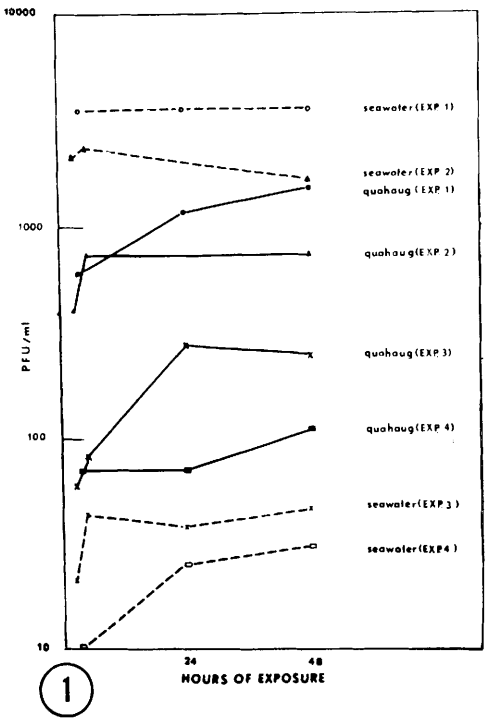
Tissue culture. Primary African Green MKTC cells were used throughout experiments. Procedures for trypsinization and preparation of 3-ounce prescription bottles were essentially those described by Hsiung (10).

Plaque assay. General procedures used were those described by Hsiung and Melnick (11).

Shellfish. The Northern quahaugs, hard clams, used in all experiments were of commercial size for 'cherry stone' in restaurants. With shells each shellfish weighed 100 $\pm$ 10 g and was approximately 2.5 $\times$ 3 inches in size. Conditions of shellfish and procedures for preparation of homogenates have been described(8).

Experimental aquarium. Seawater used for experiments was continuously pumped from Narragansett Bay, R. I., into a 900-gallon balance tank in a wet laboratory and excess seawater was returned to bay. Water flow, from near the bottom of tank, was regulated by a polyvinyl chloride ball valve. All water contact surface in the entire system was nonmetallic. Seawater from the gravity feed tank first passed through a heat exchanger which maintained water temperature at approximately 15-20°C. The water then passed through a Purdy type of UV treatment unit (12) to a mixing box of 6 $\times$ 7 $\times$ 12 inches where virus was added to inflowing seawater by a diaphragm pump and was thoroughly mixed with the water by going through several baffles in the box. Virus contaminated seawater flowed into the experimental aquarium at a rate of 2 liter/min. Each aquarium was 1 $\times$ 1 $\times$ 2 feet in size, and made of wood with polyvinyl chloride lining. Functional capacity of each aquarium was approximately 54 liters. At the above flow rate, calculated flow time through the entire aquarium was about 27 minutes.

Experimental results. Effect of virus level in water on uptake. Although viral uptake by shellfish has been repeatedly shown, it has never been defined what role, if any, might be played by levels of virus in seawater upon the degree and efficiency of uptake. To clarify this point, 4 experiments were carried out in August 1965, as follows: Each experiment involved 15-20 quahaugs polluted with a high or low level of virus in a flow-through water system. Groups of 5 quahaugs were harvested at varying intervals. During each harvest, a seawater sample was obtained by pooling 10 ml of seawater withdrawn carefully from the aquarium at each of 5 selected positions as close as possible to shellfish. Liquors and meats from the shellfish of each group were pooled and 50% homogenates in phosphate buffered water, pH 7.2 were prepared. All homogenates and seawater samples were assayed for viral content with results shown in Fig. 1. It may be observed from these data that levels of contamination achieved in quahaugs were generally less than those in seawater when high viral levels in seawater



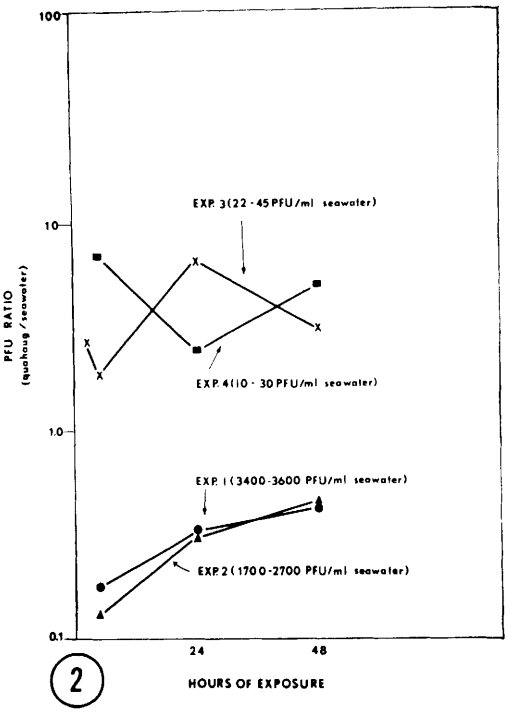


FIG. 1. Viral uptake by quahaugs in seawater polluted with different levels of Poliovirus I.
FIG. 2. Relation of viral uptake and levels of viral pollution.

were used. On the other hand, contamination of quahaugs was greater than that in water when low viral levels in water were used.

From these data, the ratio of viral content in shellfish to that in seawater was computed and is illustrated in Fig. 2. The relationship between degree of contamination of shellfish and virus level in surrounding seawater is clearly shown. A comparatively higher uptake of virus in shellfish was achieved in low viral pollution of seawater and *vice versa*. These results seem to indicate that a certain quantitative limitation was imposed on the viral uptake mechanism of shellfish. Thus, it appears that the basic mechanism of viral uptake by shellfish might be mediated by a chemical reaction.

Viral uptake in simulated natural environment. To clarify patterns of viral pollution of shellfish which might have occurred under natural conditions, one experiment was conducted by particularly using sediments. The bottom of the aquarium was covered completely and evenly with 2 inches of autoclaved sand obtained from Narragansett Beach, R. I., on which 9 quahaugs were placed far apart from one another. Running seawater was then allowed to flow slowly into the tank for a 24-hour period before artificial pollution was begun in order to condition shellfish to new environment. It was observed that within 1-2 hours all quahaugs were dug into sand with their siphons stretched out to the greatest extent and pumping actively. The next day, appropriate amounts of diluted virus were introduced continuously into inflow water. Amounts of virus were adjusted to yield a level of approximately 10 PFU/ml of seawater in the tank. One shellfish and a water sample were removed periodically for a period up to 3 weeks. All samples were stored at —20°C until further processing. Quahaugs were thawed in a petri dish at room temperature. By the end of 1 hour, the total amount of shell liquor and majority of hemolymph were drained out. Meat was then taken out of the shell and digestive diverticulum with the stomach in its center

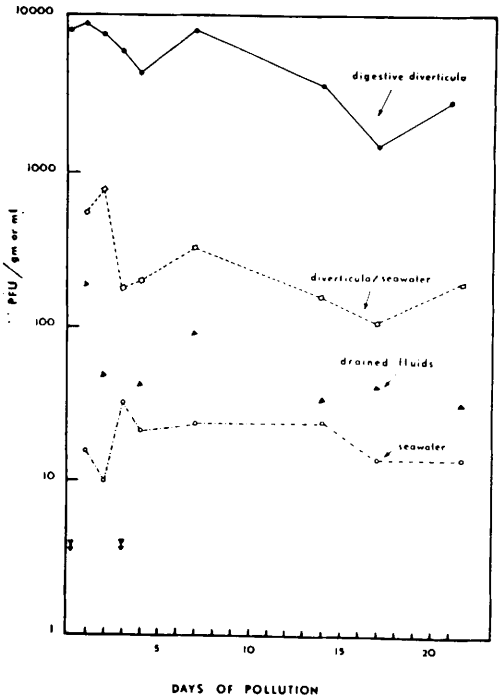


FIG. 3. Uptake of Poliovirus I by quahaugs in simulated natural conditions.

was dissected out. Twenty % homogenates in Hanks' balanced salt solution (HBSS) were prepared for this organ and remaining body. All homogenates, shellfish fluids and seawater samples were assayed at one time with results illustrated in Fig. 3. Since no virus was detected in all homogenates of remaining part of body, these values are not included in the Figure.

As shown, viral contents in drained fluids varied considerably from one specimen to another. In general, their values were not far from those of seawater. Digestive diverticula contained 3000-9000 PFU/g of poliovirus. The factor of concentration by this organ from seawater is illustrated by the ratio of viral content in diverticula to that in seawater. Such degree of viral concentration by shellfish was probably in part attributable to experimental conditions which simulated their natural environment.

Drained fluids from each of 100 ± 10 g quahaugs in this experiment amounted to 11-13.5 ml and each digestive diverticulum weighed 0.7-1.4 g. Total viral contents in drained fluids and diverticulum from each quahaug were computed from the above data with results summarized in Table I. These data seem to disclose more precisely quantitative distribution of virus in a polluted shellfish than shown in Fig. 3. On the average, 6.8% of virus was found in drained fluids whereas over 90% was found in diverticula. From these results one may surmise that naturally polluted shellfish most probably harbor practically all their viral contaminants in diverticula, provided one assumes that pollution level in the estuary may be much lower than 10 PFU/ml of water.

Adsorption of virus by shellfish mucus. One wonders what mechanism may be involved by which shellfish can pick up and concentrate minute particles such as poliovirus into their digestive system. Mucus sheet has been considered as one of the major mecha-

TABLE I. Distribution of Poliovirus in Individual Quahaugs Contaminated in Simulated Natural Conditions.

Days of pollution	Total PFU/quahaug: Drained fluids PFU	%	Digestive diverticula PFU	%	Total PFU	%
.2	<48	<.7	8250	>99.3	<8,298	100.0
1.0	2350	19.4	9790	80.6	12,140	100.0
2	614	6.7	8525	93.3	9,139	100.0
3	<46	<.6	7260	>99.4	7,306	100.0
4	483	7.9	5655	92.1	6,138	100.0
7	1242	13.4	8050	86.6	9,292	100.0
14	374	9.7	3467	90.3	3,841	100.0
17	462	23.5	1500	76.5	1,962	100.0
21	384	16.1	1995	83.9	2,379	100.0
Geom. mean	366	6.8	4998	93.2	5,364	100.0
Range	<46–2350	<.6–23.5	1500–9790	76.5–99.4	1,962–12,140	100.0

TABLE II. Adsorption of Poliovirus on Pseudofeces.

Diluent of virus	Temperature of adsorption, °C	Preparation	PFU/ml	%
Seawater	4	Supernate	12	12.0
		Sediment	88	88.0
"	18	Supernate	16	15.9
		Sediment	85	84.1
"	37	Supernate	14	15.2
		Sediment	78	84.8
"	4	No pseudofeces	140	100.0
HBSS	4	Supernate	50	42.4
		Sediment	68	57.6
"	18	Supernate	32	36.7
		Sediment	58	63.3
"	37	Supernate	36	33.9
		Sediment	70	66.1
"	4	No pseudofeces	90	100.0

nisms for shellfish to feed(13). To explore the possibility that mucus sheet may entrap virus, an experiment was conducted as follows: One g of pseudofeces (wet wt) was collected from clean quahaugs and homogenized in 30 ml HBSS. The suspension was portioned into 6 5-ml aliquots. Each of 3 aliquots was mixed with an equal volume of poliovirus diluted in filtered seawater. Each of another 3 aliquots was mixed with an equal volume of poliovirus diluted in HBSS. One mixture from each set was incubated at 4°C, 18°C and 37°C, respectively, for 1 hour with frequent agitation. Following this, all tubes were centrifuged at a low speed. Supernates were collected and sediments resuspended in original volume of MKA medium(14). One tube each of poliovirus in respective media, HBSS and filtered seawater, diluted 2-fold in MKA medium and incubated at 4°C for 1 hour, served as a control of total viral content before adsorption. All preparations were assayed with results given in Table II.

As shown, when virus in HBSS was mixed with pseudofeces suspension, approximately 60% of virus was adsorbed onto the mucus, after 1 hour of incubation. There is a slight increase of adsorption at higher temperatures, but these differences can hardly be considered significant. When virus in seawater was mixed with pseudofeces suspension, more than 80% of virus was adsorbed onto it at all temperatures tested. These data seem to support the hypothesis that viruses may be entrapped or adsorbed on mucus sheet. Virus laden mucus threads may then be ingested, pass through the entire digestive system, and finally be eliminated in fecal material of shellfish.

Discussion. Various studies showed a definite uptake of human enteric viruses by several species of shellfish. In view of the high concentrations of virus used in polluting seawater in all experiments, one would still wonder what is happening in a natural environment when the level of viral pollution in estuarine environment is probably very low. How, then, may shellfish acquire a sufficient number of viruses to produce an infection in man under these cicumstances? Recent work by Mitchell *et al*(7) shed some light on this problem in showing that Eastern oysters can concentrate poliovirus when a flow-through water system was used. By using a similar system, the present study reveals at least two basic facts: (a) Viral uptake by shellfish appears more efficient when surrounding water is contaminated with low levels of virus. (b) Shellfish can concentrate more virus in their organs when they are provided with a more suitable experimental environment. Exact natural conditions of shellfish growing areas have not yet been duplicated in laboratory, thus it is felt that shellfish may even have greater ability in concentrating virus from their natural environment.

Patterns of viral accumulation and retention in shellfish as shown in Fig. 3 have certain implications in epidemiologic situations. Maximal pollution of shellfish was achieved very rapidly in simulated natural conditions in which a minimum number of virus were present. Thereafter, this level of contamination in shellfish was more or less maintained so long as there was sufficient virus present in seawater. As shown previously (8), shellfish unload their contaminants rapidly if viral level in water recedes. This may mean that contaminated shellfish sold in the market were harvested at the exact time when estuarine water was polluted. As known, most shellfish-associated hepatitis outbreaks took place in winter and early spring and lasted for

several months(2,4). Then one may wonder whether the estuaries were polluted continuously with hepatitis virus during the entire period of outbreaks. If so, a large number of hepatitis cases, clinical or subclinical, should have existed in the community and served as a source of water pollution. Mosley and his associates(15) had considered this possibility, but failed to substantiate it by surveying these communities. Another explanation for the epidemiologic observations may be as follows. It was shown in our laboratory(16) that among several factors studied water temperature plays a major role in affecting shellfish in accumulation, retention and elimination of viruses. It seems more likely that, during various outbreaks, shellfish were polluted in the late fall when water temperature was optimal for viral uptakes, *i.e.*, 10 to 20°C. The temperature then dropped suddenly to below 5-6°C. At this temperature, shellfish probably stopped feeding completely. Thus, these shellfish retained their contaminants as long as the temperature remained in same range, and they served as a constant source for dissemination of hepatitis viruses.

The finding of quantitative distribution of virus in shellfish is also of practical importance. First, as shown in Fig. 3 and Table I, one quahaug picked up an amount of virus equivalent to that present in approximately one liter of seawater. This seems to indicate that a hepatitis outbreak associated with hard clams would occur if pollution level of estuarine water reached approximately one infectious unit of the virus per liter of seawater. Since little is known as to what constitutes a minimal infectious dose for hepatitis virus(17, 18), one can not estimate what level of viral pollution existed in water during these outbreaks. Secondly, most viral contaminants were localized in the digestive system of shellfish. This lends strong support to the conviction that a depuration process may be a means of obtaining clean shellfish for human consumption. The rationale is that since total intestinal content in Eastern oysters has been shown to be eliminated within 1-2 hours (19), during this process it is most likely viral contaminants will be discharged along with fecal material. The rapid rate of viral depuration by Eastern oysters has been shown by Mitchell *et al*(7) and that by quahaugs by ourselves(8,9).

Data presented in Table II are preliminary. They do, however, shed light on basic mechanism of viral uptake by shellfish. It is important to note that pseudofeces from shellfish, containing chiefly mucus, was capable of adsorbing a human virus at all temperatures tested. If one agrees that there is a limitation of sites of adsorption for virus in a given amount of mucus, data illustrated in Fig. 1 and 2 would be easily acceptable. However, several points deserve further study: (a) Could this be due to some other ingredients present in pseudofeces? (b) What is the basic ingredient(s) in mucus which can adsorb virus? (c) Could this ingredient be related to enteric virus receptors as described by Holland(20)? (d) What other viruses can also be adsorbed by the mucus?

Summary. Several aspects concerning viral uptake by shellfish have been investigated by using the Northern quahaug and a strain of attenuated poliovirus type I as working models. In a running seawater system, it was shown that the degree of viral uptake by shellfish was inversely related to the viral levels of their surrounding seawater. Under simulated natural conditions, uptake was considerable and majority of virus was recovered from digestive diverticula with stomachs. The basic mechanism of viral uptake by shellfish was explored by using pseudofeces obtained from normal quahaugs. It was shown that poliovirus was capable of adsorbing onto the mucus material very efficiently regardless of temperature used, 4-37°C.

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Cardiac Output in Ducks.* (31521)

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Changes in arterial blood pressure, venous pressure(1,2) and regional distribution of blood flow in diving ducks(3) have been reported. Although changes in cardiac output have been inferred, based mainly on central venous pressure, right atrial pressure, and distribution of flow, no actual determinations on cardiac output have been made. The object of this experiment was to determine cardiac output of ducks restrained on their backs and not in a diving position.

Materials and methods. Cardiac outputs were determined during the summer on male and female White Pekin ducks (8-14 months of age) by the dye dilution technique adapted for the chicken(4,5), employing a densitometer and indocyanine dye. The dye was injected into the right heart by means of a catheter placed in the right wing vein and pushed near the entrance of right atrium(4). Dye concentration curves were derived from arterial blood flowing from carotid artery through a Waters densitometer and amplified and recorded on an oscilloscope (Electronics for Medicine). Blood flow was calculated from the area under the curves(4). Blood pressure was determined directly from a carotid artery by means of a Statham gauge(4). Total peripheral resistance (TPR) (in units) was calculated by dividing mean blood pressure (mm Hg) by cardiac output (ml/min).

Results and discussion. The pertinent data are presented in Table I. The body weight of the male ducks was significantly higher than that of females, and the cardiac output per bird was likewise higher (approaching statistical significance), but not on a body weight basis, where the difference was not statistically significant. However, the actual values on a body weight basis appear to be somewhat higher than for chickens and about the same as for turkeys(6). There was no statistically significant sex difference in blood pressure and heart rates of the ducks as there is in chickens(6). This confirms the work of Ringer *et al*(7). The actual level of blood pressure of ducks is of the same magnitude as of male chickens but higher than that of female chickens. The heart rate of ducks is considerably lower than in chickens or turkeys.

Because of the higher cardiac output in relation to mean blood pressure of ducks as compared to chickens, the TPR is appreciably lower in ducks.

Summary. Cardiac output of adult White Pekin ducks restrained on their backs was determined by the dye dilution technique. Mean flows in ml/min/kg were 286.8 and 253.4 for males and females respectively, and

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