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Survival of Human Enteroviruses in the Hawaiian Ocean Environment: Evidence for Virus-Inactivating Microorganisms†

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The stability of certain human enteroviruses in the Hawaiian ocean environment was examined. The present data indicated that the time for 90% reduction of poliovirus type 1 at 24 ± 1°C in seawater samples obtained from different sites in Hawaii ranged from 24 to 48 h, and complete inactivation occurred within 72 to 96 h. The accumulated evidence also strongly indicated that a virus-inactivating agent(s) of a microbiological nature was present in both clean and sewage-polluted seawaters, but not in fresh, mountain stream waters. The antiviral activity was lost when the seawater samples were subjected to boiling, autoclaving, or filtration through a 0.22- or 0.45-μm, but not a 1.0-μm, membrane filter. That the antiviral activity of the seawater was related to the growth activities of microorganisms was corroborated by the observed effects of added nutrients, a lower temperature of incubation, and the presence of certain antibiotics. Other enteric viruses, such as coxsackie virus B-4 and echo virus-7, were also shown to be similarly inactivated in seawater.

Hawaii, an island state, like most coastal and island communities, disposes its sewage into the ocean. Our previous studies have reported the presence of human enteroviruses in the Hawaiian ocean environment as a result of the discharge of raw sewage (6a, 9). These sewage-borne human enteric viruses become a potential public health problem only if they are able to survive in the marine environment and be disseminated to recreational and shellfish-producing coastal waters. Seawater from various sources (Mediterranean Sea, Red Sea, Baltic Sea, North Sea, Atlantic Ocean, and the Gulf of Mexico) has been reported to contain antiviral activity (1, 2, 6-8, 10). However, there appears to be no consensus as to the component of these waters responsible for the antiviral activity. Furthermore, no comparable studies have been carried out in seawater obtained from the semitropical zone of the Pacific Ocean. The present report describes the stability of human enteroviruses in the seawaters surrounding Oahu, the major island in the state of Hawaii, and also characterizes the virus-inactivating agent(s) present in these waters.

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MATERIALS AND METHODS

Cell culture, virus, and virus assay. The African green monkey kidney cell line BGM was obtained from G. Berg, Environmental Protection Agency, Cincinnati, Ohio, and was passaged, grown, and maintained as previously described (3). Enteroviruses used included coxsackie virus B-4 and echovirus-7, originally isolated from sewage (3), and poliovirus type 1 (strain LSc2ab), which served as the model human enteric virus. All virus stocks were grown in BGM cells, purified by differential centrifugation, genetron treatment, and CsCl banding as previously described (3). The purified virus preparations were stored in phosphate-buffered saline (PBS) at −70°C. All titrations were made by the plaque technique, and the results are reported as plaque-forming units.

Seawater samples. Seawater samples were obtained in sterile containers from the southern (Mamala Bay), western (Waianae Bay), and eastern (Kailua Bay) coasts of Oahu. Water samples from these sites were obtained either at the surface or at a depth of 1 to 2 ft (ca. 30.48 to 60.96 cm), 0.5 to 1 mile (ca. 0.805 to 1.609 km) from shore, and away from the sewage outfall unless specifically stated. One sample, taken 25 miles (ca. 40.225 km) off the island of Maui, was obtained with the assistance of the U.S. Navy. Another sampling site was the middle loch of Pearl Harbor, which receives sewage discharge and stream and storm water runoffs. As compared with the above coastal water samples, water from Pearl Harbor is characterized by high turbidity, high nutrient level, and in-

creased flora. Surface water samples at this site were obtained from a dock approximately 0.5 mile from the sewage outfall. Freshwater samples were obtained from a mountain stream (Nuuanu Stream) at an elevation of 600 feet (ca. 182.880 m) above sea level. Estuary water samples refer to the zone of mixing of Nuuanu Stream water and ocean water. All water samples were used either on the day of sampling or after overnight storage at 4°C unless otherwise specified.

Virus survival studies. Replicate flasks containing 100 ml of any water sample to be tested were inoculated with a suspension of the purified virus preparation so as to yield a final titer of 10^4 to 10^6 plaque-forming units per ml. Preliminary experiments indicated that the volume of the water sample was inconsequential since similar results were obtained regardless of whether the final volume was as small as 50 ml or as large as 19 liters. As controls, PBS or filter-sterilized samples of the test water or both were inoculated with a similar amount of virus. All samples were continually mixed with a magnetic stirrer at 24 ± 1°C, a temperature similar to the ocean water surrounding the island of Oahu. To determine the initial concentration of virus, 0.5 ml of sample was removed from the mixture 3 min after the addition of virus, and residual virus was determined by taking similar samples at various time intervals post-inoculation. All samples were mixed into 4.5 ml of Melnick B medium containing 100 U of penicillin per ml and 0.1 mg of streptomycin per ml and were either immediately assayed for virus or stored for up to 4 days at 4°C. Survival of the virus in the test waters was determined by calculating the log V/V_0, where V_0 is the titer of the virus in the controls at time zero and V is the virus titer in the test waters at various time periods post-inoculation. The time required for a loss in titer of 90% of the input virus population is designated T_{90} and is used to compare the stability of the virus under the various experimental conditions tested. Although data from individual experiments are plotted, all experimental results described were reproduced in at least three separate experiments.

RESULTS

Stability of poliovirus in seawater. A major factor governing the recovery and distribution of infectious viruses in the ocean is their relative stability in seawater. This ability to survive in the ocean environment aids in the dissemination of the virus and may represent a potential route of viral disease transmission. To determine the stability of sewage-borne human enteroviruses after they are discharged into the marine water environment, poliovirus was added to natural seawater samples derived from various sources, and the survival of the virus in these samples at 24 ± 1°C was compared with that of the virus in PBS (see above). Seawater samples were obtained from the following sources: (i) from a site 4 miles (ca. 6.476 km) east of the ocean sewage outfall in Mamala Bay and approximately 1 mile offshore (the water here showed no evidence of fecal contamination, as evidenced by the absence of fecal coliform and of human enteric viruses); (ii) from within the sewage plume of the ocean outfall in Mamala Bay; and (iii) from a dock approximately 0.5 mile from the sewage outfall in Pearl Harbor. The stability of poliovirus in these natural marine waters and in PBS at 24 ± 1°C is shown in Fig. 1. The virus was stable for 4 days in PBS. However, in the clean and sewage-polluted Mamala Bay waters the virus was stable for only 1 day, after which time it was steadily inactivated during the next 3 days of incubation, resulting in a T_{90} of approximately 2 days. In contrast, virus inactivation in Pearl Harbor water was observed with no delay and yielded a T_{90} of less than 1 day. The immediate inactivation of poliovirus in Pearl Harbor water, as opposed to the stability of the virus for 1 day in waters obtained from Mamala Bay, was consistently observed in other experiments. These results indicated that sewage-polluted marine waters contained more antiviral activity than unpolluted seawater.

Effect of heat, filtration, nutrients, and temperature of incubation. To determine whether the antiviral activity was biological in nature, the seawater was treated by a number of procedures known to affect biological activity. Initially, seawater samples from Pearl Harbor were preheated by either boiling for 10 min or autoclaving at a pressure of 15 lb/in^2 and a temperature of 121°C for 15 min or filtered through 1.0- or 0.45-μm membrane filters. The treated waters and untreated water were then inoculated with poliovirus, and the survival of virus was determined at 24°C. The results (Fig. 2) showed that, although poliovirus was rapidly inactivated by the untreated water, the virus was essentially unaffected when the water was either boiled or autoclaved. Furthermore, when the seawater was filtered through a 0.45-μm

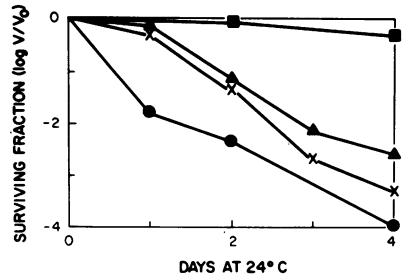

FIG. 1. *Survival of poliovirus type 1 suspended in PBS (■), seawater from unpolluted area in Mamala Bay (▲), seawater from plume formed by ocean sewage outfall in Mamala Bay (×), and seawater from Pearl Harbor (●).*

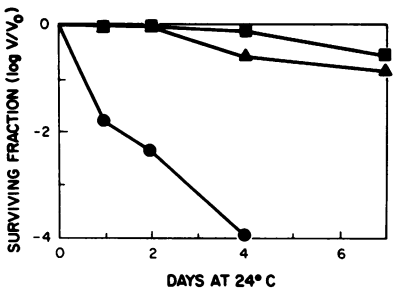

FIG. 2. *Survival of poliovirus type 1 suspended in seawater samples obtained from Pearl Harbor which were untreated (●), boiled for 10 min (■), or autoclaved (▲).*

membrane filter, the virus remained stable for 3 days, the duration of the experiment (Fig. 3). In contrast, the virus was stable for only 1 day in seawater which had been filtered through a 1.0-μm membrane filter and was subsequently inactivated during days 2 and 3 of incubation. Unfiltered seawater rapidly inactivated the virus. Similar results were obtained when Mamala Bay water was treated in the same fashion.

On the basis of the size (larger than 0.45 μm but smaller than 1.0 μm) and the susceptibility of the antiviral activity to thermal inactivation, it was postulated that the antiviral agent(s) present in marine waters was a microorganism. To test this hypothesis, poliovirus was added to samples of seawater obtained from Pearl Harbor and incubated at 4°C, a temperature at which most microorganisms do not grow, and at 24°C, the normal temperature of the coastal waters of Hawaii. The results (Fig. 4) indicated that, whereas the poliovirus was stable at 4°C, the virus was rapidly inactivated at 24°C. Parallel results were obtained when seawater from Mamala Bay was similarly tested. Poliovirus was then added to seawater samples from an unpolluted area in Mamala Bay. The microbial nutrient peptone was added to a duplicate set of water samples to a final concentration of 0.001%. The stability of the virus in the two sets of seawater samples was determined at 24°C. The results (Fig. 5) showed that the addition of peptone markedly increased virus inactivation and supported the hypothesis that a nutrient such as peptone enhanced the multiplication of microorganisms and consequently increased antiviral activity.

Restoration of antiviral activity. If the antiviral activity of seawater is due to a marine microorganism(s), then filter-sterilized seawater should support the growth of this marine microorganism and thus regain its antiviral activity. To determine whether such a phenomenon does occur, 0.25 ml of Pearl Harbor water was inoculated into each of the following: (i) 100 ml of sterile Todd-Hewitt broth and (ii) two 100-ml samples of Pearl Harbor water which had been prefiltered through a 0.22-μm membrane filter to remove antiviral activity. Poliovirus was then inoculated into all three samples and thoroughly mixed. The Todd-Hewitt broth sample and one of the filtered Pearl Harbor water samples were incubated under the usual laboratory conditions (24°C and approximately 12 h daily under fluorescent light). The remaining filtered Pearl Harbor water sample was incubated at 24°C in the dark for the duration of the experimental period. It was found that, whereas the virus was stable for 8 days in Todd-Hewitt broth, it was similarly inactivated (>99%) by the Pearl Harbor water samples maintained under both dark and light conditions (Fig. 6). On the basis of these results, it was concluded that microorganisms were responsible for the antiviral activity in Pearl Harbor water. Furthermore, they were high-salt-requiring marine microorganisms

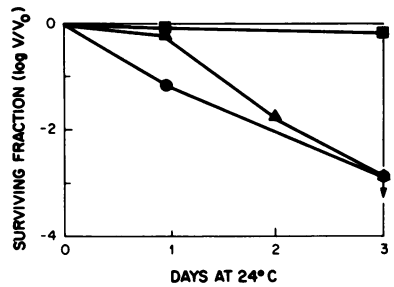

FIG. 3. *Survival of poliovirus type 1 suspended in seawater samples obtained from Pearl Harbor which were untreated (●), filtered through a 0.45-μm membrane filter (■), or filtered through a 1.0-μm membrane filter (▲). Descending arrow indicates that the surviving fraction was less than −3.*

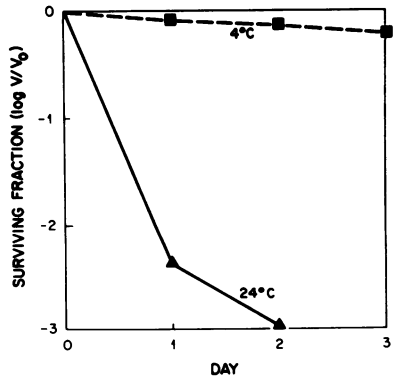

FIG. 4. *Survival of poliovirus type 1 suspended in seawater samples obtained from Pearl Harbor and incubated at 4 and 24°C.*

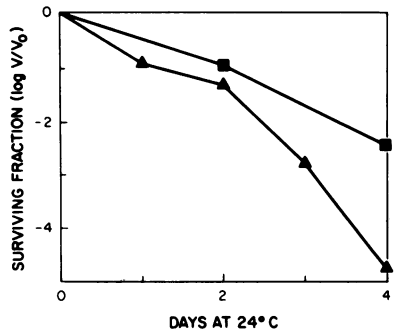

FIG. 5. *Survival of poliovirus type 1 suspended in seawater samples obtained from Mamala Bay which were untreated (■) or supplemented with 0.001% peptone (▲).*

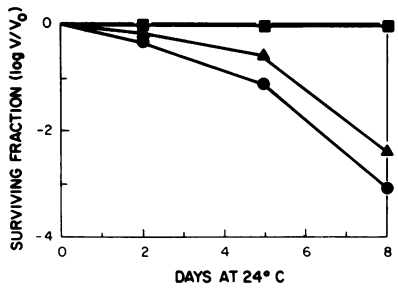

FIG. 6. *Restoration of antiviral activity after the addition of 0.25 ml of untreated seawater obtained from Pearl Harbor into 100 ml of sterile Todd-Hewitt broth or 100 ml of filter-sterilized seawater obtained from Pearl Harbor. The survival of poliovirus type 1 suspended in Todd-Hewitt broth (■) and filter-sterilized Pearl Harbor water (●) was determined in the presence of fluorescent light and compared with the survival of poliovirus suspended in filter-sterilized Pearl Harbor water and incubated in complete darkness (▲).*

which cannot grow in Todd-Hewitt broth and do not require light for their growth.

Effect of antibiotics. A more definitive test to demonstrate that microorganisms were responsible for the antiviral activity in Pearl Harbor water was to examine the effect of antibiotics such as penicillin (200 U/ml) and streptomycin (0.2 mg/ml) on the growth of the antiviral microorganisms. Poliovirus was inoculated into water samples (100 ml each) taken from Pearl Harbor. Antibiotics were added to one set of samples at the time of virus inoculation and to another set at 1 day after incubation. An untreated water sample inoculated with poliovirus served as the control. All samples were incubated at 24°C, and the amount of residual virus was determined daily. As expected, Figure 7 shows that poliovirus was rapidly inactivated in the untreated water sample from Pearl Harbor. However, the virus remained stable for the duration of the experimental period (3 days) in the water sample treated with penicillin and streptomycin at the time of the addition of virus. These results strongly indicate that the antiviral activity of Pearl Harbor water is related to the multiplication of certain marine microorganisms. That continued multiplication of the responsible microorganisms is a requirement for antiviral activity was evidenced by the observation that antibiotics added after 24 h of incubation were still capable of reducing the rate of the antiviral activity of the sample. Other tests showed that either penicillin or streptomycin alone could effectively inhibit the antiviral activity of Pearl Harbor water, as well as that of Mamala Bay water.

Stability of other enteric viruses. To determine the spectrum of antiviral activity in these marine waters, coxsackievirus B-4 and echovirus-7 were incubated at 24°C in waters obtained from Pearl Harbor and in PBS. Both viruses were stable for 2 days in PBS, whereas more than 99% of these viruses were inactivated after 2 days in the marine water. These results indicated that the antiviral activity in seawater can be extended to include the coxsackie- and echoviruses.

Distribution of antiviral activity in natural waters. In this series of experiments, the antiviral activity for poliovirus in samples of natural bodies of water taken from several sites reflecting different environmental conditions was examined under the same experimental conditions as previously described. The results (Table 1) showed that the antiviral activity was detected in all seawater samples taken from the western and eastern coasts of Oahu and from a

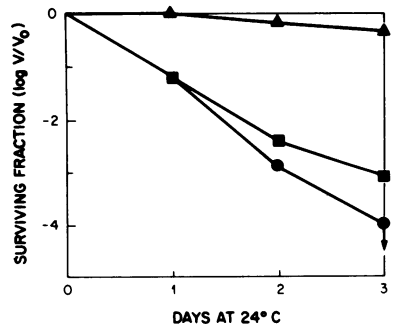

FIG. 7. *Survival of poliovirus type 1 suspended in seawater samples obtained from Pearl Harbor which were untreated (●) or treated with 200 U of penicillin per ml and 0.2 mg of streptomycin per ml at the time the virus was added (▲) or 24 h after the virus was added (■). Descending arrow indicates that the surviving fraction was less than −4.*

site in the open ocean at least 25 miles away from the coastline of the island of Maui. The rate and kinetics of poliovirus inactivation of these waters were similar to those observed in waters taken from Mamala Bay. Furthermore, the addition of penicillin and streptomycin to these waters inhibited their antiviral activity. In contrast to seawater, freshwater samples obtained from a mountain stream (Nuuanu) showed no antiviral activity. However, antiviral activity was present in estuarine water samples taken at the mouth of this stream as it flowed into the ocean. This again strongly suggested that microorganisms of marine origin were involved and that these microorganisms are normally present in the seawaters surrounding the state of Hawaii.

DISCUSSION

The T_{90} for human enteric viruses (poliovirus type 1, coxsackievirus B-4, and echovirus-7) at 24°C in seawater samples obtained from different sites in Hawaii was determined to range from 24 to 48 h, and complete inactivation occurred within 72 to 96 h. Furthermore, the accumulated evidence strongly indicated that virus-inactivating agents of a microbiological nature are present in both clean and sewage-polluted seawaters, but not in fresh mountain stream waters. The antiviral activity was lost when the seawater samples were subjected to boiling, autoclaving, or filtration through a 0.22- or 0.45-μm, but not a 1.0-μm membrane filter. That the antiviral activity of the seawater was related to the growth activities of microorganisms was corroborated by the observed effects of added nutrients, a lower temperature of incubation, and the presence of certain antibiotics. In this respect, the antiviral activity due to marine microorganisms in seawater has been reported previously for the Baltic and North seas (7), and for the Red and Mediterranean seas (10). However, the successful isolation and identification of a marine microorganism with antiviral properties has been reported by only one laboratory (5). Unfortunately, the antiviral properties of the isolated marine bacterium, identified as *Vibrio marinus,* were lost while maintaining the bacterium under in vitro conditions (5).

TABLE 1. *Stability of poliovirus type 1 in natural waters of Hawaii*

Water source	Conductivity of water (μS/cm)	Log virus survival (log V/V_0) after 4 days at 24 ± 1°C
Waianae Coast: 0.5 mile off western coast of Oahu	230,000	−1.5
Mokapu Point: 0.5 mile off eastern coast of Oahu	220,000	−2.7
Open ocean: 25 miles off coast of island of Maui	240,000	−2.7
Upper Nuuanu Stream: freshwater 600 ft above sea level in non-inhabited, forest reserve area	470	−0.7
Mouth of Nuuanu Stream: discharge of Nuuanu Stream into the ocean	200,000	−2.2
Sterile PBS	73,000	−0.5

A number of non-biological factors (heavy metals, chemicals, adsorption to colloidal particles, temperature, etc.) in seawater have also been reported to have antiviral properties (1, 4, 6, 8). The different factors reported with antiviral activity may reflect differences in either the test virus used (bacterial versus human) or the water sources examined. With the former, differences in the stability of different virus groups and serotypes, and even among strains of the same serotype, have been noted (2). In the latter instance, it is reasonable to assume that environmental factors and the compositional makeup of the seawater may differ from one geographical location to another. Furthermore, it is highly likely that all seawaters contain a variety of potential antiviral factors, and the antiviral activity observed is generally the expression of the most dominant factor(s) present in any given water source. In this regard, the present study of the seawaters surrounding the Hawaiian islands (which vary only slightly in temperature throughout the year) has provided strong evidence to indicate that virus inactivation is due to some as yet unidentified marine microorganism(s). It would be of interest to determine whether the growth of these virus-inactivating microorganisms is peculiar to or predominates in the semitropical to tropical waters of the Pacific Ocean. The isolation and characterization of the antiviral microorganisms are currently being investigated.

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