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Inactivation of Hepatitis A Virus and Indicator Organisms in Water by Free Chlorine Residuals

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Hepatitis A virus (HAV) and selected indicator organisms were mixed together in chlorine-demand-free buffers at pH 6, 8, or 10 and exposed to free chlorine residuals, and the survival kinetics of individual organisms were compared. HAV was enumerated by a most-probable-number dilution assay, using PLC/PRF/5 liver cells for propagation of the virus and radioimmunoassay for its detection. At all pH levels, HAV was more sensitive than *Mycobacterium fortuitum*, coliphage V1 (representing a type of phage common in some sewage-polluted waters), and poliovirus type 2. Under certain conditions, HAV was more resistant than *Escherichia coli, Streptococcus faecalis*, coliphage MS2, and reovirus type 3. It was always more resistant than SA-11 rotavirus. Evidence is presented that conditions generally specified for the chlorine disinfection of drinking-water supplies will also successfully inactivate HAV and that HAV inactivation by free chlorine residuals can reliably be monitored by practical indicator systems consisting of appropriate combinations of suitable indicators such as coliform and acid-fast bacteria, coliphages, the standard plate count, and fecal streptococci.

Hepatitis A virus (HAV) is one of few viruses for which conclusive evidence of water-borne transmission exists (16). Information on the behavior of HAV in water treatment processes is limited because practical technology for direct research on the virus has not been available (10, 16). Results obtained in tests with human volunteers indicate that HAV was inactivated within 30 min by breakpoint chlorination of heavily contaminated water, or 1 mg of total or 0.4 mg of free chlorine residual per liter after purification of the same water by coagulation, settling, and filtration through a diatomaceous silica filter (10, 21). Epidemiological data, extrapolations from properties of related viruses, and recent experiments with marmosets also suggest that chlorine levels in this range may, under suitable conditions of pH, turbidity, and exposure time, inactivate HAV in water (10, 16, 23).

In this study, recently developed methods for propagating HAV in various cell culture systems and detection of the virus by serological techniques (3, 7, 8, 17, 24) were used to compare the sensitivity of an HAV strain to free chlorine residuals in water with that of indicator organisms used for evaluating the microbiological quality of water. The results shed new light on requirements for water chlorination as well as methods for monitoring the virological safety of water supplies.

MATERIALS AND METHODS

Viruses. The MBB strain of HAV derived from a stool suspension of a patient, collected 5 days before onset of hepatitis A, was used after seven passages in the PLC/PRF/5 cell line derived from a primary hepatocellular carcinoma (7). PLC/PRF/5 cells (passage 90 to 120) were cultured in Eagle minimal essential medium with Earle salt solution as described previously (12). HAV was titrated by most-probable-number assays (1, 29) on threefold dilutions, using PLC/PRF/5 cells in 25-cm^2 plastic flasks (Costar, Cambridge, Mass.) or rolled glass tubes incubated at 35°C. Flasks were incubated for 6 weeks, but it was not possible to maintain cells in tubes for more than 4 weeks. HAV antigen was detected by means of a solid-phase radioimmunoassay with monospecific human convalescent serum (7) and commercial reagents and equipment (Abbott Laboratories, North Chicago, Ill.). Reovirus type 3 ("Lang" strain), the SA-11 simian rotavirus, and attenuated poliovirus type 2 (strain P712), as well as their 50% tissue culture infective dose enumeration in rolled tubes with Eagle minimal essential medium with Earle salt solution and incubation at 37°C, have been described previously (9, 14, 22). Primary vervet kidney cells were used for the former two viruses, and the BGM African green monkey kidney cell line was used for the poliovirus (14). Organisms other than viruses in samples from reaction mixtures for titration of HAV, reovirus, SA-11, or poliovirus were inactivated by adding 0.5 ml of chloroform to 10-ml samples, followed after 15 min at room temperature by centrifugation (2,600 × *g* for 15 min) and titration of the supernatant. Coliphage MS2 has been described previ-

ously (22). Coliphage V1, which produces large lytic plaques on *Escherichia coli* C603, was isolated from the Vaal River (South Africa) and has a cubical structure, a diameter of 60 nm, and a very short tail (unpublished data). Coliphages were assayed without chloroform treatment, using a double-layer agar method (11). The host specificity of these phages made it possible to separately titrate MS2 and V1 phages in the same reaction mixture because MS2 forms plaques only on *E. coli* K-12 Hfr and not on strains C603 or E25, and V1 forms plaques only on strain C603 and not on *E. coli* K-12 Hfr or E25.

Bacteria. *Mycobacterium fortuitum* DR14/1 was isolated from purified wastewater (11), and *E. coli* strains B, K-12 Hfr (22), and E25 (15) have been described previously. *E. coli* C603 (20) was kindly supplied by S. B. Primrose (University of Warwick, England). *Streptococcus faecalis* FS1 was isolated from purified wastewater (11). All bacteria were counted by membrane filtration, using an enriched Middlebrook 7H9 agar for *M. fortuitum* (11), M-FC agar without rosolic acid for *E. coli* (13), and M-Enterococcus agar for *S. faecalis* (11). *E. coli* and *S. faecalis* plates were incubated at 35°C instead of the conventional 44.5°C to limit the detrimental effect of temperature shock on chlorine-injured bacteria (13).

Chemical analyses. Chlorine levels were measured by the *N,N*-diethyl-*p*-phenyldiamine ferrous titrimetric method (1). Other chemical tests were done as described previously (1).

Chlorination experiments. We used chlorine-demand-free 0.05 M sodium phosphate buffer at pH 6 or 8 and 0.05 M borate buffer without KCl at pH 10 (6). Chlorine-demand-free equipment was used, and microorganisms were washed in the buffer concerned or in sterile distilled water before experimentation. Each reaction mixture was prepared by adding 1-ml quantities of appropriate suspensions (see Fig. 1 to 3) of *E. coli* E25, *S. faecalis*, *M. fortuitum*, and coliphages MS2 and V1, as well as either poliovirus, reovirus, SA-11, or HAV, to 200 ml of buffer in a 600-ml glass beaker stirred by a magnetic stirrer. Each reaction mixture contained only one enteric virus because it was not possible to separately enumerate different enteric viruses in the same reaction mixture. Two 10-ml samples were removed to count microorganisms before the addition of chlorine (time, 0 min). One milliliter of an appropriate dilution (see Table 1) of a commercial stock solution of sodium hypochlorite (Jik, Johannesburg, South Africa) was then added (6), and 10-ml samples taken at suitable time intervals (see Fig. 1 to 3) were added to 1 ml of a sodium thiosulphate solution (20 g/liter) to neutralize the chlorine for counting surviving organisms (6). This procedure implies that in each experiment the survival of one enteric virus was compared with that of five indicator organisms in the same reaction mixture. Each experiment was repeated at least three times. Chlorine concentrations in reaction mixtures were measured immediately after the addition of chlorine (time, 1 min) and after completion of experiments (15 min). In negative control experiments, sterile distilled water instead of chlorine was added to reaction mixtures. The temperature of reaction mixtures was kept at 25°C.

RESULTS

Inactivation of microorganisms is represented by the average survival curves in Fig. 1 to 3, and the chlorine concentrations concerned are listed in Table 1. Under all three experimental conditions, *M. fortuitum*, followed by coliphage V1, was the most resistant of all test organisms. The survival of the other organisms varied with different conditions. At pH 6, the decreasing sequence of resistance up to the level of 99% inactivation was *M. fortuitum*, coliphage V1, and poliovirus, with hardly any detectable difference among *E. coli* E25, coliphage MS2, *S. faecalis*, HAV, reovirus, and SA-11 virus (Fig. 1). However, beyond the 99% level of inactivation the survival curves displayed tailings which changed the relative picture of resistance considerably, and the extrapolated 6 log reduction data show the following sequence of resistance:

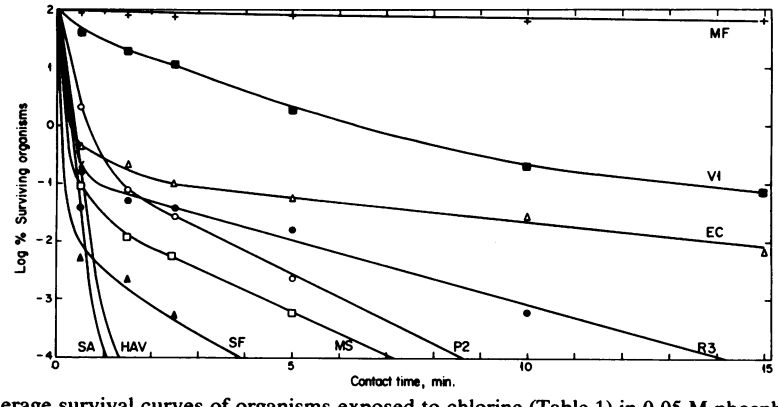

FIG. 1. Average survival curves of organisms exposed to chlorine (Table 1) in 0.05 M phosphate buffer (pH 6.0). Number of organisms per milliliter at 0 min: *E. coli* E25 (EC), 10^6; *S. faecalis* (SF), 10^6; *M. fortuitum* (MF), 10^3; coliphage MS2 (MS), 10^6; coliphage V1, 10^5; HAV, 10^3; poliovirus (P2), 10^5; SA-11 virus (SA), 10^5; reovirus (R3), 5×10^4.

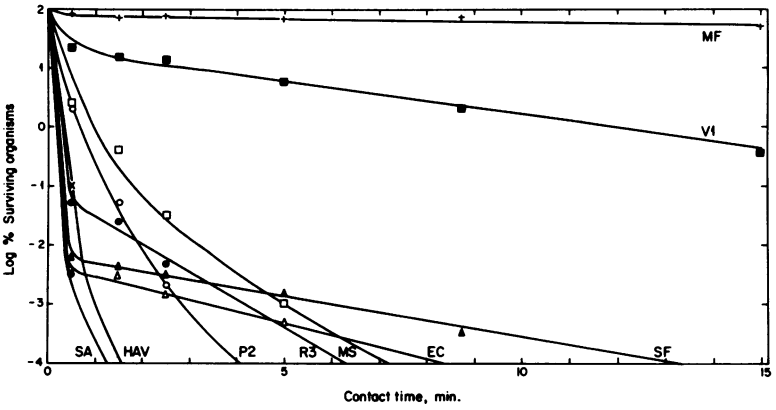

FIG. 2. Average survival curves of organisms exposed to chlorine (Table 1) in 0.05 M phosphate buffer (pH 8.0). Number of organisms per milliliter at 0 min: *E. coli* E25 (EC), 10^6; *S. faecalis* (SF), 10^6; *M. fortuitum* (MF), 10^3; coliphage MS2 (MS), 10^6; coliphage V1, 5×10^5; HAV, 10^3; poliovirus (P2), 5×10^4; SA-11 virus (SA), 3×10^5; reovirus (R3), 5×10^4.

M. fortuitum, coliphage V1, *E. coli* E25, reovirus, poliovirus, coliphage MS2, *S. faecalis*, HAV, and SA-11. *E. coli* E25 was of particular interest because its counts decreased rapidly during the first minute, after which a few organisms persisted in slowly declining numbers. *E. coli* B and K-12 Hfr behaved similarly.

Apart from *M. fortuitum* and coliphage V1, the relative survival of organisms in the pH 8 phosphate buffer differed considerably from that in the pH 6 phosphate buffer. Up to the 99% level of reduction in counts, the decreasing sequence of resistance was *M. fortuitum*, coliphage V1, coliphage MS2, poliovirus, and HAV, with virtually no detectable difference among *S. faecalis, E. coli*, reovirus, and SA-11 virus (Fig. 2). However, the survival curves of *E. coli, S. faecalis*, and reovirus displayed a tailing phenomenon which changed the decreasing order of resistance at the extrapolated 6 log reduction level to *M. fortuitum*, coliphage V1, *S. faecalis, E. coli*, coliphage MS2, reovirus, poliovirus, HAV, and SA-11.

Apart from *M. fortuitum* and coliphage V1, the relative behavior of organisms in the pH 10 borate buffer differed considerably from that in the pH 6 and 8 phosphate buffers. Up to the level of 99% reduction in counts, the decreasing order of resistance was *M. fortuitum*, coliphage V1, poliovirus, *S. faecalis*, coliphage MS2, HAV, reovirus, SA-11 virus, and *E. coli*. However, beyond this level HAV displayed a relatively pronounced tailing phenomenon, and at the extrapolated 6 log reduction level it had changed

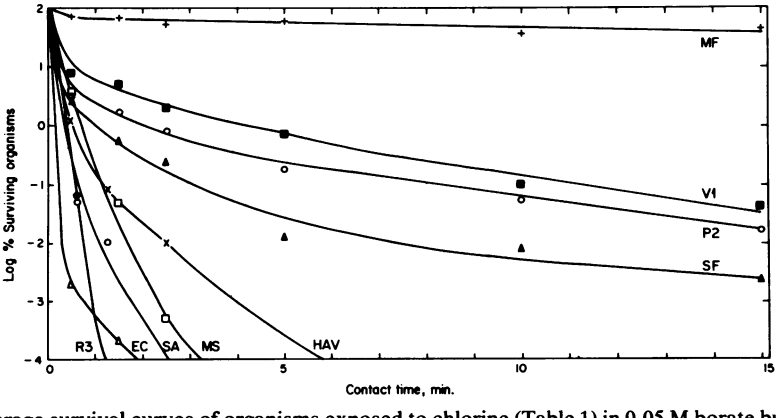

FIG. 3. Average survival curves of organisms exposed to chlorine (Table 1) in 0.05 M borate buffer (pH 10.0). Number of organisms per milliliter at 0 min: *E. coli* E25 (EC), 10^6; *S. faecalis* (SF), 10^6; *M. fortuitum* (MF), 10^3; coliphage MS2 (MS), 10^6; coliphage V1, 10^6; HAV, 10^4; poliovirus (P2), 3×10^5; SA-11 virus (SA), 10^4; reovirus (R3), 5×10^4.

TABLE 1. Chlorine concentrations in reaction mixtures represented by survival curves in Fig. 1 to 3

Buffer	Time (min)	Chlorine residuals (mg/liter)[a]			
		Free chlorine	Monochloramine	Dichloramine	Total
Fig. 1: 0.05 M phosphate (pH 6.0)	1	0.42 (0.40–0.44)	0.13 (0.10–0.15)	0.09 (0.07–0.11)	0.64 (0.62–0.68)
	15	0.06 (0.03–0.08)	0.19 (0.16–0.21)	0.15 (0.13–0.18)	0.40 (0.37–0.43)
Fig. 2: 0.05 M phosphate (pH 8.0)	1	0.41 (0.39–0.43)	0.24 (0.20–0.27)	0.15 (0.14–0.17)	0.80 (0.76–0.83)
	15	0.08 (0.06–0.09)	0.30 (0.25–0.34)	0.23 (0.20–0.26)	0.61 (0.57–0.64)
Fig. 3: 0.05 M borate (pH 10.0)	1	0.40 (0.39–0.41)	0.32 (0.30–0.34)	0.17 (0.13–0.20)	0.89 (0.86–0.92)
	15	0.28 (0.26–0.29)	0.22 (0.20–0.25)	0.26 (0.24–0.29)	0.76 (0.72–0.80)

[a] Value in parentheses is the range.

places with coliphage MS2 in the order of resistance, whereas reovirus dropped back to the lowest position. Negative controls indicated that exposure to the different pH levels for the duration of the experiments did not have a significant effect on the survival of test organisms. However, prolonged exposure (25 min or more) to pH 10 borate buffer notably reduced counts of reovirus and *E. coli*.

HAV titrations in cell culture tubes yielded results comparable to those of flasks, but flasks were eventually preferred (although more cumbersome and expensive) because the longer incubation may recover more viruses (7).

Some properties other than chlorine concentration, pH, and temperature of reaction mixtures which may affect the survival of microorganisms were, on average, as follows: turbidity, 1.4 nephelometric turbidity units; dissolved organic carbon, 5.0 mg/liter; Kjeldahl nitrogen, 0.3 mg/liter; nitrite nitrogen, <0.01 mg/liter; chlorides, 30 mg/liter.

DISCUSSION

Since the experimental procedure was primarily designed to directly compare the response of selected organisms to free chlorine residuals when mixed together in a demand-free system, no attempt was made to disintegrate clumps or aggregates of organisms which might be present because these also occur under natural conditions.

An outstanding feature of the results is the variation in the relative rate of inactivation among some organisms at different pH levels. This is probably largely due to variation in the form of chlorine present at different pH levels. At acidic pH levels free chlorine residuals occur mainly in the form of hypochlorous acid (HOCl), whereas the dissociated hypochlorite ion (OCl^-) predominates at basic pH levels. In addition, the reaction mixtures also contained various concentrations of chloramines (Table 1), and the mode of inactivation of different microorganisms depends on the form of chlorine and various other factors (W. O. K. Grabow, *in Proceedings of an International Symposium on Viruses and Disinfection of Water and Wastewater, Guildford, England*, in press). Although experimental conditions were not identical, the data on the response of *M. fortuitum*, *E. coli*, and poliovirus are basically in agreement with results published earlier and serve as a means of comparison with related studies (5, 18, 26). The prolonged survival of small numbers of *E. coli* at pH 6 (Fig. 1) and 8 (Fig. 2), and of *S. faecalis* at pH 8 (Fig. 2) and 10 (Fig. 3), has not been reported before and may be due to more successful recovery of chlorine-injured organisms in this study.

The details on the relative survival of the different organisms are important in the selection of indicator systems for evaluating the efficiency of water chlorination. The basic requirements of indicator organisms are that they should be present whenever pathogenic organisms are present, they should be present in similar or higher numbers, they should be at least as resistant to treatment processes, and they should be detectable by practical techniques (4). The survival curves in Fig. 1 to 3 show that at all pH levels tested, *M. fortuitum* was by far the most resistant. Since earlier results indicate that the group of acid-fast organisms, of which *M. fortuitum* is a member, generally outnumbers pathogenic microorganisms such as enteric viruses in wastewater and natural water environments (5, 11), these findings support views that acid-fast bacteria are valuable indicators of chlorination efficiency (4, 11). Likewise, coliphages usually outnumber enteric viruses in water, and the survival curves of coliphage V1, which represents a common type of coliphage in sewage-polluted river water, and even those of coliphage MS2, which may occur less frequently (11), support views that coliphages are valuable indicators of the virucidal efficiency of free chlorine residuals (11, 16). Even *E. coli* and *S. faecalis*, which are often not considered resistant enough to serve as indicators for the inactivation of viruses, proved more

resistant than poliovirus, reovirus, SA-11 virus, and HAV to free chlorine residuals under certain conditions. Others have also found that *E. coli* may under certain circumstances be more resistant to free chlorine residuals than are enteric viruses (19, 26), which is in agreement with data indicating that coliform specifications have rarely failed their function as indicators of water quality (16). The relatively rapid inactivation of the SA-11 virus suggests that the closely related human rotavirus (22) may also not be exceptionally resistant to free chlorine residuals. However, such extrapolations should be considered with caution because related viruses may differ considerably in their response to chlorine residuals (6).

The comparatively rapid die-off of HAV at all pH levels tested (Fig. 1 to 3) suggests that indicator organisms, such as acid-fast bacteria, coliphages, poliovirus, and under certain circumstances, also *E. coli* and *S. faecalis*, may serve as reliable indicators of the inactivation of HAV by free chlorine residuals. The validity of these indicators would of course depend on their numbers in water which contains HAV. Although there is virtually no information on the numbers in which HAV occurs in water environments, data on the quality of water associated with hepatitis A outbreaks (16) signify that at least some of the indicators will meet requirements in this regard. It should also be kept in mind that the chlorine resistance of viruses tends to decrease during repeated propagation in cell culture (16, 25). However, it appears unlikely that naturally occurring HAV may be significantly more resistant to free chlorine residuals than the naturally occurring counterparts of the indicators because the test strain of HAV had been passaged only seven times in cell culture whereas the more resistant indicators were established laboratory strains. Physicochemical properties of the HAV strain were not distinguishable from those of HAV directly recovered from the stools of hepatitis A patients (27, 28). A highly important aspect of the results of this study is that the survival data primarily represent the response to free chlorine residuals. The relative survival of the same organisms may differ extensively in water which contains predominantly chloramines, as in the case of chlorinated wastewater (2).

The variation in the relative survival of HAV and indicators at different pH levels, and uncertainty about the numbers of HAV in water environments, support the view that the efficiency of chlorination, particularly in high-risk situations such as the direct or indirect reclamation of drinking water from wastewater, should be evaluated by indicator systems consisting of appropriate combinations of indicators such as coliform bacteria, the standard plate count, coliphages, acid-fast bacteria, and fecal streptococci (4, 11, 16).

The chlorine concentrations in Table 1 and the corresponding survival curves in Fig. 1 to 3 represent direct details on the response of HAV to free chlorine residuals in water and indicate that specifications generally accepted for the disinfection of drinking-water supplies (16) will also satisfactorily inactivate HAV. These specifications require a free chlorine residual of 1 to 2 mg/liter for 1 to 2 h at a pH of less than 8 and turbidity of less than 1 unit (16).

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