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Inactivation of Bacteriophage f2 with Chlorine

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鹽素에 依한 Bacteriophage f2의 殺菌作用

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ABSTRACT

Chlorine was used for inactivation of bacteriophage f2 at *p*H 5.5, 7.5, and 10.0 at 10°C. The inactivation rate phage with chlorine varied depending on the *p*H value and reaction time. Hypochlorous acid appeared to be the major species of free chlorine for the inactivation. Survival of the phage treated with chlorine and infectivity of the RNA extracted from the chlorinated phage were examined. The RNA extracted from untreated phage was chlorinated and its infectivity was assayed. All three samples showed similar rates of inactivation at *p*H 5.5 and 7.5, but the naked RNA was more susceptible to chlorine at *p*H 10.0. The rate of inactivation was compared with specific and non-specific attachment of the phage f2. The specific attachment of the phage lagged behind the inactivation with chlorine, but non-specific attachment of the phage increased after the phage had been inactivated by extended chlorination. Chlorine may penetrate to the bacteriophage f2 by altering the strutural integrity of the protein coat, but the main target of free chlorine for inactivation of the phage appeared to be the phage RNA.

INTRODUCTION

Expanding urbanization and industrial development, together with increasing use and reuse of water for domestic and recreational purposes have caused the quality of environmental water for the public to deteriorate. Chlorine has been used almost exclusively to disinfect public water supplies and wastewater effluents. Standard water and wastewater treatment employing post-chlorination as well as coagulation and filtration has been adequate against bacterial pathogens. The reaction of chlorine with bacteria can be explained by sulfhydryl hypothesis (Allison 1962; Knox *et al.* 1948) or by inactivaon of nucleic acids(Prat *et al.* 1948).

Enteric viruses have been demonstrated to be much more resistant to chlorine than bacteria (Dahling *et al.* 1972; Shah and McCamish 1972) and extremely high dosages of chlorine must be applied to achieve adequate inactivation of the viruses in the processes of water and wastewater treatment (Lothop and Sproul 1969). Organic materials (Farrah *et al.* 1977; Moore *et al.* 1975) and minerals (Hloba *et al.* 1972) have been reported to protect enteric viruses in water from various disinfectants and to react with free chlorine and lower its capacity of disinfection. Therefore, the absence of bacteria from the treated effluent is not infallible proof of disinfection

and conceivable hazard by viruses still exisists in the environmental water that is to be reused (Hoehn 1976; Lefler and Kott 1975; Ludovici *et al.* 1975; White 1975).

The bacteriophage f2 containing RNA is chemically and physically similar to the enteric viruses and can be readily prepared, purified, and assayed by a simpler system to obtain fundamental information concerning inactivation. Since the first use of the phage f2 by Hsu as a model for inacvation studies of enteric viruses (Hsu *et al.* 1966), the phage has been used by many investigators (Cramer *et al.* 1976; Dahling *et al* 1972; Olivieri *et al.* 1975; Shah and McCamish 1972).

The phage f2 was reported to respond to chlorine in a manner similar to enteric viruses (Hsu *et al.* 1966). The phage has been reported to be more sensitive to free chlorine (Dahling *et al.* 1972), but more resistant to combined chlorine than poliovirus type 1 (Shah and McCamish 1972; Sproul 1976). Cramer *et al.* (1976) reported that the phage was equally or more resistant to chlorine and iodine than poliovirus type 3. Carlson *et al.* (1976) reported that the ability of free chlornie to inactivate viruses was better indicated by the oxidation-reduction potential of the chlorine than by its concentration. The free chlorine was reported not only to inactivate viral RNA, but also to bleach viral coat protein (Olivieri *etal.* 1975). Sufficient evidence has not been available on the fundamental mechanism and reaction of chloine responsible for inactivation of viruses.

This study concentrates on the effect of free chlorine, the principal virucidal agent (Olivieri *et al.* 1975; Scarpino *et al.* 1975; Sproul 1976), on the components of bacteriophage f2 and presents a possible mechanism of chlorine inactivation of the phage.

MATERIALS and METHODS

1. Bcteriophage and Bacteria

Bacteriophage f2 (ATCC #15766-B) and its host *Escherichia coli* K-13 (ATCC #15766) were obtained from the American Type Culture Collection. The phage f2 was grown and prepared according to the method of Loeb and Zinder (1961) with some modifications. The phage was assayed by the agar overlay techniqued described by Adams (1959). *E. coli* K-13 was grown in tryptone broth (Loeb and Zinder 1961) and used as the host for preparation and specific attachment of the phage f2. *E. coli* K-15 T^- and *E. coli* C-3000-38 were obtained from Dr. Olivieri at The Johns Hopkins University. *E. coli* K-15 T^- is not a host for the phage and was grown in tryptone broth and used for non-specific attachment experiments. *E. coli* C-3000-38 required uridine, thymine, and the amino acids, arginine, lysine, and histidine for growth and was cultivated in the chemically defined TPG medium (Oeschger and Nathans 1966). This mutant strain was used as the host for incorporation of sulfur-35 radionuclieotde into the phage f2.

2. Preparation and Assay of Infectious Phage RNA

Infectious RNA was prepared from bacteriophage f2 by the method of Hofschneider and Delius (1968). The infectious RNA to be treated with chlorine was extracted five times with ether to remove traces of phenol. After the final extraction, the residual ether was removed by bubbling nitrogen gas through the mixture. The RNA concentration of the preparation was 0.23mg/*l* as detemined at a wave length of 260 nm by the method of Engelhardt and Zinder

(1964). A 100-fold dilution of the RNA was used in the chlorination experiments. The infectivity assay of the RNA was performed with spheroplasts of *E. coli* K-13 by the method described by Hofschneider and Delius (1968).

3. Incorporation of Sulfur-35 into Phage f2

Sulfur-35 was incorporated into phage f2 by the method of Oeschger and Nathans (1966) with some modifications. 200m*l* of TPG medium containing a limited amount of sulfur (0.009g/*l*) was inoculated with 10m*l* of an overnight culture of *E. coli* C-3000-38 grown in the low-sulfur TPG medium. When the culture reached an absorbance of 0.15 at a wave length of 525nm, 4mCi of [^{35}S] -sulfuric acid was added. At an absorbance of 0.4, phage f2 was added at 0.1 multiplicity of infection (MOI) and growth was allowed to continue until lysis was observed. The sulfur-35 labeled phage f2 was purified according to the polyethylene glycol(PEG) procedure described by Yamamoto *et al.* (1970).

4. Attachment of Phage f2 to Bacteria

Attachment and filtration of the sulfur-35 labeled phage f2 were performed by the method of Brinton and Beer (1967) with a slight modification. The specific attachment of the phage to the host *E. coli* K-13 was evaluated by non-specific attachment studies with a female strain *E. coli* K-15 T$^-$. 1.0m*l* of the appropriate bacterial suspension in tryptone broth containing 0.4g/*l* calcium choride was dispensed in test tubes. At zero time, 1.0m*l* of chlorine-treated and untreatedf 2 samples were added to the bacterial suspension medium and allowed to incubate. The entire content of each tube was filtered through 0.45μm type HA Millipore filters. Each filter was precoated with 1.0m*l* of 3% bovine serum albumin to limit attachment of free f2 to the filters. The filters were washed four times with 2.0m*l* of tryptone broth containing 0.2g/*l* calcium chloride, dried for 15min at 85°C, and counted on a Packard model 3375 liquid scintillation spectrophotometer in Biofluor cocktail (New England Nuclear).

5. Preparation of Chlorine Stock Solution and Determination of Chlorine Concentration

Chlorine stock solutions were prepared by bubbling pure chlorine gas though a system of serially connected three gas washing bottle containing organic-free distilled water. The chlorine collected in the second bottle was used for the stock solution. The resulting chlorine solution was 2 to 4g/*l* and was stored in a brown glass bottle at 4°C.

The amperometric titration procedure of Standard Methods (1976) was used to compare standard curves for the spectrophometric method used for determination of chlorine. Direct measurement of hypochlorous acid in solution at *p*H 5.5 was performed at a wave length of 238 nm with a Beckman spectrophotometer according to the method of Granstrom *et al.* (1964). Chlorine concentration was directly calculated from absorbance value at 238 nm using a molar adsortion coefficient 102 *l*/cm mole.

6. Experimental Methods

The reaction system was provided according to Benarde *et al.* (1965). Inactivation experiments of the phage were performed in organic-free distilled water containing phosphate(0.35g/*l* K_2HPO_4 and 0.27 g/*l* KH_2PO_4) at 10°C and at *p*H 5.55, 7.5, and 10.0 to obtain information on the mode of action for different species of free chlorine. A 250m*l* aliquot of the phosphate buffer was placed in the system

and f2 phage or phage RNA was added to the desired titer and allowed to mix. Immediately prior to each trial, the chlorine stock solution was checked and adjusted to the proper reaction *p*H. After 5m*l* of control sample was removed for phage assay at the zero time, the chlorine stock solution was rapidly added to make 2.0 to 3.0mg/*l* chlorine dosage in the reaction system and mixed. At specified intervals, samples were removed into sterile tubes containing nutrient broth to stop the action of chlorine and examined for phage survival and chlorine concentration.

RESULTS

1. Inactivation of f2 with Chlorine

The preliminary tests showed that the buffer diluent and f2 preparation had low levels of chlorine demand. The reaction system in the absence of the phage was also evaluated for chlorine demand. The chlorine residual of the solution decreased by about 13 to 29% of the initial concentration in 300 sec of reaction. Less chlorine was lost at *p*H 10.0 in the system, since the mere stable hypochlorite ion was the predominant species of chlorine at that *p*H.

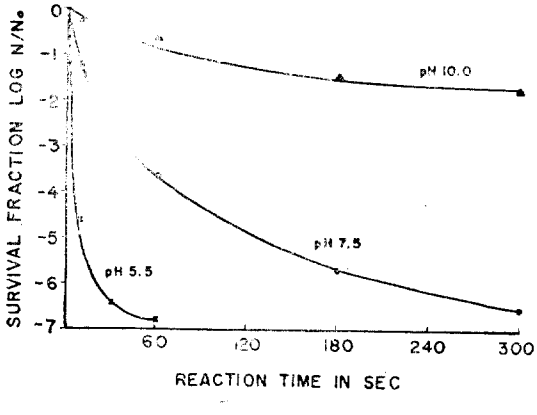

Fig. 1. Inactivation of bacteriophage f2 with 2.0 to 3.0mg/*l* chlorine at *p*H 5.5, 7.5, and 10.0 at 10°C

The inactivation of the phage f2 with chloine at different *p*H and reaction time can be seen in Fig. 1. Each point represents an average of three replicate trials. The phage was inactivated at different rates, depending on the *p*H value and reaction time. At *p*H 7.5, three to four logs were inactivated in 60 sec. At *p*H 10.0 about two logs of inactivation required 300 sec.

2. Effect of Chlorine on f2 RNA

Two types of RNA preparation were examined to evaluate the effect of chlorine on f2 phage RNA. After the phage f2 was treated with chlorine, phage survival was assayed and RNA was extracted from the same sample. The RNA was designated "RNA" and assayed for infectivity to the spheroplasts. This experiment provided information on the effect of chlorine on phage RNA in the chlorine-treated phage. Another experiment was performed with naked f2 phage RNA which was designated "n-RNA". The naked RNA was prepared from the f2 phage, then treated with chlorine, and assayed for infectivity.

The effects of chlorine on the intact phage f2, the RNA extracted from the chlorinated phage, and the naked RNA are compared in Fig. 2. The logs of the survival fraction (N/N_0) of the three different samples during the first 60 sec of reaction were plotted against reaction time at three different *p*H values. At *p*H 5.5, the rates of chlorine inactivation of the intact phage, its RNA, and the naked RNA were similar. All three were rapidly inactivated within 5 sec. At *p*H 7.5, the three samples showed the same rate of inactivation. However, the naked RNA was more rapidly inactivated than either the intact phage or its RNA at *p*H 10.0.

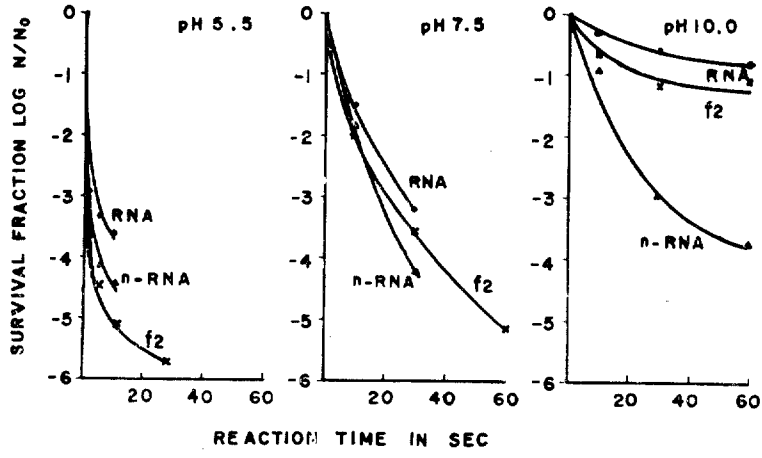

Fig. 2. Inactivation of 2.0 to 2.0 to 3.0mg/*l* chlorine at 10°C on phage f2, RNA extracted from chlorinated phage (RNA), and phage RNA (n-RNA) at *p*H 5.5, 7.5, 10.0.

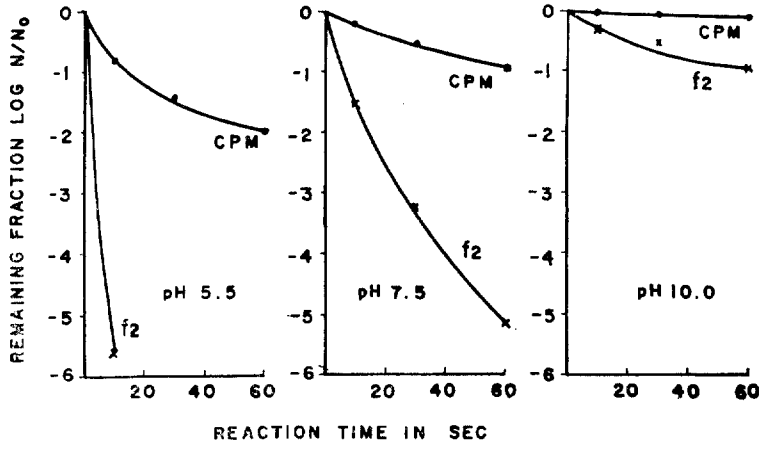

Fig. 3. The relationship between the inactivation of phage f2 with 2.0 to 3.0mg/*l* chlorine and specific attachment of the phage to bacteria *p*H 5.5, 7.5, and 10.0 at 10.0 at 10°C.

3. Effect of Chlorine on f2 Attachment

The sulfur-35 labeled phage f2 was treated with chlorine for 300 or 600 sec at different *p*H values of 5.5, 7.5, and 10.0 at 10°C. Residual chlorine and survival of the phage in the samples were examined during the reaction time. The chlorine-treated phage samples and untreated controls were assayed for their abilities to attach to the host and non-host bacteria. The results are shown in Table 1. The radioactivity of the untreated phage associated with the host bacteria was 6638 to 8337 and with non-host bacteria 63 to 83. Significant difference was observed between the specific and non-specific attachments of the control phage. At *p*H 5.5, about seven logs of the chlorinated phage were inactivated and specific attachment decreased by 60 sec. At *p*H 7.5, five logs of inactivation and decreasing attachment were observed by 180 sec. Survival and specific attachment of the phage decreased gradually at *p*H 10.0.

Table 1. Inactivation and attachment of sulfur-35 labeled bac teriophage f2

*p*H	Reaction time (sec)	Free chlorine (mg/*l*)	Phage survival (PFU/m*l*)	Radioactivity (CPM)	
				E. coli K-13 (host)	*E. coli* K-15T⁻ (non-host)
5.5		Control	5.9×10^{9}	7,232	63
	10	2.31	1.7×10^{4}	1,303	53
	30	2.24	6.4×10^{2}	368	40
	60	2.13	2.3×15^{2}	179	56
	180	2.07	2.1×10^{2}	364	128
	300	1.97	1.8×10^{2}	968	424
7.5		Control	4.2×10^{9}	6,638	83
	10	2.46	1.3×10^{8}	4,836	61
	30	2.33	2.4×10^{6}	2,559	52
	60	2.29	3.3×10^{4}	934	48
	180	2.21	1.8×10^{4}	480	146
	300	2.11	1.5×10^{3}	986	492
10.0		Control	7.3×10^{9}	8,337	71
	10	2.72	4.2×10^{9}	8,163	63
	30	2.48	2.8×10^{9}	7,885	37
	60	2,39	8.1×10^{8}	7,215	19
	180	2.24	4.8×10^{6}	5,531	89
	300	2.16	3.5×10^{4}	3,978	188
	600	2.02	2.3×10^{4}	1,575	856

However, non-specific adsorption of the phage to non-host bacteria remarkably increased after the phage had been inactivated with extended chlorination at each *p*H value.

Attachment data of the chlorinated phage were corrected for specificity by subtracting by non-specific adsorption and plotted as log N/N_0 for each sample to compare them with the survival fractions of the phage. The comparison of the inactivation rate and the specific attachment of the phage during the first 60 sec can be seen in Fig. 3. At each *p*H value, the specific attachment of the phage lagged noticeably behind the inactivation.

DISCUSSION

The chlorinie demand in a reaction system is particularly important in the study of disinfection. The demand produced by the high reactivity of chlorine with organic materials reduces the levels of active chlorine species (Scarpino *et al.* 1974). However, the evaluation of low levels of chlorine demand (up to 0.1mg/*l*) in a reaction system is extremely difficult. The volatile nature of the chloine and the opportunity for loss resulting from the necessary handling of low level chlorine solutions can offer reasonable explanations of the observed difference in this study.

The effect of *p*H on the species of chlorine present in an aqueous system is well understood. The inactivation of bacteriophage f2 at 10°C was directly related

to the presence of hypochlorous acid. The phage were rapidly inactivated at *p*H 5.5, where greater than 99% of the chlorine exists as hypochlorous acid. The inactivation proceeded at a slow rate at *p*H 10.0, where about 0.1% hypochlorous acid exists. At the intermediate *p*H of 7.5 (54.8 % hypochlorous acid), an intermediate rate of phage inactivation was observed. These results were consistent with the findings of decreased bactericidal and virucidal effect of chlorine with increased *p*H (Kelly and Sanderson 1958; Longley *et al.* 1974; Olivieri *et al.* 1975; Sproul 1976).

The inactivation rate of the intact phage with chlorine was similar to the rate of the RNA extracted from the chlorinated phage at each *p*H value. The inactivation rate of the naked RNA by hypochlorous acid at *p*H 5.5 and 7.5 was almost the same as that of the intact phage and its RNA. These results are well supported by those of Olivieri *et al.* (1975). On the other hand, the naked RNA was more susceptible to hypochlorite ion (the ionized form of hypochlorous acid at *p*H 10.0) than either the intact phage or its RNA. This indicates that hypochlorous acid was the major disinfecting species of chlorine to the intact phage f2 and that hypochlorite ion appeared to react less with viral RNA in the phage or to bleach the viral coat protein less, as suggested by others (Olivieri *et al.* 1974).

Bacteriophage f2 attaches to the side of F pili appended onto the surface of the male bacteria (Loeb and Zinder 1961). From the data of inactivation and attachment, the specific attachment of the chlorinated phage lagged behind the inactivation. This suggest that the inactivation of the phage with chlorine is not directly related to the A-protein playing a role of attachment and that the phage can still attach to the host after inactivation occurs. These results are supported by the postulation provided by Olivieri *et al.* (1975). However, the increase in nonspecific attachment of the phage upon extended chlorine may alter the structural integrity of the protein coat by reacting with the coat proteins of the phage. As a result, the free chlorine appeared to penetrate to the bacteriophage f2 through the protein coat and to inactivate the phage RNA as the main target.

摘 要

鹽素를 使用하여 bacteriophage f2의 殺菌作用을 *p*H 5.5, 7.5 그리고 10.0에서 各各 實驗하였다. 鹽素의 殺菌效果는 試驗溶液의 水素 이온 濃度와 反應時間에 따라 달랐으며 遊離鹽素中 hypochlorous acid가 殺菌作用의 主成分이었다. 鹽素로 處理한 phage f2의 不活性率과 그로부터 分離한 RNA의 不活性率 및 鹽素處理를 하지 않은 phage f2로 부터 RNA를 分離한 후 鹽素로 處理하여 그 不活性率을 함께 試驗하였을때 *p*H 5.5와 7.5에서는 세가지 試料가 比等한 成績을 보였으나 *p*H 10.0에서는 分離後 處理한 RNA가 鹽素에 對하여 더 빠른 不活性率을 나타냈다. 放射性 同位元素 S-35로 附着한 phage f2의 宿主細菌에 對한 特異性 吸着率과 非特異性 吸着率을 鹽素에 依한 殺菌效果와 比較試驗하였을때 鹽素에 依한 殺菌率의 急格한 增加에도 不拘하고 特異性 吸着率은 조금 減少하였으나 鹽素處理를 繼續함으로서 phage f2가 죽은 後에는 非特異性 吸着率이 顯著히 增加하였다. 따라서 鹽素에 依한 bacteriophage f2의 殺菌作用은 그 phage의 構成 蛋白質을 變化시켜 內部로의 浸透를 助長하는 同時에 主로 그 phage의 RNA를 不活性化 시킴으로서 이루어지는 것으로 評價되었다.

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