Bacteriophages of *Clostridium saccharoperbutylacetonicum*

Part XIV Effect of Mono- and Divalent Cations on HM 2 Phage*

By Motoyoshi HONGO, Akira MURATA and Seiya OGATA

*Laboratory of Applied Microbiology, Department of Agricultural
Chemistry, Kyushu University, Fukuoka, Japan*

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Monovalent cations such as Cs⁺, Rb⁺, K⁺ and Na⁺, and, to a lesser extent, NH₄⁺ had an inactivating effect on HM 2 phage (group I) of *Cl. saccharoperbutylacetonicum*. These cations had no effect on HM 3 phage (group II) and HM 7 phage (group III). The critical concentration of Na⁺ for inactivation of the phage was between 0.4 and 0.02 M with its maximum at 0.1 M. Divalent cations such as Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, Mn²⁺, Co²⁺ and Ni²⁺, and, to a lesser extent, Cd²⁺ and Zn²⁺, had a protective effect on the inactivation of the phage by monovalent cations. The molarity of Na⁺ for maximum inactivation of the phage was shifted by increasing concentration of Mg²⁺ added (5×10^{-4} , 1×10^{-3} , 2.5×10^{-3} , 5×10^{-3} and 1×10^{-2} M, respectively) from 0.1 M to 0.3, 0.5, 0.7, 0.9 and 1.5 M, respectively. Polyamines such as putrescine and spermidine also had a similar protective effect.

In the first paper of this series,¹⁾ the stability of HM-phages in salt solutions was briefly described. HM 2 phage (group I)²⁾ was strikingly unstable when diluted in 0.1 M NaCl without Mg²⁺, whereas HM 3 phage (group II)²⁾ and HM 7 phage (group III)²⁾ were fairly stable when diluted in the same solution. However, the detailed studies on the stability of HM-phages were not done.

The effect of the ionic environment on phages had been the subject of many investigations. However, there were few reports about the inactivation of phage by monovalent cation. Burnet and McKie³⁾ reported that monovalent cations enhanced the inactivation

of phage of *coli* and *staphylococcus* by heat and the addition of divalent cations partially or completely prevented the phage inactivation. Adams⁴⁾ and Lark and Adams⁵⁾ examined the inactivating effects of temperature on T5 coliphage in NaCl solution. Friedman⁶⁾ found that some phages of *Bacillus megaterium* were inactivated rapidly by NaCl or KCl of about 0.1 M. However, the mechanism of the inactivation of phage by monovalent cations has remained obscure. Therefore, the study on the mechanisms of inactivation of phage by monovalent cations was done using HM-phage systems. The study yielded some informations about a particular property of HM 2 phage.

The present paper deals with the effect of various monovalent cations on HM 2 phage and the protective effect by divalent cations on

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1) M. Hongo and A. Murata, *Agr. Biol. Chem.*, **29**, 1135 (1965).

2) M. Hongo, T. Aono and A. Murata, *ibid.*, **30**, 399 (1966).

3) F. M. Burnet and M. McKie, *Australian J. Exp. Biol. and Med. Sc.*, **7**, 183 (1930).

4) H. M. Adams, *J. Gen. Physiol.*, **32**, 579 (1949).

5) K. G. Lark and M. H. Adams, *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 171 (1953).

6) M. Friedman, *J. Bacteriol.*, **66**, 501 (1953).

the inactivation of the phage by monovalent cations. Succeeding reports will be published in this Journal.

MATERIALS AND METHODS

Phage and bacterial strain. Phage HM 2 (group I) and its host, *Clostridium saccharoperbutylacetonicum* N1-4, were used.^{1,2)}

Media. TYA medium¹⁾ was used throughout the work. Modified TYA broth was also used for preparing HM 2 phage.⁷⁾

Buffer solution. As a basal buffer, 0.005 M Tris-HCl buffer solution (pH 7.2) was used unless otherwise mentioned, in which the phage was stable.

Assay of phage. The phage was assayed by the modified double layer method described previously.⁸⁾

Preparation of phage. Phage lysates were prepared by the procedures previously described,^{8,9)} and purified by repeated centrifugation. The purified phages ($5 \sim 10 \times 10^{12}$ PFU/ml) in buffer containing 0.007 M Mg^{2+} (pH 7.2) were stored in an ice-jar until used. For long storage, the phage in buffer containing 0.007 M Mg^{2+} and 5% w/v glycerol (or 10% w/v dimethylsulfoxide) was stored at $-20^{\circ}C$ in a deep freezer.⁸⁾

Experiments of inactivation of free phage. The phages were diluted in the basal buffer (pH 7.2) to give a concentration of $5 \sim 10 \times 10^9$ PFU/ml at $0^{\circ}C$. They were further diluted in the basal buffer containing various testing cations of special reagent grade to give a concentration of $5 \sim 10 \times 10^7$ PFU/ml at $30^{\circ}C$ and removed at intervals for assay of phage survivals. The glass wares used in this experiment were cleaned with acid dichromate and repeatedly rinsed with hot distilled water.

RESULTS

Effect of concentration of sodium ion on phage inactivation

The stability of HM 2 phage in saline had

been described.¹⁾ The phage was strikingly unstable when diluted in 0.1 M saline. The stability of the phage increased as the concentration of saline decreased.

For detailed studies, the solutions of NaCl were prepared at various concentrations between 2 M and 0.001 M, in which the stability of phage was examined. The results are shown in Fig. 1. The phage was relatively stable at the concentration of NaCl, higher than 0.8 M and lower than 0.01 M. The critical concentration of NaCl for inactivation of the phage was between 0.4 M and 0.02 M, and the maximal inactivation was presented at 0.1 M. The result was differed from Adams',⁴⁾ but similar to Friedman's⁶⁾ in the existence of its max. critical concentration.

Experiments were then performed to determine whether the detrimental effect of NaCl to the phage was attributable to sodium ion, chloride ion, or both ions. The similar experiments were carried out with Na_2SO_4 and Na_3PO_4 . As shown in Fig. 2, the pattern of inactivation of the phage by these salts was the same as that by NaCl. Also, whether the anion used was chloride, phosphate or sulfate, the rate of inactivation was not sig-

FIG. 1. Inactivation of HM 2 Phage by Exposure to Various Molarities of NaCl.

▲ Incubation for 15 min. ○ Incubation for 60 min. ● Incubation for 24 hr.

Temp: $30^{\circ}C$.

Buffer: 0.005 M Tris-HCl buffer (pH 7.2).

Initial concentration of phage: 5×10^7 PFU/ml.

7) M. Hongo and S. Ogata, *Agr. Biol. Chem.*, **32**, 459 (1968).

8) M. Hongo and A. Murata, *ibid.*, **29**, 1149 (1965).

9) M. Hongo and A. Murata, *ibid.*, **30**, 292 (1966).

FIG. 2. Inactivation of HM 2 Phage by Exposure to Various Molarities of Na_2SO_4 and Na_3PO_4 .

○ Na_2SO_4 . ● Na_3PO_4 . — Incubation for 60 min. ---- Incubation for 24 hr.

Same explanations are used as in Fig. 1.

nificantly different in all cases. Therefore, it was clear that the inactivating effect of the salt was associated with the monovalent cation, sodium ion, and not with the anion.

Inactivation of phage by various monovalent cations other than sodium

The influence of monovalent cations other than Na^+ on the phage was examined. Sodium chloride was replaced by potassium, rubidium, cesium, lithium and ammonium chloride. Similar results were obtained with K^+ , Rb^+ , Cs^+ , and NH_4^+ as shown in Fig. 3. However, the potency of inactivation of the phage by NH_4^+ was weaker than that by Na^+ , K^+ , Rb^+ or Cs^+ , but their patterns of inactivation were similar one another. On the other hand, the pattern of inactivation of the phage by Li^+ was remarkably different from that by the other. The rate of inactivation of the phage increased according as the concentration of Li^+ increased, but at all concentrations employed the rate of inactivation by Li^+ was very low.

For comparing the power of the action of various monovalent cations on the phage one another, the molarity of cation for the max. inactivation was used and the time course of

FIG. 3. Inactivation of HM 2 Phage by Exposure to Various Molarities of K^+ , Cs^+ , Rb^+ , Li^+ and NH_4^+ .

○ K^+ . ▲ Cs^+ and Rb^+ . □ Li^+ . ■ NH_4^+ .

Same explanations are used as in Fig. 1.

FIG. 4. Time Course Curves of Inactivation of HM 2 Phage by Cs^+ , Rb^+ , Na^+ , K^+ , Li^+ and NH_4^+ .

▲ Cs^+ . △ Rb^+ . ● Na^+ . ○ K^+ . □ Li^+ . ■ NH_4^+ . Temp.: 30°C .

Initial concentration of phage: 1×10^8 PFU/ml. All monovalent ions used were at 0.1 M concentration.

inactivation of the phage was followed. The results are shown in Fig. 4. Among K^+ , Na^+ , Rb^+ and Cs^+ , the difference of the power of their action on the phage was not observed, and the power of NH_4^+ was weaker than that of the formers. However, the rate of inactivation by Li^+ did not appear to be ex-

ponential. The power of action of 0.1 M monovalent cations on the inactivation of HM 2 phage stand $\text{Cs}^+ = \text{Rb}^+ = \text{Na}^+ = \text{K}^+ > \text{NH}_4^+ > \text{Li}^+$ in line.

Prevention of phage inactivation by divalent metal ions and polyamines

HM 2 phage was completely stabilized by Mg^{2+} alone at more than $5 \times 10^{-3} \text{ M}^*$ at 30°C for 48 hr. It had been reported¹¹ that the stability of the phage in saline was increased by low concentration of Mg^{2+} . Now, divalent metal ions other than Mg^{2+} and polyamines were further examined.

To a basal buffer containing 0.1 M Na^+ , various divalent metal ions and polyamines were added at various concentrations. The results are shown in Tables I and II. The addition of $1 \times 10^{-3} \text{ M}$ Ca^{2+} , Mg^{2+} , Sr^{2+} , Ba^{2+} , Co^{2+} , Ni^{2+} or Mn^{2+} prevented completely the inactivation of the phage by 0.1 M Na^+ during

TABLE I. EFFECT OF VARIOUS DIVALENT CATIONS ON INACTIVATION OF HM 2 PHAGE BY SODIUM ION

Divalent cation added	Phage survivals (%) at time		
	2 min	2 hr	24 hr
None*	30	0	0
Be^{2+} ($1 \times 10^{-3} \text{ M}$)	30	0	0
Mg^{2+} ($1 \times 10^{-3} \text{ M}$)	100	100	75
Ca^{2+} ($1 \times 10^{-3} \text{ M}$)	100	95	60
Sr^{2+} ($1 \times 10^{-3} \text{ M}$)	100	95	75
Ba^{2+} ($1 \times 10^{-3} \text{ M}$)	100	100	70
Zn^{2+} ($1 \times 10^{-3} \text{ M}$)	85	40	2
Cd^{2+} ($1 \times 10^{-3} \text{ M}$)	92	76	33
Co^{2+} ($1 \times 10^{-3} \text{ M}$)	99	99	70
Ni^{2+} ($1 \times 10^{-3} \text{ M}$)	99	99	76
Cu^{2+} ($1 \times 10^{-3} \text{ M}$)	60	2	0
Mn^{2+} ($1 \times 10^{-3} \text{ M}$)	100	98	75

* 0.005 M Tris-HCl buffer containing 0.1 M Na^+ (pH 7.2).

Temp.: 30°C .

Initial concentration of phage: 5×10^7 PFU/ml.

* The molarity of divalent cation and polyamine is represented by signs of power, in order to distinguish from that of monovalent cation at a glance.

TABLE II. EFFECT OF POLYAMINES ON INACTIVATION OF HM 2 PHAGE BY SODIUM ION

Polyamine added	Phage survivals (%)
None*	0
Putrescine ($5 \times 10^{-2} \text{ M}$)	62 (70)**
($1 \times 10^{-2} \text{ M}$)	80 (90)**
($5 \times 10^{-3} \text{ M}$)	75 (90)**
($1 \times 10^{-3} \text{ M}$)	7 (90)**
Spermidine ($5 \times 10^{-2} \text{ M}$)	35 (36)**
($1 \times 10^{-2} \text{ M}$)	65 (60)**
($5 \times 10^{-3} \text{ M}$)	76 (90)**
($1 \times 10^{-3} \text{ M}$)	65 (90)**

* 0.005 M Tris-HCl buffer containing 0.1 M Na^+ (pH 7.2).

** Without 0.1 M Na^+ .

Incubation: for 60 min at 30°C .

Initial concentration of phage: 5×10^7 PFU/ml.

2 hr. The preventive effect of Zn^{2+} and Cd^{2+} was lower than that of these metal ions. In spite of killing agent against the phage, Cu^{2+} appeared to be an inhibitor against the inactivation of the phage within early phase of the reaction. But Be^{2+} did not prevent the inactivation. Putrescine and spermidine also could block the action of 0.1 M Na^+ , but at the same time showed their action of direct killing on the phage.

Effect of increasing concentration of divalent cation on inactivation of phage by various concentrations of monovalent cation

The effect of divalent cation was further determined with Mg^{2+} . HM 2 phage was incubated in Na^+ between 2 M and 0.001 M containing $5 \times 10^{-4} \text{ M}$ to $1 \times 10^{-2} \text{ M}$ Mg^{2+} . The results are shown in Figs. 5 and 6. After 60 min, $5 \times 10^{-4} \text{ M}$ Mg^{2+} prevented completely the inactivation of the phage by Na^+ at the concentration of 0.05 M and less, and the max. critical concentration of Na^+ for the phage inactivation shifted from 0.1 M to 0.3 M. $1 \times 10^{-3} \text{ M}$ and $2.5 \times 10^{-3} \text{ M}$ Mg^{2+} blocked the action of 0.4 M Na^+ and less, and $5 \times 10^{-3} \text{ M}$ Mg^{2+} did completely as high as 2 M Na^+ . After 24 hr, the protective effect of Mg^{2+} became lower

FIG. 5. Effect of Various Concentrations of Magnesium Ion on Na^+ -Inactivation of HM 2 Phage for Short Period.

○—○ no Mg^{2+} . $\triangle$ — $\triangle$ 5×10^{-4} M Mg^{2+} . $\blacksquare$ — $\blacksquare$ 1×10^{-3} M Mg^{2+} . $\triangle$ — $\triangle$ 2.5×10^{-3} M Mg^{2+} . $\blacktriangle$ — $\blacktriangle$ 5×10^{-3} M Mg^{2+} .
Incubation: for 60 min at 30°C .
Initial concentration of phage: 5×10^7 PFU/ml.

FIG. 6. Effect of Various Concentrations of Magnesium Ion on Na^+ -Inactivation of HM 2 Phage for Prolonged Period.

●—● no Mg^{2+} . $\triangle$ — $\triangle$ 5×10^{-4} M Mg^{2+} . $\blacksquare$ — $\blacksquare$ 1×10^{-3} M Mg^{2+} . $\triangle$ — $\triangle$ 2.5×10^{-3} M Mg^{2+} . $\blacktriangle$ — $\blacktriangle$ 5×10^{-3} M Mg^{2+} . $\square$ — $\square$ 1×10^{-2} M Mg^{2+} .
Incubation: for 24 hr at 30°C .
Initial concentration of phage: 5×10^7 PFU/ml.

in all concentrations of Na^+ than after 60 min. Further, by prolonging the incubation time, it became clear that the concentration of Na^+ for max. rate of inactivation was shifted; it was shifted by 1×10^{-3} , 2.5×10^{-3} and 5×10^{-3}

M Mg^{2+} from 0.1 to 0.5, 0.7 and 0.9 M, respectively. 1×10^{-2} M Mg^{2+} blocked completely the action of 0.8 M Na^+ and less during 24 hr, and shifted the max. critical concentration of Na^+ for the phage inactivation to about 1.5 M.

It was ascertained that the higher concentration of Mg^{2+} was more effective against the action of Na^+ than the lower concentration.

When Ca^{2+} , Ba^{2+} , Co^{2+} or spermidine was employed, the shifting of max. critical concentration of Na^+ for the phage inactivation was recognized as when Mg^{2+} was employed.

The protective profile of Mg^{2+} against the various concentrations of K^+ , Rb^+ or Cs^+ on the phage inactivation was very similar to Mg^{2+} - Na^+ profile in the shape of phage survival curve and in the shifting of it. Further the similar examinations were done about NH_4^+ which did not act so vigorously on HM 2 phage or about Li^+ which inactivated the phage in different pattern from that of Na^+ in the absence of Mg^{2+} . The results are shown in Fig. 7. The rate of inactivation after 24 hr was a little larger than that after 60 min, and the max. critical concentration of NH_4^+ for the inactivation was about 0.5 M, similar to

FIG. 7. Effect of Magnesium Ion on Inactivation of HM 2 Phage by Li^+ and NH_4^+ in Various Concentrations.

$\square$ Li^+ . $\blacksquare$ NH_4^+ . — Incubation for 60 min. --- Incubation for 24 hr.
Temp: 30°C .
Initial concentration of phage: 5×10^7 PFU/ml.
Concentration of Mg^{2+} : 1×10^{-3} M.

that in the case of Na^+ . On the other hand, the max. critical concentration of Li^+ for the phage inactivation was not seen and the rate of inactivation became larger as with increasing the concentration of Li^+ in spite of the presence of Mg^{2+} . The action of Li^+ on the phage was considered to be essentially different from that of Na^+ and the other monovalent cations.

The phenomena stated above might be

interpreted on the basis of ion antagonism between mono- and divalent cation. In a following paper,¹⁰⁾ it will be discussed.

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10) M. Hongo and S. Ogata, in preparation.