The role of bivalent ions in the inactivation of bacteriophage ϕ X174 by lipopolysaccharide from *Escherichia coli* C

Elizabeth ROWATT

Department of Medical Microbiology, Faculty of Clinical Sciences, University College, University Street, London WC1E 6JJ, U.K.

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1. The need for Ca^{2+} in the inactivation of bacteriophage ϕ X174 by lipopolysaccharide from *Escherichia coli* C was confirmed. 2. Ca^{2+} could be replaced almost completely by Na^+ , but the concentration of Na^+ needed was greater by more than an order of magnitude. 3. Other bivalent ions caused inactivation in the same way as Ca^{2+} , and the degree of inactivation varied according to the ion. 4. At 50% inactivation of bacteriophage, the relation between the concentrations of NaCl and of bivalent or trivalent ions (M^{x+}) fitted the conception that NaCl was neutralizing electrostatic repulsion between virus and lipopolysaccharide by an ionic-strength effect: that is, $\log[\text{M}^{x+}]$ varies inversely with $\sqrt{[\text{NaCl}]}$. 5. The variation in effect of bi- and ter-valent ions and the low concentration needed show that this is not an ionic-strength effect but likely to involve binding to more than one site.

The bacteriophage ϕ X174 is inactivated by lipopolysaccharide from its host *Escherichia coli* C so that it no longer forms plaques on lawns of the host organism. At the same time the viral DNA becomes susceptible to deoxyribonuclease, as it does at the beginning of infection of the whole bacterium. Ca^{2+} or Mg^{2+} is necessary for this reaction (Fujimura & Kaesberg, 1962; Jazwinski & Marco, 1973; Incardona & Selvidge, 1973).

In the present work, NaCl has been found to replace most of the Ca^{2+} required for the reaction. Other bivalent ions and one trivalent ion are shown to replace Ca^{2+} completely, and Na^{2+} will reduce the concentration necessary with all ions.

Estimation of the relative concentrations of NaCl used with a given concentration of a particular bivalent or trivalent ion to cause 50% inactivation of bacteriophage has shown that the activity of these ions varies considerably and the order of activity in low concentrations of NaCl differs from the order of activity in high concentrations of NaCl. The evidence points to more than one site of action of the ions and a role of NaCl and of the bi- and ter-valent ions in low $[\text{NaCl}]$ of neutralizing charges on virus and lipopolysaccharide.

Materials and methods

Micro-organisms

Bacteriophage ϕ X174 (wild type) and *E. coli* C (A.T.C.C. 13706) were obtained from Dr. A. B. Stone, formerly of the Department of Botany and Microbiology, University College, London, U.K.

Preparation of lipopolysaccharide

Bulk quantities of *E. coli* C wet-cell paste (100g bags) were supplied by the Microbiological Research Establishment, Porton, Wilts., U.K. The cells had been washed in 10mM-Tris buffer, pH 7.4, containing 10mM-magnesium acetate. Cell walls were prepared by breaking the cells, suspended in 0.1M-phosphate buffer, pH 7.4, in a Manton-Gaulin homogenizer (model 15M-8 BA; APV Co., Crawley, Sussex, U.K.) by the method of Follows *et al.* (1971).

Lipopolysaccharide was extracted by 45% (w/v) phenol (Osborn, 1966) from cell walls treated with chloroform/methanol (2:1, v/v) (Key *et al.*, 1970a). To prepare the sodium salt, the crude lipopolysaccharide was separated from the aqueous phase by centrifuging at 20000g for 1 h. The deposit was treated with Chelex 100 (Bio-Rad analytical-grade

chelating resin; Bio-Rad Laboratories, Richmond, CA, U.S.A.) to give a solution of the sodium salt (A. P. MacLennan, personal communication). This was centrifuged again at 20000g for 1 h to remove peptidoglycan (Key *et al.*, 1970b), leaving a clear solution of sodium lipopolysaccharide. The lipopolysaccharide was pelleted from this by centrifuging at 100000g, giving a clear jelly which was dissolved in water to give a concentration of 10–15mg/ml. This was kept at +4°C and its concentration was estimated by its heptose content (Osborn, 1963), allowing 3mol/mol of lipopolysaccharide (Jansson *et al.*, 1981). The Ca^{2+} concentration was determined with an atomic absorptiometer (by kind permission of Professor Peter Baker, King's College, London, U.K.).

Growth of bacteriophage

Bacteriophage ϕX174 was grown by a modification of the method used by Sinsheimer (1966) for the growth of lysis-defective mutants of phage ϕX174 . Phage ϕX174 was purified by precipitation at pH4 and extraction with Arcton 113 (ICI, London S.W.1, U.K.; Epstein, 1958), followed by differential centrifugation in sucrose and tartrate density gradients (Newbold & Sinsheimer, 1970); potassium tartrate was used instead of CsCl.

Assay of bacteriophage

Bacteriophage was assayed using serial tenfold dilutions in a modified Miles & Misra (1938) determination on lawns of *E. coli* C growing on Tryptone Soy Agar (London Analytical and Bacteriological Media, London EC3R 7QB, U.K.). Glass pipettes delivering 0.02ml were used for adding phage to the lawns. Plaques were counted in dilutions containing 10–100 plaques/0.02ml after a 2 h incubation at 37°C.

Measurement of phage inactivation

Phage inactivation was carried out in 0.5ml of 10mM-Tris acetate buffer, pH7.4 (10mM-Tris/5mM-acetic acid) containing $(2-4) \times 10^5$ p.f.u. (plaque-forming units) of phage/ml, lipopolysaccharide and other ions as stated. The mixtures were incubated with shaking in a water bath at 37°C for 21 min and the residual phage was titrated. All experiments were carried out with one preparation of *E. coli* C lipopolysaccharide at a concentration of 0.18 μM containing approx. 0.01 μM - Ca^{2+} . Ca^{2+} contamination was reduced by using double-distilled water deionized by passing through Dowex MR-3C nuclear-grade mixed resin (Sigma Chemical Co., St. Louis, MO, U.S.A.) to give a resistance of more than 2M Ω . Plastic vials, containers and pipettes were used except for the final 0.02ml drops in the phage assay (see above). All solutions were stored in plastic except 1M-

CaCl_2 solution. The general level of Ca^{2+} contamination in the inactivation system was approx. 0.1 μM .

Expression of phage inactivation

The degree of inactivation was expressed as $(P_0 - P_t)/P_0$, where P_0 is the phage titre with all components except lipopolysaccharide, and P_t is the phage titre with all components, including lipopolysaccharide, both after 21 min incubation. In the text, inactivation is generally referred to as a percentage (the degree of inactivation is multiplied by 100). The amounts of added ions which give 50% inactivation are related to the binding constant of virus and lipopolysaccharide, assuming reversible binding. The titre in the absence of lipopolysaccharide was the same before and after 21 min incubation with the ions at the concentrations used and did not differ from that with lipopolysaccharide before incubation. Regression lines in Table 1 and Figs. 1, 2 and 3 were calculated from means ($\pm$ S.D.) of results from three or more experiments. In Table 2 the concentration of NaCl giving 50% phage inactivation was calculated for each bi- or ter-valent-ion concentration in each experiment, and these values (with S.E.M.) were used for calculation.

Chemicals

Chloride salts were used, and all chemicals were BDH AnalaR Reagents, except NaCl, which was Grade 2 (High Purity Materials; Johnson Matthey Chemicals, London E.C.1, U.K.); hexamine cobaltic chloride $[\text{Co}(\text{NH}_3)_6\text{Cl}_3]$, which was a BDH Laboratory Reagent; and pentammine cobaltic chloride $[\text{CoCl}(\text{NH}_3)_5\text{Cl}_2]$, which was a gift from Professor M. L. Tobe (Department of Chemistry, University College, London, U.K.). The CaCl_2 was a standard 1M-solution and it was used to standardize the other salts by AgNO_3 titration with K_2CrO_4 as indicator, except for the complex cobalt ions. $\text{CoCl}(\text{NH}_3)_5\text{Cl}_2$ was washed with conc. HCl and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ was recrystallized from HCl and both were dried *in vacuo*. These were weighed without further drying for fear of decomposition. In some early experiments NaCl was dried to constant weight and MgCl_2 was prepared by weighing MgO and dissolving it in HCl.

Results

Effect of Ca^{2+} and Na^+ on phage inactivation

The preparation of lipopolysaccharide used in these experiments gave 50% inactivation of phage with 0.9mM- Ca^{2+} (Fig. 1). The addition of Na^+ reduced the concentration of Ca^{2+} needed, 30mM- Na^+ , for example, causing 50% inactivation with

Fig. 1. Effect of Ca^{2+} on inactivation of bacteriophage by lipopolysaccharide

Bacteriophage $[(2-4) \times 10^5 \text{ p.f.u./ml}]$ was incubated with lipopolysaccharide ($0.18 \mu\text{M}$) in 10mM-Tris/acetate buffer, pH 7.4, at 37°C for 21 min with various concentrations of CaCl_2 . Points represent means for two (0.8mM, 1.5mM), four (0.7mM, 0.9mM) or six (1.0mM, 1.2mM) experiments with S.D. values of 0.08 (two values) or less than 0.04 (four values). Open circles represent calculated values. Regression line has $r^2 = 0.984$.

0.5mM-Ca^{2+} . The higher the concentration of Na^+ the lower the concentration of Ca^{2+} needed, and plotting the relation between inactivation and $[\text{Na}^+]$ with a constant $[\text{Ca}^{2+}]$ gave a series of straight lines (Fig. 2), which were parallel until the $[\text{Ca}^{2+}]$ fell below 0.1mM. At 0.05mM-Ca^{2+} , 400mM-Na^+ gave only 30–40% inactivation and, if Ca^{2+} was not added, the phage was not inactivated. At 50% inactivation the two ion concentrations do not vary inversely; the $[\text{Na}^+]$ needed per unit of Ca^{2+} rises as the $[\text{Ca}^{2+}]$ falls (see Fig. 2).

K^+ , NH_4^+ and Tris ions replaced Ca^{2+} almost quantitatively in the same way as Na^+ , and this suggested that all their effects were due to ionic-strength changes. Tris was present as the buffer of the reaction medium and its effect on the reaction was tested by using 10, 20 or 30mM-Tris, each with a range of Na^+ concentrations. Tris was slightly less active than Na^+ , 10mM-Tris having the same action as 8.5mM-Na^+ . This means that the Ca^{2+} activity recorded in Fig. 1 was assisted by the

equivalent of 8.5mM-Na^+ . In calculations of all other experiments, including those recorded in Fig. 2, 8.5mM-NaCl representing the Tris buffer has been added to the concentration of NaCl used.

Effect of other bivalent ions on phage inactivation

Other bivalent ions were tested in place of Ca^{2+} in the phage inactivation. All caused inactivation and the inactivation was increased by NaCl , but the amount of inactivation varied according to the ion used.

Their activity is recorded in Table 1(a) as the concentration of NaCl necessary for 50% inactivation when a series of concentrations of bivalent ions was used. The order of activity depends on whether a high or a low concentration of the ion is considered: Ca^{2+} is more active than Ni^{2+} at a low concentration, but Ni^{2+} becomes more active than Ca^{2+} as the concentration rises, having more than three times the activity at 0.5mM. The selectivity of the effect shows that the bivalent ions do not simply change the ionic strength. Binding of ions must be involved.

The tervalent hexammine cobalt(III) ion was also tested. As shown in Table 1(b), this ion was much more active than the bivalent ions. This is strong evidence that there is an electrostatic component to the ion binding at one site, as expected from the nature of the lipopolysaccharide.

Discussion

Bacteriophage ϕX174 has an isoelectric point of pH 6.6 (Aach, 1963), so that at pH 7.4 it is negatively charged. The *E. coli* lipopolysaccharide contains carboxy and phosphate groups, which are also negatively charged (Jansson *et al.*, 1981), and before it reacts with the virus the like charges on each must be neutralized. Let us therefore consider the reaction of two negatively charged surfaces (effectively polyelectrolytes) which we shall call P^{n-} (lipopolysaccharide) and V^{m-} (virus):

The reaction is opposed by electrostatic repulsion, which is reduced by the addition of salt, so that binding can be written

$$\log K = \log K_0 + A\sqrt{\mu} \quad (1)$$

where μ is the ionic strength, K_0 is the value of K at $\mu = 0$, and A is a constant.

The effectiveness of P against V then increases with μ , since the sign of A is that for two like charges. This is observed.

Now the effectiveness of P is also observed to increase at very low concentrations of bivalent

Fig. 2. Replacement of Ca^{2+} by Na^+ in phage inactivation by lipopolysaccharide

The procedure was that described in Fig. 1, except that $[\text{NaCl}]$ was varied with constant concentrations of Ca^{2+} . Regression lines were calculated from results from at least three experiments with s.d. of never more than 0.1 and in more than half the results less than 0.06. Open circles represent calculated values. Regression lines had $r^2 = 0.997$ –0.998, except that for 0.1 mM- Ca^{2+} , which had $r^2 = 0.981$.

cations, M^{x+} , which could not be an ionic-strength effect since it has a high degree of metal-ion specificity, but must be related to the stabilization of PV by M^{x+} . To describe this binding we need a second constant, K_M , for the reaction:

This will also have an ionic-strength-dependence:

$$\log K_M = \log K_{M(0)} - B_M \sqrt{\mu} \quad (2)$$

where B_M is a constant and the change of sign reflects the fact that the reaction is between oppositely charged species.

If we study the effect of changes of salt (NaCl), i.e. of μ , on the effectiveness of P in the presence of different M, then we must sum eqn. (1) and eqn. (2) to get PVM information:

$$\begin{aligned} \log(K \cdot K_M) &= \log(K_0 \cdot K_{M(0)}) + (A - B_M) \sqrt{\mu} \\ &= \log(\text{effectiveness}) \\ &= \log \frac{[\text{PVM}]}{[\text{P}][\text{V}]} + \log \frac{1}{[\text{M}]} \end{aligned}$$

In Fig. 3 we plot 50% inactivation of V action at different NaCl concentrations (represented by μ)

using Ca^{2+} at fixed concentrations, $[\text{M}]$. Points represent equivalent activity of $\sqrt{[\text{salt}]}$ at a variety of $\log[\text{M}]$ values. Since P and V are fixed (50% inhibition), we expect a straight line of slope $A - B_M$ and intercept $\log(K_0 \cdot K_{M(0)})$. Data for other ions tested are listed in Table 2, which gives slopes and x-intercepts. The slopes depend on the metal ion used, which means that B_M may not refer to the same binding sites, i.e. there are differing B_M values for the different metals.

We note that the tervalent cation, $[\text{Co}(\text{NH}_3)_6]^{3+}$, is most effective, as expected, since it should have a large $K_{M(0)}$, assuming that binding has a large electrostatic component. At $\sqrt{\mu} = 0$, small bivalent ions are slightly more effective than large bivalent ions, but all bivalent ions fall in the small range of 0.9–4 mM. The peculiarity is the effect of salt, which greatly increases the spread of the effects of different metal ions, so that at high salt, Cd^{2+} is especially effective and Ca^{2+} becomes much more effective. This suggests that $K_{M(0)}$ is the sum of bindings to different sites which are differently ionic-strength-dependent, i.e. they have different B_M values. One explanation could be that Ca^{2+} and Cd^{2+} (large cations) can bind to $-\text{SO}_3^-$ anions in regions of high negative charge. It is

Table 1. Concentrations of NaCl required to give 50% inactivation of phage by lipopolysaccharide with (a) various bivalent ions (b) the trivalent ion $[Co(NH_3)_6]^{3+}$. The mean inactivation was obtained for each concentration of M^{2+} and NaCl (at least three experiments per value) and the concentration of NaCl giving 50% inactivation of phage calculated from the regression lines for each ion. r^2 was 0.98 or 0.99 for 80% of the results and 0.91 was the lowest value.

(a)	$[M^{2+}]$ (mm)	[NaCl] giving 50% inactivation (mm)						(b) $[Co(NH_3)_6]^{3+}$		[NaCl] giving 50% inactivation (mm)
		M^{2+} ... Cd^{2+}	Mn^{2+}	Co^{2+}	$[CoCl(NH_3)_5]^{2+}$	Ni^{2+}	Sr^{2+}	Ca^{2+}	Mg^{2+}	
	0.025	92.53								94.08
	0.040	67.08								61.51
	0.050	55.82								45.68
	0.075	45.55						97.26		43.06
	0.10	41.47						83.49		30.91
	0.150	28.57	54.77	58.62	85.60	98.91				
	0.20		42.49	51.27	46.20	73.30	67.12	62.67	101.2	
	0.25		30.37	36.28		56.75				
	0.30			33.26						
	0.40		18.71	15.66		28.35	57.11	52.60	61.64	
	0.45				14.41	15.35	40.21		54.32	
	0.50					9.08		32.03		
	0.60								35.50	
	0.70							17.93		
	0.80						16.95		15.04	
	0.90							9.23		
	1.00								8.99	

Table 2. Relation between $\log M^{x+}$ and NaCl concentrations giving 50% inactivation of phage, and characteristics of regression lines

Data in this Table are those given in Table 1 but, in order to give a more meaningful standard error, concentrations of NaCl giving 50% phage inactivation at each M^{x+} concentration were calculated for each experiment. Values in column 3 are expressed as the reciprocal of $A-B_M$. To obtain the values in columns 2 and 3, all concentrations were expressed in molar units; values in columns 4 and 5 are given in millimolar units. (Sr^{2+} results were from four experiments, all others from six or more.)

Ion	From x-intercept, $K_0 K_{M_{ion}} (\pm \text{S.E.M.})$ $= \frac{1}{[M^{x+}]}$	Slope $\frac{1}{A-B_M} (\pm \text{S.E.M.})$ $= \frac{\sqrt{[NaCl]}}{\log [M^{x+}]}$	[M^{x+}] (mm) at:	
			$\sqrt{[NaCl]} = 0$	$\sqrt{[NaCl(M)]} = 0.392$
$[Co(NH_3)_6]^{3+}$	$1.78 \times 10^4 (1.75 \times 10^3)$	-0.296 (0.016)	0.0563	0.00496
Cd^{2+}	$6.12 \times 10^2 (1.56 \times 10^2)$	-0.161 (0.012)	1.63	0.00609
Mn^{2+}	$6.80 \times 10^2 (1.07 \times 10^2)$	-0.204 (0.015)	1.47	0.0178
Co^{2+}	$5.31 \times 10^2 (7.94 \times 10^1)$	-0.196 (0.013)	1.88	0.0189
Ca^{2+}	$2.44 \times 10^2 (2.52 \times 10^1)$	-0.186 (0.007)	4.11	0.0323
$[CoCl(NH_3)_5]^{2+}$	$1.10 \times 10^3 (1.76 \times 10^2)$	-0.300 (0.029)	0.908	0.0449
Sr^{2+}	$3.09 \times 10^2 (4.95 \times 10^1)$	-0.217 (0.016)	3.24	0.0501
Ni^{2+}	$1.02 \times 10^3 (8.64 \times 10^1)$	-0.325 (0.018)	0.979	0.0608
Mg^{2+}	$5.13 \times 10^2 (4.99 \times 10^1)$	-0.325 (0.020)	1.95	0.1207

Fig. 3. Concentrations of Na^+ and Ca^{2+} giving 50% inactivation of phage with lipopolysaccharide

Data are from Fig. 2; 50% end points were found from regression lines and plotted as $\log[Ca^{2+}]$ against $\sqrt{[NaCl]}$.

known that large cations bind more selectively to larger strong acid anions, e.g. $-SO_3^-$, than do smaller cations (Williams, 1970), whereas the smaller cations, and especially the transition-metal cations, bind better to weak acid anions, e.g. $-CO_2^-$, and even neutral centres. Strain *et al.*, (1983), using n.m.r. studies, have found evidence indicating that bivalent and trivalent metals such as Ca^{2+} , Cd^{2+} and La^{3+} interact strongly with a

high-affinity binding site in lipopolysaccharide. We propose that there are at least two chemically different binding sites in the system for bivalent-metal ions.

The binding of $[Co(NH_3)_6]^{3+}$ shows a slope unlike that of the other large cations. It must be remembered, however, that this ion and the $[CoCl(NH_3)_5]^{2+}$ ion are quite unusual in that they do not lose their co-ordination shell ligands on combination with anions. It is difficult, therefore, to make a simple comparison with the series of hydrated ions in Table 2.

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