The inhibition of infectivity of bacteriophage ϕ X174 by high-valency metal cations and cyclic polyamines

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It has been shown previously that many aliphatic polyamines and metal cations decrease the infectivity of bacteriophage ϕ X174 when lipopolysaccharide from *Escherichia coli* C is present. Cations of higher charge are more effective. In the present paper it is shown that certain of the metal cations and cyclic polyamines diminish phage infectivity without lipopolysaccharide. The relation of cation concentration to loss of infectivity is different for the two types of reaction. In the absence of lipopolysaccharide the inhibition increases with the charge of the cation, but that by cyclic polyamines depends also on the hydrocarbon chains and their conformation. Some characteristics of the reactions are discussed.

INTRODUCTION

The bacteriophage ϕ X174 is inactivated by interaction with lipopolysaccharide from its host *Escherichia coli* C in the presence of metal cations or organic polyamines of the type $\text{NH}_3^+ \{ [\text{CH}_2]_m \text{NH}_2^+ \}_n [\text{CH}_2]_m \text{NH}_3^+$ (Rowatt & Williams, 1985). The effect of the cations is mainly proportional to their charge and consists, at least in part, of neutralization of the negative charge on both macromolecules, which then allows their interaction. The activity of bivalent ions varies with the cation and was shown to be related to their binding capability. Whereas most bivalent cations, the trivalent cation hexamine cobaltic ion and the linear aliphatic polyamines do not affect the bacteriophage without lipopolysaccharide, we show here that some metal cations of high valency, such

as La^{3+} , one bivalent cation, Cd^{2+} , and some cyclic polyamines can inhibit the bacteriophage activity in the absence of lipopolysaccharide. In fact addition of lipopolysaccharide reduces the inhibition in many of these cases. These effects will be described and discussed with reference to Scheme 1, which shows the multiplicity of interactions which can occur in a complex system of the three interacting species: lipopolysaccharide, viral surface and cations.

MATERIALS AND METHODS

The methods used were those reported previously (Rowatt & Williams, 1985), except that phage was diluted for assay in 10 mM-Tris/5 mM-acetate buffer,

Scheme 1. Interactions of phage, cations and lipopolysaccharide

Inhibition by metal ions is produced in two ways: (1) by the direct action of M^{n+} removing phage (rate constant K_b) and (2) by a combined action of M^{n+} and lipopolysaccharide (LPS) (K_I). Lipopolysaccharide can also interact with M^{n+} (K_a) (Figs. 1 and 3). Note that $K_x K_I = K_b$, $K_{III} = K_a K_{II}$.

Table 1. Inhibition of phage infectivity by metal cations and polyamines in the presence and absence of lipopolysaccharide

Bacteriophage ϕ X174 (2×10^5 plaque-forming units/ml) was incubated at 37 °C for 20 min in 10 mM-Tris/acetate buffer, pH 7.4, with or without lipopolysaccharide (LPS) from *E. coli* C (0.45 μ M). Dilutions for phage titration were made in 10 mM-Tris/acetate buffer, pH 7.4, except the last three cyclic polyamines, which were diluted in 0.01 M-borate buffer. Results are means $\pm$ s.d. obtained from regression lines calculated from three or more experiments and are expressed as $-\log [\text{cation}]$, that is, the first set of values, 5.12 ± 5.75 , represents the values $7.58 \times 10^{-6} \text{ M} \pm 1.77 \times 10^{-6} \text{ M}$. Cd^{2+} has a biphasic response in the presence of lipopolysaccharide: (1) represents the value at $\log [\text{Cd}^{2+}]$ values below -4.3 , and (2) represents the value above -4.3 . The purity of the Ruthenium Red is uncertain.

Cation	Average charge at pH 7.4	$-\log \{[\text{Cation}] (\text{M})\}$ at $P_0 - P_t = P_t$	
		- LPS	+ LPS
1,4,7-Triazacyclodecane	1.1	5.12 ± 5.75	3.70 ± 5.10
1,4,8,12-Tetra-azacyclopentadecane	2.0	3.68 ± 4.44	—
1,4,7,10,13,16-Hexa-azacyclo-octadecane	3.0	6.42 ± 7.85	4.97 ± 6.16
1,4,7-Triazacyclononane	1.2	< 3	
1,4,7,11-Tetra-azacyclododecane	2.0	< 2	
Cd^{2+}	2	4.30 ± 4.97	(1) 4.51 ± 5.22 (2) 4.32 ± 5.49
La^{3+}	3	5.82 ± 6.24	5.38 ± 7.10
$[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	5	7.29 ± 7.92	5.84 ± 7.40
$[(\text{NH}_3)_5\text{RuORu}(\text{NH}_3)_4\text{ORu}(\text{NH}_3)_5]^{6+}$	6	7.46 ± 7.92	5.82 ± 6.60

pH 7.4, the buffer used for incubation, instead of 0.01 M-borate buffer, pH 7.4, which was used in the previous experiments. Tris was used because with Cd^{2+} and La^{3+} , for example, the decrease in phage activity was lower in the borate buffer, and it is known that such cations can give insoluble borates. The phage titre was not altered by incubation without cations for 20 min.

The lipopolysaccharide (from *E. coli* C) was the second preparation described by Rowatt & Williams (1985) and was used at the same dilution as in that paper. This concentration as estimated by galactose content by Dr. S. G. Wilkinson (Department of Chemistry, University of Hull, Hull, U.K.) is now shown to be 0.45 μ M. For precipitation by cations a preparation of higher concentration (6.75 μ M) was used.

In previous work the fall in phage titre was recorded as the fraction of phage infectivity lost, $(P_0 - P_t)/P_t$, where P_0 is the initial phage titre and P_t the titre at time t (Rowatt, 1984). In the present experiments, the reduction in infectivity was recorded as $(P_0 - P_t)/P_t$ or the ratio of non-infective to infective phage. In experiments with cation alone this is proportional to the concentration of cation (see below). The errors in this method of presentation appear larger than in that used previously, especially at large values of P_t , but the response curves obtained are more easily appreciated in terms of the equilibrium constants of Scheme 1.

The source of most chemicals was as reported by Rowatt & Williams (1985). Ruthenium Red (48% pure) was obtained from Sigma Chemical Co., Poole, Dorset, U.K.; 1,4,8,12-tetra-azacyclopentadecane and 1,4,7,10,13,16-hexa-azacyclo-octadecane from Aldrich Chemical Co., Gillingham, Dorset, U.K. The other cyclic polyamines were gifts from Professor M. Tobe (University College London, London, U.K.). $(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5\text{Cl}_5$ was a gift from Professor A. G. Sykes (University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.). The Ruthenium Red contained 98% of the

theoretical concentration of ruthenium ion, but the concentration as assessed by the colour of the product was only 48% of the theoretical [according to the suppliers (Sigma)].

RESULTS

The new observations in the present paper are, firstly, that some metal cations of high charge reduce the infectivity of bacteriophage ϕ X174 in the absence of lipopolysaccharide (Table 1). The cations most active at low concentration are, in order: $[(\text{NH}_3)_5\text{RuORu}(\text{NH}_3)_4\text{ORu}(\text{NH}_3)_5]^{6+}$ (Ruthenium Red) and $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+} > \text{La}^{3+} > \text{Cd}^{2+} \gg [\text{Co}(\text{en})_3]^{3+}$ (where en is ethylenediamine). The effect of $[\text{Co}(\text{en})_3]^{3+}$ decreases above 0.1 mM (Fig. 1). $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$ has not been tested at higher levels. The results for Ruthenium Red almost coincide with those for $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$. Since the Ruthenium Red is of somewhat uncertain purity, it is not included when the absolute level of concentration is significant.

The second new observation is that cyclic polyamines of the general formula $\{\text{NH}[\text{CH}_2]_m\}_n^{q+}$ also reduce phage infectivity on incubation. The formulae of these compounds are given in Fig. 2. The effect is not solely related to charge, although the most active, 1,4,7,10,13,16-hexa-azacyclo-octadecane, is the most highly charged, with an average positive charge at pH 7.4 of 3.0 (Fig. 1; Table 1). The next most active is 1,4,7-triazacyclodecane, with an average charge of 1.0 at pH 7.4, and then 1,4,8,12-tetra-azacyclopentadecane with a charge of 2 at this pH. The compound 1,4,7-triazacyclononane, with an average charge of 1.2 at pH 7.4, is inactive at 1 mM, and 1,4,7,11-tetra-azacyclododecane (charge 2.0) is inactive at 8 mM (Table 1; pK values from Kodama & Kimura, 1976a,b, 1978; Kimura *et al.*, 1981). The last two compounds contain ethylene groups in place of the propylene groups in the other compounds (Fig. 2).

Fig. 1. Inhibition of phage infectivity by cations

Data from experiments shown in Table 1, except that reduction in infectivity is expressed as the mean of all results at a given concentration of cation in all experiments. Regression lines were calculated from the mean of the values (mean of 5–14 results per [cation], with an average s.d. of less than 25%). Symbols: Δ , $[\text{Co(en)}_3]^{3+}$; $\square$, Cd^{2+} ; $\blacksquare$, La^{3+} ; $\circ$, $[(\text{NH}_3)_6\text{CoNH}_2\text{Co}(\text{NH}_3)_6]^{5+}$; $\blacktriangledown$, 1,4,8,12-tetra-azacyclopentadecane; ∇ , 1,4,7-triazacyclodecane; $\bullet$, 1,4,7,10,13,16-Hexa-azacyclo-octadecane.

The effect of lipopolysaccharide on the reaction varies with the cation. The metal cations Cd^{2+} and La^{3+} cause 50% loss of infectivity at similar concentrations with or without lipopolysaccharide. Lipopolysaccharide does not alter loss of infectivity caused by Cd^{2+} up to the 50% level, but above that the loss with lipopolysaccharide is very much greater than that without. With La^{3+} infectivity remains constant in the presence of lipopolysaccharide up to the cation concentration giving 50% loss without lipopolysaccharide; at higher concentrations the infectivity falls rapidly (Figs. 1 and 3). With the other cations lipopolysaccharide increases the concentration of cation needed for a given loss in infectivity 30 or more times (Figs. 1 and 3). 1,4,8,12-Tetra-azacyclopentadecane was not tested with lipopolysaccharide. Increasing the concentration of lipopolysaccharide scarcely altered the concentration of Cd^{2+} needed to reduce infectivity, but with La^{3+} and Ruthenium Red the concentration of cation needed was proportional to that of lipopolysaccharide added. The concentration of 1,4,7,10,13,16-hexa-azacyclo-octadecane needed was increased, but to a lesser extent (Table 2). The many effects of the various cations over different concentration ranges can only be explained by a complex set of reactions (see Scheme 1).

The above four cations and $[(\text{NH}_3)_6\text{CoNH}_2\text{Co}(\text{NH}_3)_6]^{5+}$ and Ca^{2+} all precipitate lipopolysaccharide on incubation at 37 °C for 20 min. The observable precipitate (obtained using the higher concentration of 6.75 μM -lipopolysaccharide in 10 mM-Tris buffer) is only visible after centrifugation [3000 rev./min (r 16 cm) for 20 min in an MSE Centaur centrifuge], except in the case of 1,4,7,10,13,16-hexa-azacyclo-octadecane, which shows a flocculent precipitate before centrifugation. With

Ruthenium Red, the red precipitate was resuspended in water and the absorption measured at 530 nm. The lipopolysaccharide was precipitated at 10 μM -Ruthenium Red, but apparently not when the concentration was 8 μM . This corresponds to approx. 2 cationic groups per lipopolysaccharide molecule, but firstly the concentration of Ruthenium Red is uncertain and secondly it may be contaminated with other ruthenium cations which can combine with lipopolysaccharide. With the other cations, the presence of a precipitate was judged by opalescence and the results, which are less accurate, are shown in Table 3.

DISCUSSION

The above experiments concerning the effect of high-valency metal cations and cyclic polyamines on the infectivity of phage with and without lipopolysaccharide have shown that: (1) some cations will reduce infectivity without lipopolysaccharide; (2) a limited number of these reduce infectivity almost equally with lipopolysaccharide; and (3), for a final group of cations, inhibition is reduced by lipopolysaccharide. Assuming simple binding equilibria, we can examine these effects by using a plot of $\log [\text{cation}]$ against $\log [(P_0 - P_i)/P_i]$, which is an equivalent expression to that used in the study of enzyme inhibitors.

In general, inhibition due to cation (M) + lipopolysaccharide (LPS) can be written:

$$\text{Inhibition} = \sum_0^n k_1 K [\text{M}]^n + \sum_0^n k_2 K [\text{M}]^n [\text{LPS}] - \sum_0^n K' [\text{M}]^n [\text{LPS}] \quad (1)$$

where the first term is inhibition by M alone and that in the second term is by M only when LPS is present. The

Fig. 2. Formulae of cyclic polyamines

Key to numbered compounds: (I) 1,4,7-triazacyclonane; (II) 1,4,7-triazacyclodecane; (III) 1,4,7,11-tetra-azacyclododecane; (IV) 1,4,8,12-tetra-azacyclopentadecane; (V) 1,4,7,10,13,16-hexa-azacyclo-octadecane.

third term is one for the removal of M by LPS. k is an effective action constant when M is fully bound, and K is a binding constant. We can then compare plots of $\log [(P_0 - P_t)/P_t]$ against $\log [M]$ for (a) cation alone and (b) cation in the presence of LPS.

From eqn. (1): $\text{Effect} \propto [M]^n$

where n , the number of cations involved in the complex under consideration, gives the slope of the line. In the absence of lipopolyaccharide, where the second and third

terms are zero, the slope of the line (n) is close to unity, except for 1,4,7,10,13,16-hexa-azacyclo-octadecane and 1,4,7-triazacyclodecane, where the slope is nearer 2 (Fig. 1 and Table 4). In most cases, therefore, $n = 1$, so that either (1) only one site of binding of M to the phage is necessary, or (2) several sites are recognized, but they are totally independent of each other. The reaction under consideration is described by the equilibrium constant K_b in Scheme 1.

At high concentrations, the effect on phage of the

Fig. 3. Inhibition of phage infectivity by cations and lipopolysaccharide

Data, as in Fig. 2, are from Table 1. Regression lines for Cd^{2+} are calculated from the values between 10 and 75 μM and between 40 and 150 μM . The key to symbols is as in Fig. 2.

complex ion $[\text{Co}(\text{en})_3]^{3+}$ falls (Fig. 1), pointing to a reaction of the type:

$$\text{Effect} = \Sigma K_n \{[\text{Co}(\text{en})_3]^{3+}\}^n X$$

with the phage anion, X, forming a series of complexes $\{\text{Co}(\text{en})_3X + [\text{Co}(\text{en})_3]_2X + [\text{Co}(\text{en})_3]_3X \dots\}$ of which the later, but not the earlier, ones are infective. Scheme 1 shows this reaction; the cations react with the phage to give the inactive phage- M^{n+} complex (constant K_b), followed by reaction with more than one cation to give phage which is again infective (constant K_c). A similar

Fig. 4. Relation of charge to activity of cations in reducing phage infectivity in the presence of lipopolysaccharide

Data are from Fig. 2 of Rowatt & Williams (1985) and Fig. 3 of the present paper. Cations active without lipopolysaccharide: ○, metal cations; □, cyclic polyamines. Cations only active with lipopolysaccharide: ●, metal cations; ■, aliphatic polyamines.

effect of changing cation concentration has been found by Sullivan & Lilley (1987) in the formation by cations of cruciform structures of DNA, another polyelectrolyte.

When lipopolysaccharide is present, eqn. (1) leads us to expect that there can be many different observations, since at low lipopolysaccharide concentrations there could be no effect, but the effect would be apparent as either lipopolysaccharide concentration increases or if n in the second term is greater than in the first (as with

Table 2. Effect of [lipopolysaccharide] on the action of cations on phage infectivity

Data are from the experiments shown in Table 1, with associated experiments using different concentrations of lipopolysaccharide. Values are given in μM instead of $-\log [\text{concn. (M)}]$, for ease of comparison. Regression lines are calculated from the means at each [cation]. The first of the pairs of values for Cd^{2+} relates to concentration and slope below 50 μM and the second to those above 50 μM .

Cation	[LPS] (μM)	[Cation] at $P_0 - P_t = P_t$ (μM)	Slope
Cd^{2+}	0	50	0.6
	0.22	32, 46	0.8, 1.9
	0.45	32, 40	0.8, 2.1
	0.89	27, 31	0.7, 2.1
La^{3+}	0	3.25	0.7
	0.45	2.56	11
	0.89	4.14	24
1,4,7,10,13,16-Hexa-azacyclo-octadecane	0	0.24	2
	0.45	9.01	4
	0.89	16.6	8
$[(\text{NH}_3)_5\text{RuORu}(\text{NH}_3)_4\text{ORu}(\text{NH}_3)_5]^{6+}$	0	0.017	1.6
	0.22	0.72	12
	0.45	1.39	16

Table 3. Precipitation of lipopolysaccharide by cations

Lipopolysaccharide (6.75 μM) was incubated for 20 min at 37 °C in 4 ml of 10 mM-Tris/acetate, pH 7.4, with the stated cations. Solutions were then centrifuged for 20 min at 3000 rev./min (r 16 cm) in an MSE Centaur centrifuge. The supernatant was removed and the deposit suspended in a drop of supernatant, transferred to a 2 ml vial and the turbidity examined. With Ruthenium Red the deposit was made up to 1 ml with water and the absorption measured at 530 nm. Data are for 100% loss of infectivity from Fig. 3 and an unpublished (E. Rowatt & R. J. P. Williams) experiment for Ca^{2+} .

Cation	[Cation]:		
	Giving 100% loss of infectivity at 0.45 μM -LPS	Giving precipitation at 6.75 μM -LPS	Not giving precipitation at 6.75 μM -LPS
Ca^{2+}	1.2 mM	5 mM	2 mM
Cd^{2+}	0.2 mM	2 mM	1 mM
La^{3+}	3 μM	40 μM	20 μM
1,4,7,10,13,16-Hexa-azacyclo-octadecane	15 μM	250 μM	100 μM
$[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	1.40 μM	25 μM	—
$[(\text{NH}_3)_5\text{RuORu}(\text{NH}_3)_4\text{ORu}(\text{NH}_3)_5]^{6+}$	1.8 μM	10 μM	8 μM

Table 4. Slope of the [cation]-versus-loss-of-infectivity line in the presence or absence of lipopolysaccharide

Data are from Figs. 1 and 3.

Cation	Slope of regression line:	
	Without LPS	With LPS (0.45 μM)
1,4,7-Triazacyclodecane	1.6	3.0
1,4,7,10,13,16-Hexa-azacyclo-octadecane	2.2	4.4
Cd^{2+}	0.6	0.8, 2.1
La^{3+}	0.7	10
$[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	1.4	24
$[(\text{NH}_3)_5\text{RuORu}(\text{NH}_3)_4\text{ORu}(\text{NH}_3)_5]^{6+}$	1.6	16

Cd^{2+} ; Table 2). Again we can expect some cases where the first term is zero, as with most bivalent metal cations and the aliphatic polyamines. With high-valency metal cations and cyclic polyamines the third term is the greatest, as shown by the proportionality between cation and lipopolysaccharide concentration needed for loss of infectivity (Table 2). Here the reaction of the second term does not occur until that described by the third term is satisfied, because K' is greater than K . If the second term is higher than the capacity of the lipopolysaccharide for the cation in question, the loss of infectivity may be reduced only minimally by lipopolysaccharide (La^{3+}), or it may not be reduced at all, giving rise to a biphasic curve (Cd^{2+} ; Figs. 1 and 3). With these two cations lipopolysaccharide still increases the value of n (Table 2).

The effect of lipopolysaccharide can now show any slope, n , depending on the degree of cation co-operativity. $n = 1$ is a simple $\text{M} + \text{LPS} + \text{phage}$ reaction, whereas $n \rightarrow \infty$ is a precipitation of $\text{M} + \text{LPS} + \text{phage}$. This explains the high values of n , which are only of notional meaning in Fig. 4. Precipitation of lipopolysaccharide by cations in the absence of phage is actually seen at concentrations of lipopolysaccharide not much higher than those found in the phage-infectivity experiments (Table 3). These concentrations of the respective cations

may neutralize the lipopolysaccharide and cause it to aggregate into structures which then interact with the phage. The bivalent cations are necessarily less active in this respect than those with higher valency. This interaction of cation with lipopolysaccharide would therefore be the first step in the reaction with phage (constant K_a) in Fig. 1. The greater activity in the presence of lipopolysaccharide of cations able to interact with phage alone (Fig. 4), suggests that phage interaction with cation is also an integral part of the reaction.

The findings with cyclic polyamines stress the importance of the separation of charges in the molecules for the interaction of organic cations with organic anions (see Tam & Williams, 1984). The effect of these compounds on phage in the absence of lipopolysaccharide bears little relation to their charge: 1,4,7,10,13,16-hexa-azacyclo-octadecane, with a charge of $3+$, is the most active tested, but 1,4,7-triazacyclodecane is more active than metal cations or aliphatic amines of similar charge (Fig. 4), whereas the corresponding compound 1,4,7-triazacyclononane (Fig. 2 and Table 1) is inactive at 1 mM. 1,4,8,12-Tetra-azacyclopentadecane is less active than 1,4,7-triazacyclodecane, although it has a higher charge, and the corresponding 1,4,7,11-tetra-azacyclodecane is again inactive. The activity of more compounds of this type should give information on the nature and dimensions of the active combining site of phage. The importance of an understanding of the differential competitive effects of charges spread on organic frameworks against the effect of simple inorganic cations cannot be overstressed. It could, for example, form the basis of the design of a series of drugs.

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REFERENCES

- Kimura, E., Sakonaka, A., Yatsunami, T. & Kodama, M. (1981) *J. Am. Chem. Soc.* **103**, 3041–3045
- Kodama, M. & Kimura, E. (1976*a*) *J. Chem. Soc. Dalton Trans.* 116–120
- Kodama, M. & Kimura, E. (1976*b*) *J. Chem. Soc. Dalton Trans.* 2341–2345
- Kodama, M. & Kimura, E. (1978) *J. Chem. Soc. Dalton Trans.* 1081–1085
- Rowatt, E. (1984) *Biochem. J.* **223**, 23–29
- Rowatt, E. & Williams, R. J. P. (1985) *Biochem. J.* **231**, 765–768
- Sullivan, K. M. & Lilley, D. M. J. (1987) *J. Mol. Biol.* **193**, 397–404
- Tam, S.-C. & Williams, R. J. P. (1984) *J. Chem. Soc. Faraday Trans. 1* **80**, 2255–2267

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