The interaction of cations with lipopolysaccharide from *Escherichia coli* C as shown by measurement of binding constants and aggregation reactions

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Lipopolysaccharide from *Escherichia coli* C interacts with polyvalent cations at low ionic strength at more than one site. The first site has high affinity with a K_D value of 10^{-8} M for Ca^{2+} and even stronger binding for $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$ and La^{3+} . The high-affinity site for the latter cations is beyond the sensitivity of the assay method. The second, low-affinity, site for bivalent cations has a K_m of 10^{-3} M, whereas, for trivalent and quinquivalent metal cations and spermine and hexacyclen (1,4,7,10,13,16-hexa-azacyclo-octadecane), this constant has a value of 10^{-5} M. Binding of cations to the high-affinity site does not alter the aggregation state of the lipopolysaccharide, but combination with the low-affinity site gives particles twice the size of those of the sodium salt. Very high Ca^{2+} concentrations (30 mM) give particles eight times the size of those of the sodium salt.

INTRODUCTION

Research into the effect of the lipopolysaccharide from *Escherichia coli* C on the infectivity of bacteriophage ϕX174 has shown that the interaction of a cation with the lipopolysaccharide is required for the reaction with phage (Rowatt & Williams, 1987). Cations are also known to affect the aggregation of the lipopolysaccharide, as seen by the examination of samples using the electron microscope. Work on large particles of purified lipopolysaccharide showed that they dissociated when treated with EDTA and reassociated when dialysed against Mg^{2+} (De Pamphilis, 1971). Again, in the ultra-centrifuge equivalent concentrations of triethylamine salts of rough lipopolysaccharide had an $s_{20,w}$ value of 10 S, while sodium and potassium salts had an $s_{20,w}$ value of 100 S. Magnesium salts were said to be not open to study, since they were insoluble (Galanos & Luderitz, 1975). The rough lipopolysaccharides used in all these experiments (M_r 5000; see Shands & Chun, 1980) had lost the smooth polysaccharide from their surface, and that used by Galanos & Luderitz (1975) was precipitated by approx. 10 atoms of Mg^{2+} /molecule. Binding of cations to rough lipopolysaccharides was measured in the presence of 50 mM-Tris by Schindler & Osborn (1979), who found two binding sites for Ca^{2+} and Mg^{2+} using dansylated lipopolysaccharide, one with a K_D value of about 10 μM and one with a K_D of about 1 mM.

The present paper investigates further the reaction of cations with the rough lipopolysaccharide from *E. coli* C by estimation of cation binding at low cation concentrations with the dye arsenazo III, and by estimation of the (higher) cation concentration necessary

to aggregate lipopolysaccharide so that it is deposited by centrifugation at 2000 and 10000 g. The size of particles in these systems as seen in the electron microscope is discussed.

MATERIALS AND METHODS

Lipopolysaccharide

Lipopolysaccharide was prepared from *E. coli* C obtained from the PHLS Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wilts., SP4 0JC, U.K.) (Rowatt, 1984; Rowatt & Williams, 1985, 1987) and used as the sodium salt at concentrations (based on galactose content; Rowatt & Williams, 1987) of 20 μM in the cation-binding experiments and 6.75 μM in the electron-microscopic study and in tests of aggregation by cations. The preparation of lipopolysaccharide had similar properties to others made previously with respect to glucose/galactose and phosphate/galactose concentration ratios, Ca^{2+} -combining activity and phage-inactivation activity.

Chemical analysis of the lipopolysaccharide

An inductively coupled plasma-emission-spectroscopy analysis of the lipopolysaccharide was carried out by Dr. M. Thompson and Dr. M. Ramsay (Applied Geochemistry Research Group, Imperial College of Science and Technology, South Kensington, London S.W.7, U.K.). The lipopolysaccharide contained 13.96 ± 0.24 g-ions of Na^+ /mol of lipopolysaccharide and 5.786 ± 0.007 g-atoms of phosphorus. Lipopolysaccharide from *E. coli* or *Salmonella* contains three CO_2^- (from 2-keto-3-deoxyoctonate) and a variable number of phosphate

Abbreviation used: hexacyclen, 1,4,7,10,13,16-hexa-azacyclo-octadecane { = cyclo[-NH-CH₂-CH₂]₆ }.

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(*P*) and pyrophosphate (*PP*) groups with a maximum of seven phosphate groups (Mühlradt *et al.*, 1977). The total anionic charge of the *E. coli* C lipopolysaccharide of 14 is fully explicable as being due to $4P^{2-} + PP^{3-} + 3CO_2^-$. Ca^{2+} at 0.011 g-ion/mol was the next most abundant element, and Mg^{2+} was too low to be detected, i.e. < 0.007 g-ion/mol. Treatment with Chelex-100 is more efficient at removing bivalent cations than the electrodialysis method recommended by Galanos & Luderitz (1975), and allowance need not be made for cations other than Na^+ in the lipopolysaccharide. Lipopolysaccharide also contains two NH_2 groups in glucosamine residues. These are substituted with fatty acids (see Rietschel *et al.*, 1972) and may well be in a hydrophobic region of the molecule and uncharged.

Chemicals

Chemicals used were as reported in previous papers (Rowatt, 1984; Rowatt & Williams, 1985, 1987). $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$ was specially prepared by Professor A. G. Sykes (School of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.), to whom we are grateful. Arsenazo III was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) at 98% purity. Bovine serum albumin used in electron microscopy was supplied by Taab Laboratories (Emmer Green, Reading, Berks., U.K.) and phosphotungstic acid (AnalaR; $H_3PO_4 \cdot 12WO_3 \cdot xH_2O$) by BDH, Poole, Dorset, U.K.

Arsenazo III was dissolved in water at 1 mM and stored frozen. The cation concentration in any solution was estimated as described by Rowatt & Williams (1989). In the presence of lipopolysaccharide the procedure was as follows.

Cations at a given concentration were allowed to react with lipopolysaccharide in 0.6 ml of Tris/acetate buffer, pH 7.4, for 30 min at room temperature in cuvettes. Arsenazo III (0.4 ml) was added to give final concentrations of 25 μM -arsenazo III, 20 μM -lipopolysaccharide and 2 mM-Tris/1 mM-acetate buffer. The Tris concentration is high enough to stabilize the ionic strength. After a few minutes, absorption was measured at 654 nm (Ca^{2+} and La^{3+}) or 634 nm $\{[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}\}$ with a Cecil 505 double-beam spectrophotometer. The solution of buffer + arsenazo III contained from 0.05 to 0.1 μM impurity cation estimated as Ca^{2+} . Since the identity of the contaminating cation is not known and the molar absorption coefficient varied with the cation, it is not possible to measure the degree of contamination accurately, and cation levels can only be measured where this contamination is a relatively insignificant fraction of the total.

A standard curve was obtained for each experiment using four or more concentrations of cation, and the resulting regression line was used to calculate free cation in the presence of lipopolysaccharide. The value of r was 0.997 ± 0.005 .

Aggregation of lipopolysaccharide

Lipopolysaccharide (6.75 μM) was incubated with cations in 10 mM-Tris/5 mM-acetate buffer, pH 7.4, at 37 °C for 30 min, and any aggregate was removed by centrifuging on an MSE Centaur centrifuge at high speed (10000 g) for 10 min or at low speed (2000 g) for 10 min. Unaggregated lipopolysaccharide in the supernatant was

estimated as carbohydrate by addition to the supernatant (0.5 ml) of 0.5 ml of 5% (w/v) phenol and 2.5 ml of conc. H_2SO_4 and the resulting absorption was read at 485 nm (Dubois *et al.*, 1956). Aggregated lipopolysaccharide was found by difference.

Electron microscopy

Cations were incubated at 37 °C for 20 min with lipopolysaccharide (6.75 μM) in 5 ml of 10 mM-Tris/5 mM-acetate buffer, pH 7.4, and centrifuged at 300000 g for 45 min. The greater part of the supernatant was removed, leaving some 0.1 ml into which the deposit was resuspended giving a 50 $\times$ concentration of the preparation. This suspension (10 μl) was mixed with 20 μl of 0.05% bovine serum albumin and 10 μl of 3% phosphotungstic acid (adjusted to pH 6.5 with 10% KOH) was added. The mixture was applied to a Formvar/carbon-coated grid. The surplus liquid was removed with filter paper and the grid air-dried before examination in a Jeol 1200 EX electron microscope. Micrographs were taken at magnifications of $\times 37000$ and $\times 60000$. Microscope magnifications were calibrated by using the spacing on negatively stained catalase crystals. Measurements were made on three-times-enlarged prints.

RESULTS

Cation binding to lipopolysaccharide at low cation concentration: Scatchard plots from spectrophotometry

Reaction of cation with lipopolysaccharide was analysed by the Scatchard (1949) equation, the concentration of bound over free cation being plotted against the concentration of cation bound per molecule of lipopolysaccharide. Scatchard plots for Ca^{2+} and $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ are shown in Fig. 1, and the values for K_D ($-1/\text{slope}$) and number of cations n combining with lipopolysaccharide (intercept on x -axis) are shown in Table 1. The number of charges involved is greater and the K_D is lower for $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$.

Values for La^{3+} are included in Table 1, but they are not very accurate, with a K_D of 0.0094 ± 0.0047 μM , both because the free La^{3+} concentration is low and because the arsenazo III, with a K_D approximately as low as that of the lipopolysaccharide, removes some of the cation from the lipopolysaccharide. This is shown by the alteration of colour from reddish-purple (the low-cation colour) to greenish-blue (the high-cation colour) immediately after the addition of the dye to the cation/lipopolysaccharide mixture (see Rowatt & Williams, 1989).

Low concentrations of Mg^{2+} are not estimatable by arsenazo III, but, with 8 μM of each cation, Mg^{2+} inhibited Ca^{2+} binding to lipopolysaccharide by about 40%, showing that it binds with approximately the same strength as Ca^{2+} .

Cation binding at high concentration: aggregation of lipopolysaccharide

Higher concentrations of cation caused aggregation of lipopolysaccharide, and the K_m or concentration causing 50% aggregation was calculated from the regression line of the percentage carbohydrate deposited against [cation] (Table 2). The effect of Ruthenium Red was also tested, although its purity was uncertain.

Fig. 1. Scatchard plot of cation binding of lipopolysaccharide

Lipopolysaccharide was incubated with CaCl_2 or $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]\text{Br}_5$ in 0.6 ml of Tris/acetate buffer, pH 7.4, for 30 min at room temperature. Arsenazo III (0.4 ml) was added, giving final concentrations of 25 μM -arsenazo III, 20 μM -lipopolysaccharide and 2 mM-Tris/1 mM-acetate buffer, and free cation was estimated by the change in absorption at 654 or 634 nm. Free cation concentration was calculated by using a fresh dose-response curve for each experiment. Values giving an absorption reading of less than 0.03 at one-tenth concentration were not used. Three experiments with Ca^{2+} (●) and four with $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$ (▲) are plotted. The s.d. was mostly less than one-tenth the mean of readings of duplicates with three spectrophotometer readings and, since it affects both axes, it is not recorded.

Concentrations of La^{3+} and $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$ aggregating lipopolysaccharide are low enough for estimation of the free cation in the supernatant with arsenazo III; those of Ca^{2+} and Cd^{2+} are not. When lipopolysaccharide was aggregated by La^{3+} 5.2 g-ions/molecule were bound (16 ± 1 charges) and when aggregation was by $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$, 3.4 g-ions/mol were bound (17 ± 1 charges).

At Ruthenium Red concentrations at which most of the lipopolysaccharide was removed by centrifugation at 10000 g, the supernatant was colourless; at lower

Table 1. Dissociation constants of lipopolysaccharide at low cation concentrations

Data are as for Fig. 1. K_D and n have been calculated from the Scatchard plot for each experiment (at least three per cation).

Cation	n	$-\log[K_D \text{ (M)}]$
Ca^{2+}	2.52 ± 0.06	7.77 ± 0.10
$[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	3.03 ± 0.12	8.30 ± 0.10
La^{3+}	2.92 ± 0.27	8.03 ± 0.27

Table 2. Aggregation of lipopolysaccharide by cations

Lipopolysaccharide (sodium salt at 6.75 μM) was incubated in 10 mM-Tris/5 mM-acetate buffer, pH 7.4, at 37 °C for 30 min, centrifuged at 10000 g for 10 min, and the decrease in carbohydrate in the supernatant measured [absorption at 483 nm after interaction of 0.5 ml of supernatant + 0.5 ml of 5% (w/v) phenol + 2.5 ml of conc. H_2SO_4 , as described by Dubois *et al.* (1956)].

Cation	Concn. giving 50% aggregation (K_m)
Ca^{2+}	$5.55 \pm 0.69 \text{ mM}$
Cd^{2+}	$2.23 \pm 0.20 \text{ mM}$
$[\text{CoCl}(\text{NH}_3)_5]^{2+}$	$2.15 \pm 0.27 \text{ mM}$
Mg^{2+}	$4.97 \pm 0.62 \text{ mM}$
La^{3+}	$32.6 \pm 0.81 \mu\text{M}$
$[\text{Co}(\text{NH}_3)_6]^{3+}$	$45.3 \pm 2.9 \mu\text{M}$
$[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	$21.2 \pm 1.1 \mu\text{M}$
Spermine	$50.5 \pm 0.50 \mu\text{M}$
Ruthenium Red	$17.4 \pm 0.14 \mu\text{M}$
Hexacyclen	$73.5 \pm 9.5 \mu\text{M}$

concentrations of Ruthenium Red, some colour was left in the supernatant, possibly attached to dye aggregate too small to be pelleted at that centrifugal force.

Aggregates formed with Cd^{2+} and Ruthenium Red could also be pelleted at 2000 g, but with Ca^{2+} the concentration had to be increased to 12.5 mM (from 5.6 mM) before the lipopolysaccharide was deposited at this g value. Initially Ca^{2+} must form a smaller aggregate than Cd^{2+} and Ruthenium Red.

Electron microscopy

Electron microscopy was used to see whether a change in concentration of cations caused a parallel change in particle size of the lipopolysaccharide. The basic lipopolysaccharide as the sodium salt with 14 atoms of Na^+ /molecule of lipopolysaccharide was studied first. When spun down at 300000 g, this lipopolysaccharide (6.75 μM in 10 mM-Tris/5 mM-acetate buffer, pH 7.4) contained small electron-dense rod-shaped particles and less dense circular and near-circular forms with a maximum diameter (12.5 nm) in the same size range as the rod length (Fig. 2a). Such particles were not seen in control preparations consisting of Tris buffer with bovine plasma albumin or bovine plasma albumin alone. Addition of Ca^{2+} at concentrations from 0.5 mM to 30 mM increased the size of both forms (Figs. 2b, 2c and 2d). At the highest concentration (30 mM) the discs were stacked (Fig. 2d). From their appearance in all these experiments the particles are seen to be discs lying flat on the support film (the less dense circular forms) or standing on edge to a greater or lesser extent (the dense rods with parallel sides when the disc was on edge or bowed sides when the disc was angled). There was no significant difference between the diameter of the circular forms and the length of the rods, supporting this interpretation. It is therefore reasonable to sum the numbers of rods and discs so as to obtain the extent of aggregation (Table 3). The summed totals have been plotted to give irregular distribution curves which are characterized by a mean with an s.d. (Fig. 3). The relation of length of long axis to concentration is shown in Table 4.

Fig. 2. Morphology of lipopolysaccharide

Lipopolysaccharide (sodium salt, $6.75 \mu\text{M}$) was incubated with the stated concentration of cation in 10 mM-Tris/5 mM-acetate buffer, pH 7.4, for 30 min at 37°C and centrifuged at $300\,000\text{ g}$ for 45 min. The deposit was resuspended in a drop of supernatant (giving a final lipopolysaccharide concentration of about $300 \mu\text{M}$), mixed with 2 vol. of 0.05 % bovine serum albumin and 1 vol. of 3 % phosphotungstic acid, pH 6.5, and air-dried on a Formvar/carbon-coated grid. Preparations were examined electron-microscopically as described in the Materials and methods section. (a) No cation; (b) $12.5 \mu\text{M-Ca}^{2+}$; (c) 5 mM-Ca^{2+} ; (d) 30 mM-Ca^{2+} ; (e) 2.5 mM-Cd^{2+} ; (f) $10 \mu\text{M-Ruthenium Red}$. The bar represents 100 nm.

Table. 3. Size of particles of lipopolysaccharide with various added cations

Methods used were as for Fig. 2. Up to 100 particles were measured from each print, and the percentage lying in each size range recorded, equating diameter of circular forms with long axis of rods.

Size range (nm)	Percentage of particles in each size range														
	[Ca ²⁺]						0.1 M-Na ⁺ with:		[Cd ²⁺]		[(NH ₃) ₅ CoNH ₂ - Co(NH ₃) ₃] ³⁺ (25 μM)	Hexacyclen (250 μM)	La ³⁺		Ruthenium Red (10 μM)
	No addition		12.5 μM	5 mM	30 mM	No addition	0.5 mM-Ca ²⁺	12.5 μM	2.5 mM	6 μM			20 μM		
	0	0	13.33	6.66	0	0	0	2.50	0	0	0	0	0	0	0
< 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5.1-10	17.94	13.33	6.66	0	0	2.84	2.50	10.00	0.84	0	0	3.33	0	0	1.67
10.1-15	73.02	72.50	40.83	5.00	41.52	40.00	40.00	77.00	22.50	18.81	18.81	21.67	66.67	21.67	32.92
15.1-20	9.04	13.33	35.83	9.16	43.22	45.00	45.00	13.00	30.00	51.90	51.90	28.33	33.33	63.33	36.79
20.1-25	0	0.84	13.34	25.00	11.24	11.24	9.17	0	35.83	22.14	22.14	35.00	0	13.33	11.79
25.1-30	0	0	2.50	35.00	1.18	1.18	2.50	0	7.50	7.14	7.14	6.67	0	0	4.35
30.1-35	0	0	0.84	16.66	0	0	0.83	0	3.33	0	0	5.00	0	0	4.17
35.1-40	0	0	0	5.84	0	0	0	0	0	0	0	0	0	0	4.17
40.1-45	0	0	0	0.84	0	0	0	0	0	0	0	0	0	0	1.67
45.1-50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50.1-55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
55.1-60	0	0	0	0.84	0	0	0	0	0	0	0	0	0	0	1.67
60.1-65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
> 65	0	0	0	1.66	0	0	0	0	0	0	0	0	0	0	0.84
No. of micrographs ...	3	2	2	2	2	2	2	1	2	1	1	1	1	1	2
No. of rods...	47	60	60	60	51	32	32	30	60	14	14	30	30	30	58
No. of spheres ...	29	60	60	60	40	60	60	25	60	30	30	30	15	30	60

Table 4. Effect of cation concentration on size of lipopolysaccharide particles

Data are taken from Table 3, and mean length and s.d. calculated as shown in Fig. 3. Size is defined as the diameter of circular form, or length of rod, in nm.

Cation	Concn.	Size of lipopolysaccharide particles (mean length $\pm$ s.d.) (nm)								
		0	6 μ M	10/12.5 μ M	20/25 μ M	40 μ M	250/500 μ M	2.5 mM	5 mM	30 mM
Ca ²⁺				12.6 $\pm$ 2.7			14.4 $\pm$ 5.2		15.8 $\pm$ 5.2	25.4 $\pm$ 6.1
Cd ²⁺				12.7 $\pm$ 2.4				19.3 $\pm$ 5.2		
La ³⁺			14.2 $\pm$ 2.4		16.9 $\pm$ 3.2		15.4 $\pm$ 4.1			
[Co(III)] ⁵⁺					18.4 $\pm$ 3.3	19.8 $\pm$ 3.9				
Hexacyclen							19.3 $\pm$ 5.7			
Ruthenium Red			19.2 $\pm$ 8.8							
No addition		12.4 $\pm$ 2.6								
0.1 M-NaCl + Ca ²⁺		15.8 $\pm$ 3.5					16.1 $\pm$ 4.2			
* Abbreviation: [Co(III)] ⁵⁺ , [(NH ₃) ₅ CoNH ₂ Co(NH ₃) ₅] ⁵⁺ .										

* Abbreviation: [Co(III)]⁵⁺, [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺.

The width of the rods (and thickness of the discs) varied from 7 to 9 nm, with particles with low cation concentration generally narrower than those in the higher range.

Other cations also caused an increase in size of particles, but the concentrations necessary to bring this aggregation about were less than those with Ca²⁺ (Table 3). The particles with Cd²⁺ (Fig. 2e) and [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺ (Table 3) were 20 nm in diameter compared with the 15 nm particles with Ca²⁺. The very large particles were only found with Ca²⁺, although there was some stacking with Cd²⁺ at 2.5 mM (Fig. 2e).

Addition of Ruthenium Red gave rope-like forms as well as discs, which were often embedded in the rope-like material (Fig. 2f). Massed material was also observed with La³⁺, Cd²⁺ (Fig. 2e) and the higher Ca²⁺ concentrations, but appeared to be aggregates of flat discs rather than rope-like forms.

DISCUSSION

In the present paper we have estimated binding of

Fig. 3. Relation of [Ca²⁺] to size of lipopolysaccharide found in electron micrographs

Data are taken from Tables 3 and 4. The bars represent the s.d. ●, Control; ○, 5 mM-Ca²⁺; △, 30 mM-Ca²⁺.

cations to lipopolysaccharide by two methods: estimation of free cation in cation/lipopolysaccharide mixtures by spectrophotometry, and aggregation of lipopolysaccharide by cations as seen in electron-microscopic studies. The arsenazo III spectrophotometric analysis used the Scatchard (1949) procedure to give the number of atoms bound and the approximate strength of their binding. The data show that the number of cations taken up is related to their charge (Table 1). We discuss strength of binding below.

The aggregation method should also give an estimate of the numbers of cations bound and of their affinity. The numbers could only be measured for those cations with high affinity, namely La³⁺ and [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺, and here the total charge bound is the same for the two cations (16–17; Table 5). This value is similar to that found by titration of free [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺ in the presence of lipopolysaccharide. With La³⁺ the value is lower (9+), although it is still above the five charges bound by Ca²⁺. The value with La³⁺ may be affected by the high affinity of arsenazo III for La³⁺ { $-\log[K_D(\text{M})] > 8$; Rowatt & Williams, 1989}, which, in a titration in the presence of the La³⁺-lipopolysaccharide complex, could remove La³⁺ from the lipopolysaccharide and thus give too low a value for n (see the Results section). We therefore conclude that the affinity site measured by titration of free cation in the presence of lipopolysaccharide and Ca²⁺ concerns the high-affinity site, whereas those measured in the presence of La³⁺ or [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺ concern the low-affinity site, which has a $-\log K_m$ of 2.26 for Ca²⁺ (Table 2). The affinity of the high-affinity site of La³⁺ and [(NH₃)₅CoNH₂Co(NH₃)₅]⁵⁺ is too high to be measured by arsenazo III, and the relative $-\log K_D$ values are $\gg 8$ and, again, higher than the value for Ca²⁺ or Mg²⁺. The affinity of both high- and low-affinity sites of the lipopolysaccharide is thus related to charge and, from the data given in the present paper, size or shape of cations is not important.

Previous work on these precipitated forms of the lipopolysaccharide are not directly comparable with our own. Thus except for the forms found in the electron microscope by De Pamphilis (1971) of lipopolysaccharide (0.2–1 mM) in 0.01 M-Tris buffer with EDTA (5×10^{-3} M), the structures found by us are not like those found by others (De Pamphilis, 1971; Van Alphen *et al.*, 1980; Coughlin *et al.*, 1983; Kato *et al.*, 1986), possibly because

Table 5. Number of combining sites for cations in *E. coli* C lipopolysaccharide

Data for the number of cation sites were obtained as follows: (1) Na^+ from the number of Na^+ ions combined with lipopolysaccharide from the inductively coupled-emission-spectroscopy analysis (see the Materials and methods section); (2) the value for n from the Scatchard plot shown in Fig. 1; and (3) the value for cations removed by the complete aggregation of lipopolysaccharide (LPS) (estimation with arsenazo III).

Cation ...	No. of combining sites			
	Na^+	Ca^{2+}	La^{3+}	$[(\text{NH}_3)_5\text{CoNH}_2\text{-Co}(\text{NH}_3)_5]^{5+}$
Constituent cation content of lipopolysaccharide	14			
n from Scatchard plot	2.5	~3		3.0
Total charge	5.0	9		15.0
n from g-ions			5	3.4
cations removed/mol of LPS at 10000 g				
Total charge		16		17

the concentrations of cations used by them are much higher than ours. We avoided the use of high cation concentrations because of the danger of salt precipitation during drying on to the grids. The forms we show may be relevant in bacteria in aqueous solutions of low ionic strength.

Using the molecular mass given by Shands & Chun (1980) and a density of 1 for lipopolysaccharide gives an estimate of numbers of molecules in the particles shown in Fig. 2 (Table 6). The particles containing Cd^{2+} and $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$ contain approximately twice as many molecules as those containing Ca^{2+} , and the high $[\text{Ca}^{2+}]$ aggregates have eight times the number of molecules in the sodium salt of the lipopolysaccharide. The particles presumably have a double layer of molecules with the fatty acids inside.

A comparison of Tables 1 and 2 with Table 4 shows that: (1) the high-affinity site is not implicated in aggregation; (2) Ca^{2+} aggregates which pellet at 10000 g

Fig. 4. Relation of [cation] causing loss of infectivity of phage ϕX174 by lipopolysaccharide to [cation] causing aggregation of lipopolysaccharide

The concentration of cation causing 50% aggregation of lipopolysaccharide (K_m , Table 2) was converted into $-\log [K_m (\text{M})]$ and plotted against the concentration of cation causing loss of infectivity of phage ϕX174 from data in Tables 1 and 2 in Rowatt & Williams (1985) and Table 1 in Rowatt & Williams (1987). The concentration of Cd^{2+} quoted for loss of phage infectivity is the value found with the lipopolysaccharide used in 1987. Without the Cd^{2+} values (point at $x = 2.65$, $y = 4.32$), the slope is 1.091. The s.d. of lipopolysaccharide aggregation is given in Table 2, and that of loss of phage infectivity in Rowatt & Williams (1985), Tables 1 and 2, and in Rowatt & Williams (1987), Table 1.

are found at similar concentrations to those giving particles of 15 nm in the electron microscope; (3) large aggregates of 30 nm and those pelleting at 2000 g are found at similar concentrations; and (4), finally (Fig. 4), the cation concentration causing aggregation of lipopolysaccharide has a 1:1 relationship with the cation concentration causing loss of phage infectivity with

Table 6. Relative number of molecules in each particle size

The M_r of lipopolysaccharide is approx. 5000 (Shands & Chun, 1980); 1 mol contains 6×10^{23} molecules. Therefore, one molecule weighs $5000/6 \times 10^{23}$ g or 0.84×10^{-20} g. If we assume the density of lipopolysaccharide is 1 g/cm³; then one molecule occupies 8 nm³ (8.3×10^3 Å³).

Cation	Particle			No. of molecules/particle
	Radius (r) [nm (Å)]	Height (h) [nm (Å)]	Volume ($= \pi r^2 h$) [nm ³ (Å ³)]	
Low [cation]	6 (60)	7 (70)	791 (7.91×10^5)	95
5 mM- Ca^{2+}	7.5 (75)	8 (80)	1410 (1.41×10^6)	170
30 mM- Ca^{2+}	15 (150)	9 (90)	6360 (6.36×10^6)	766
2.5 mM- Cd^{2+}	10 (100)	9 (90)	2830 (2.83×10^6)	340
40 μM - $[(\text{NH}_3)_5\text{CoNH}_2\text{Co}(\text{NH}_3)_5]^{5+}$	10 (100)	8 (80)	2510 (2.51×10^6)	302
0.1 M-NaCl	8 (80)	9 (90)	1810 (1.81×10^6)	217

lipopolysaccharide (Rowatt & Williams, 1987). These two measurements are therefore an expression of the same reaction, that is, aggregation of lipopolysaccharide precedes interaction with phage.

Some interest attaches to the relative effects of inorganic cations and polyamines, since these two very different types of cation are in competition in biological systems. Here we note that the strongest sites, presumably those associated with the pyrophosphate of the lipopolysaccharide and which have a high charge density, are bound preferentially by inorganic ions, whereas the weaker sites are bound preferentially by the organic amines. The latter are probably spread-out charges as in Arsenazo III. Comparable observations have been made on these systems (see Wilson & Williams, 1987).

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