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Electrokinetic properties of endotoxins and their significance for the limulus amoebocyte lysate test

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The electrokinetic properties of the endotoxins of *Escherichia coli* and *Serratia marcescens* have been examined. Both endotoxins are negatively charged, with the zetapotential being increased by the presence of cations whose relative influence resembles the Schulze-Hardy rule for colloid stability. Fe^{3+} and Th^{4+} ions are capable of reversing the negative charge of the endotoxin particles to positive. These cations were found to have a strong inhibitory effect on the activity of endotoxins in the limulus amoebocyte lysate test but the inhibitory effect did not parallel changes in the zetapotential because the effect occurred at concentrations too low to affect this parameter.

Pyrogenic reactions arising from the infusion of large volume parenterals (LVPs) are caused by endotoxins that are cell wall fragments of Gram-negative bacteria if they are present in sufficient amounts. To remove such contaminants, LVP production usually includes a filtration procedure based on the use of adsorbent-containing depth filters or specially treated membrane filters.

However, the efficacy of these two filter types has been shown to be influenced by the composition of the solution being filtered (Baggerman et al 1981; Carrazzone et al 1985) in terms of changes in the electrokinetic properties of the endotoxins, which themselves are aggregates of lipopolysaccharide molecules that have colloidal properties (Baggerman et al 1985a).

Changes in electrokinetic properties of the endotoxins might also affect the sensitivity of the limulus amoebocyte lysate test (LAL test) (Guilfoyle & Munson 1982; Weary et al 1982; Kannegieter & Baggerman 1984).

We have investigated further the electrokinetic properties of endotoxins and their relationship with the activity of endotoxins in the LAL test.

MATERIALS AND METHODS

Electrophoresis experiments

Endotoxins of *Escherichia coli* 0111:B4 and *Serratia marcescens*, prepared by the Westphal procedure, were obtained from Sigma Chemicals. Electrolyte solutions containing different concentrations of NaCl, $CaCl_2$, $FeCl_3$ or $Th(NO_3)_4$ as well as LVPs were prepared in our hospital pharmacy, except Vamin-N (an amino acid preparation, Kabi-Sweden). Endotoxins were dispersed in these solutions to a final concentration of 0·5% w/v except for 1 mM Th^{4+}, 10 mM Th^{4+}, 10 mM Fe^{3+} and 25 mM Fe^{3+} in which the concentration was 0·1% (w/v) owing to the decreased endotoxin solubility caused by the low pH of these solutions. Solutions for studying the influence of various pH values were based on 10 mM NaCl solutions adjusted to the pH required with 0·1 M HCl or 0·1 M NaOH; the endotoxin concentration of these solutions was 0·5% (w/v) except for pH 3 when it was 0·1% (w/v). All final solutions were neutral except for $FeCl_3$ and $Th(NO_3)_4$ solutions which had pH values ranging from 6–3.

The electrophoretic mobility of the endotoxins in these solutions was determined using a Malvern Zetasizer II. This apparatus is based on the principle of two coherent beams of light, derived by splitting the output of a low-power helium-neon laser (λ = 632·8 nm), which are focused and made to cross within a precision quartz capillary cell containing the sample. A pattern of interference fringes is then formed at the crossing region. Particles moving in this region scatter light whose intensity fluctuates with a frequency which is related to the particle velocity. To measure the sign as well as the magnitude of the mobility, an electrical field (a.c.) is applied across the cell and a modulation is applied to one of the laser beams causing the optical fringe pattern to oscillate with a known frequency. Thus, particles with zero zetapotential, and hence zero mobility, scatter light that has a frequency equal to the modulation frequency. Charged particles,

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however, cause a Doppler shift of this frequency depending on the sign and magnitude of the zeta-potential. A photo-multiplier detecting the scattered light is connected to a digital correlator which numerically analyses the correlation function to determine the frequency spectrum.

The quartz capillary cell is mounted between two PTFE electrode chambers, thereby reducing sample contamination by electrode reaction products. To avoid electrode polarization, the voltage is applied as a reversing square wave, being within the range of 50–110 V, measured accurately for each sample. All measurements (five-fold for each sample) for different concentrations of the same electrolyte were made with the same adjustment of the stationary level in the capillary cell. A similar procedure was followed for the pH series and the LVP series. However, because of slightly different stationary level adjustments, small differences between baseline mobility values are observed. Electrophoretic mobilities may be transformed into zetapotentials using the Henry equation (Hunter 1981)

$$\mu_e = \frac{\varepsilon\zeta 2}{3\eta} f(\kappa R)$$

where ε is the dielectric constant of the solution, ζ is the zetapotential of the particles, and η is the viscosity of the solution. The term $f(\kappa R)$, also called the Henry factor, accounts for retardation effects caused by opposite movements of a charged particle and its surrounding electrical double layer, and depends on the relative thickness of double layer and particle in such a way that (if ζ is small)

$$1 \leqslant f(\kappa R) \leqslant 1{\cdot}5$$

with κ being the Debije parameter related to double layer thickness and thus to the ionic strength of the solution as follows:

$$\kappa = \left(\frac{2000e^2 N_A I}{\varepsilon kT}\right)^{\frac{1}{2}} m^{-1}$$

e being the unit charge, N_A being Avogadro's number, I being the ionic strength of the solution: $I = \frac{1}{2}\Sigma_i z_i^2 M_i$, k being Boltzmann's constant and T the absolute temperature, and R being equal to the particle radius. The particle radii of endotoxins were the subject of a previous study (Baggerman et al 1985a) and were established to be approximately 50 nm for *E. coli* endotoxins and 40 nm for *S. marcescens* endotoxins.

A graphical description of the relation between f and (κR) was given by Wiersema et al (1966) and was used to evaluate the f values for our systems. As a consequence of the low zetapotentials of endotoxin particles, no correction was necessary for relaxation effects. Furthermore, on the basis of the above mentioned particle radii and a hypothetical partially specific volume of $\bar{v} = 1{\cdot}1 \times 10^{-3}$ m^3 kg^{-1}, it can be calculated that electrical double layer overlap is absent, thus allowing the use of these formulae.

LAL measurements

The influence of environmental factors on the sensitivity of the LAL test was evaluated quantitatively using the LAL-Chromogenic substrate test described previously (Kannegieter & Baggerman 1984). In this test a peptide *p*-nitroaniline is added to the activated lysate resulting in the release of *p*-nitroaniline which can be measured spectrophotometrically at 405 nm.

E. coli endotoxin (Byk Mallinckrodt) was dispersed and diluted in 1 : 10 steps in polyethylene tubes (Greiner) resulting in a final concentration of 50 pg ml^{-1} in solutions with increasing concentrations of NaCl, $CaCl_2$ or $Th(NO_3)_4$. (Solutions containing $FeCl_3$ were not included because of interference due to the yellow colour of these solutions.) Samples (50 µl) of these solutions were preincubated with 70 µl of the reconstituted lysate for 45 min at 37 °C. Then 50 µl of this preincubated mixture was added to 500 µl of a mixture of 1·4 ml chromogenic substrate solution (3 mM) and 8·6 ml buffer solution (0·4 M Tris, 4·7 mM $MgCl_2$, pH 9) followed directly by the monitoring of the rate of *p*-nitroaniline formation spectrophotometrically at 405 nm at 37 °C. Finally, the increase in absorption min^{-1} was calculated.

RESULTS

The zetapotentials of both endotoxins in solutions containing different overall concentrations of various electrolytes are shown in Fig. 1. At low overall concentrations of the multivalent ions (i.e. below 1 mM), the bulk equilibrium concentration may deviate significantly from the initial concentration of these ions. The composition of the equilibrium liquid is therefore not exactly known and hence there is an inaccuracy in the value to be assigned to κR leading to an error in the Henry factor f (κR). Consequently, the calculated zetapotentials at low electrolyte concentrations are more in error than at higher concentrations; however, the qualitative trends observed are not obscured by this effect. The data in Fig. 1 clearly demonstrate the strong influence of the valence of the cation upon the magnitude of the increase of the zetapotential with increasing concen-

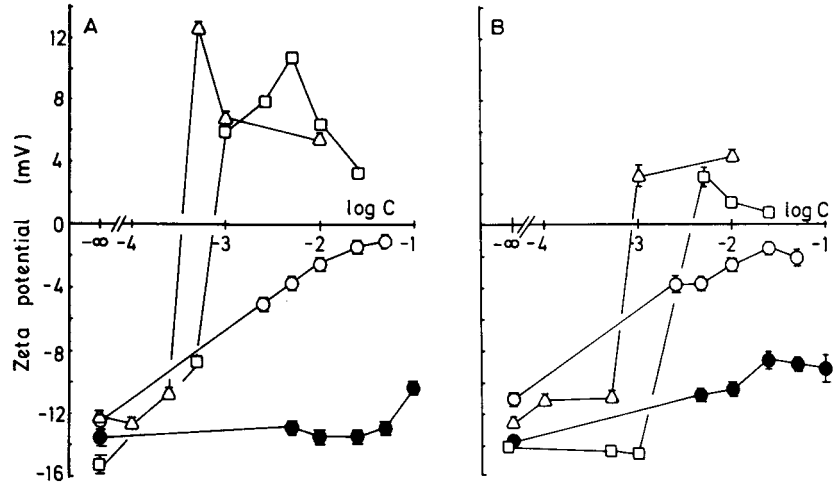

FIG. 1. The influence of different cations on the zetapotential of endotoxins. A, *E. coli* endotoxin. B, *S. marcescens* endotoxins. All values represent mean ± s.d. of five measurements. Key: (△) Th^{4+}, (□) Fe^{3+}, (○) Ca^{2+}, (●) Na^{+}.

tration. Both Fe^{3+} ions and Th^{4+} ions are able to reverse the negative zetapotential of the endotoxin particle to positive due to specific adsorption onto its surface. The magnitude of this reversal decreases with increasing cation concentration because of the screening affect of the electrical double layers due to the increasing ionic strength.

The relative influence of the various cations resembles the Schulze-Hardy rule for colloid stability which postulates that the valence of the ion of opposite charge to the colloid has the principal effect on colloid stability. In the classical picture this stability is directly related to the balance between electrostatic repulsion and van der Waals' attraction (Kruyt 1952). Fig. 2 shows the relationship between pH and zetapotential for both endotoxins. The negative zetapotentials confirm the anionic character of the surface charges on the endotoxin particles and hence the anionic nature of the lipopolysaccharide molecules constituting these particles. The zetapotential decreases with decreasing pH with a point of zero potential below pH 3.

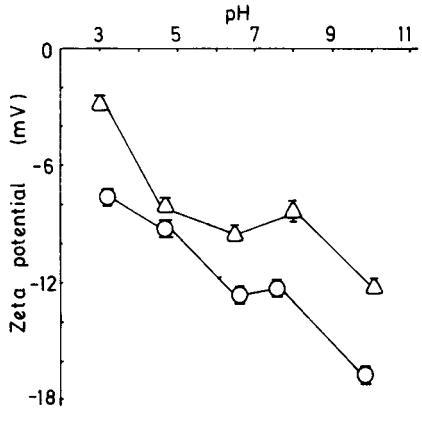

FIG. 2. The zetapotential of endotoxins as a function of the pH. All values represent mean ± s.d. of five measurements. Key: (△) *S. marcescens*, (○) *E. coli*.

The zetapotentials of endotoxins in various LVPs are shown in Table 1, the non-ionogenic solutions resulting in lower values, and Ca^{2+}-containing solutions (Vamin-N and Ringer's) resulting in higher values, i.e. being less negative.

The influence of electrolytes on the activity of endotoxins in the LAL test is shown in Fig. 3, which demonstrates that the inhibiting effects increase with the valence of the cation. However, a comparison of Fig. 1 and Fig. 3 conclusively shows a lack of correlation between endotoxin zetapotential and activity in the LAL test.

DISCUSSION

The electrokinetic properties of colloidal particles such as the electrophoretic mobility are governed by

Table 1. Zetapotentials of endotoxins in large volume parenterals.

LVP	*E. coli*	*S. marcescens*
Water for injections	−15·3 ± 0·6	−16·1 ± 1·5
Dextrose 10%	−14·4 ± 0·2	−15·3 ± 0·2
NaCl 0·9%	− 9·6 ± 0·5	−10·2 ± 0·3
Ringer's solution	− 8·5 ± 0·2	− 6·3 ± 0·6
Vamin-N	− 6·1 ± 0·2	− 4·7 ± 0·3

All values are mean ± s.d. of five measurements.

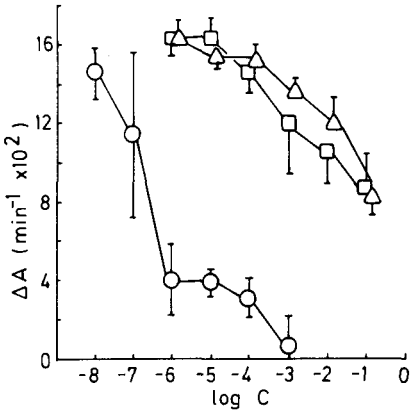

FIG. 3. The influence of different cations on the activity of *E. coli* endotoxin (50 pg ml^{-1}) in the limulus amoebocyte lysate—chromogenic substrate test. All values represent mean ± s.d. of five measurements. Key: (△) Na^+, (□) Ca^{2+}, (○) Th^{4+}.

their zetapotential. This potential is, by definition, the electrical double layer potential at the surface of shear which develops when the particle moves with respect to the surrounding liquid.

The properties of colloidal particles that are related to the zetapotential, and that are of interest to our investigations concerning endotoxins, are flocculation behaviour (precipitation resulting from the interaction of equally charged particles) and adsorption onto oppositely charged particles, which resembles heteroflocculation (Kruyt 1952; Hiemenz 1977). Furthermore, because of the charged colloidal nature of both endotoxins and proteins it is likely that the interaction between endotoxins and proteins of the limulus amoebocyte lysate is also related to this parameter.

The negative zetapotential of endotoxins is believed to arise from the dissociation of protons from the phosphate groups on the lipopolysaccharide molecule (Galanos 1977) leaving behind a negatively charged particle. The fact that the zetapotential is still decreasing well above the intrinsic pK of the phosphate groups, indicates the presence of a strong polyelectrolyte effect shifting the dissociation of the surface groups towards higher pH values. Also, weak acidic groups such as carboxyl groups may be present. Although the literature on endotoxins, including their physicochemical properties, is enormous, no data can be found on this important parameter except one study (Schramm et al 1952) which mentions an electrophoretic mobility (in Veronal buffer pH 8·2) half as low as indicated by our study. Since in a previous study (Baggerman et al 1985a) we investigated the size parameters of endotoxins, we were able to transform electrophoretic mobilities into zetapotentials and to demonstrate the influence of electrolytes thereupon. In particular, the potential reversing effects of Fe^{3+} and Th^{4+} ions and the maximum in the potential concentration curve in both cases deserve further attention. This maximum most likely arises from specific adsorption of these ions onto the particle surface or at least within the surface of shear. It is caused by two competing effects: at low concentrations it is the increasing specific adsorption which causes a strong increase in the zetapotential and charge reversal, while at higher ionic strength the screening effect of the electrical double layer lowers the zetapotential. The occurrence of specific adsorption of multivalent cations is supported by the fact that endotoxins may be radioactively labelled with trivalent or hexavalent ^{51}Cr ions resulting in very stable ^{51}Cr-endotoxin bonds (Braude et al 1955). The increase in the zetapotential due to increasing concentrations of Fe^{3+} or Th^{4+} ions is partly caused by the concomitant decrease of the pH of the solution, but as Fig. 2 shows, the contribution of this effect is of minor importance compared with the contribution of the specific adsorption.

In the case of Ca^{2+} ions the behaviour of the zetapotential concentration curve is at least qualitatively in accordance with the Gouy-Chapman picture of the double layer. In the case of Na^+ ions this is not so, since there the zetapotential is almost constant with increasing salt concentration over a large section of the curve. The displacement of the surface at which shear occurs as a result of the presence of charged macromolecules at that surface may be responsible for this effect (Goff & Luner 1984; Van den Hoven 1984). With Ca^{2+}, Fe^{3+} and Th^{4+} ions, ion bridges between these ions and the phosphate groups may prevent this shrinkage or swelling of the surface layer as a function of the salt concentration.

The results of potential measurements in large volume parenterals are in agreement with the above-mentioned influence of cations since the lowest potentials are measured in those solutions containing Ca^{2+} ions (Ringer's and Vamin-N); moreover, potentials in these solutions agree with potentials measured in solutions containing equivalent Ca^{2+} concentrations. Because lipopolysaccharides also constitute the outer layer of Gram-negative bacteria, a comparison of electrokinetic properties of these bacteria and endotoxins is obvious. A review of studies concerning the electrophoretic mobilities of bacteria has been made by James (1982), while those of *E. coli* bacteria were described earlier (Davies et

al 1956), demonstrating the influence of positively charged counterions, which our results support. Again, both authors report a marked influence of Th^{4+} ions and the relative influence of mono- and divalent cations with which we concur. The magnitude of the zetapotential of intact bacteria and their endotoxins, however, differs with respect to each other; that of *E. coli* bacteria was determined to be approximately −60 mV in water.

Comparison of these electrokinetic data for bacteria with the adsorption profiles of endotoxins onto positively charged asbestos particles (Baggerman et al 1985b) shows a close parallelism between the two phenomena thereby demonstrating the zetapotential to be the principal parameter governing endotoxin adsorption. It also explains the observed decrease in efficacy of various filters when electrolytes are involved, particularly, the coincidence of a sharp decrease in adsorption with endotoxin potential reversal in the case of Th^{4+} ions.

A similar, clear relationship, however, appears not to exist between the zetapotential and the activity of endotoxins in the LAL test. A strong influence of Th^{4+} ions in particular, but also of Na^{+} and Ca^{2+} ions, is noticeable at low concentrations not affecting the zetapotential of the endotoxins. This might indicate that the interaction between the cations and LAL proteins is more important than changes in endotoxin parameters.

Another possible explanation for this discrepancy might be the existence of several cation binding sites on the endotoxin particle. One such site could be involved in the endotoxin-LAL interaction but by its nature not contribute much to changes of the zetapotential. This particular binding site would then show distinct affinities for different cations with Th^{4+} ions showing inhibitory effects at extremely low concentrations. This highly interesting relation between specific cation binding and the activity of endotoxins in the LAL test, as well as the electrokinetic properties of LAL proteins and the influence of electrolytes thereupon, needs further investigation.

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