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Endotoxin removal from Large-Volume Parenterals by various adsorbents

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Summary

To perform endotoxin-adsorption studies, the suitability of ^{51}Cr-labelled endotoxins for this purpose was investigated. These labelled endotoxins did not exhibit important changes in physicochemical, biochemical or biological properties; in particular the adsorption behaviour of labelled and unlabelled endotoxins has been found to be identical. The adsorption of endotoxins from Large-Volume Parenterals using various adsorbents differs with respect to the solution being studied. These differences appear to result from shifts in electrokinetic forces between adsorbent and endotoxin, since the inhibiting effect of cations on endotoxin adsorption increases with increasing valency. These results suggest that the negative zetapotential of endotoxins is increased by cations according to the Schulze–Hardy rule, resulting in a different adsorption behaviour.

Introduction

Parenteral-dosage forms, like infusion fluids or Large-Volume Parenterals (LVPs), are subject to special requirements regarding the absence of pyrogenic contaminants.

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By far, the most important pyrogenic contaminants are endotoxins, the outer cell wall fragments of gram-negative bacteria, chemically characterized as lipopolysaccharides (Galanos et al., 1977). Various adsorbents constitute the active component of depth filters used for endotoxin removal in LVP-production. Asbestos-containing depth filters have been used widely until the FDA prohibited their use (Federal Register, 1976). From that moment on, attention has been focused on alternative materials, such as alumina or charge-modified (CMF) perlite, and several studies have shown these new depth filters to be equally effective (Woog et al., 1977; Gerba et al., 1980; Baggerman et al., 1981). These studies also revealed a different behaviour of all types of depth filters depending on the solution being filtrated, the presence of electrolytes leading to decrease in filtration efficacy.

In the present study, we further investigated the binding of ^{51}Cr-labelled endotoxins to various adsorbents with particular interest in the influence of electrolytes thereupon.

Materials and Methods

Labelling procedure

Endotoxins of two different micro-organisms, *E.coli* B 111 : 04 and *S.ab.aequi*, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and labelled according to the procedure described by Braude et al. (1955). Endotoxin (50 mg) was suspended in 10 ml sterile phosphate buffer (0.353 g NaH_2PO_4 + 0.639 g Na_2HPO_4 + 0.172 g NaCl in 1.00 litres water for injection). To this suspension, 100 μC $^{51}CrCl_3$ (Radiochemical Centre, Amersham, U.K.) were added. After incubation at 37°C for 24 h, ethanol 96% was added until a final concentration of 68% ethanol was reached followed by ultracentrifugation for 20 min at 35,000 rpm. The precipitate was shaken twice with 10 ml ethanol 96% for 2 h to remove unbound ^{51}Cr, dried at 37°C for 7 days and consequently dispersed in sterile water for injection to a concentration of 0.8 mg.ml^{-1}. These dispersions were stored at 4°C.

The labelled endotoxins were investigated thoroughly to establish both the absence of significant changes in properties as a consequence of labelling as well as the similarity in adsorption behaviour between labelled and unlabelled endotoxins.

(i) The labelling procedure for *E.coli* endotoxin resulted in a recovery of 66 ± 6% (mean ± S.D.; n = 7) and for *S.ab.aequi* in a recovery of 82 ± 6% (mean ± S.D.; n = 6). The activity of the labelled material was 1.9 ± 0.4 μC.mg^{-1} for *E.coli* endotoxin (mean ± S.D.; n = 6) and 1.6 ± 0.3 μC.mg^{-1} for *S.ab.aequi* endotoxin (mean ± S.D.; n = 6). These data agree well with those described by Braude et al. (1955). Starting from endotoxin-particle weight equal to 10^8 (Baggerman et al., 1985) and the activity of the $^{51}CrCl_3$ equal to 100 mC.mg^{-1}, it can be calculated that the number of Cr atoms per endotoxin particle will be approximately 10^2.

(ii) The absence of unbound ^{51}Cr in the labelled material was established by carrying out a third washing procedure using ethanol 96%. After reprecipitation, no activity could be detected in the supernatant.

(iii) The stability of the binding of ^{51}Cr was established by dialyzing labelled

endotoxin dispersions against sterile water for injection for 3 weeks at 37°C (*S.ab.aequi*), respectively 4 weeks at 37°C (*E.coli*). The percentage of the activity recovered in the dialysis fluid was 3 and 10%, respectively. Dialysis of an equal quantity of free ^{51}Cr resulted in the absence of a concentration gradient across the membrane.

(iv) We also investigated the binding of ^{51}Cr to impurities present in the endotoxins by incubating the endotoxins at 37°C for 1 h with an equal volume of Limulus Amebocyte Lysate (Byk-Mallinckrodt) according to the procedure described by Biondi et al. (1984). After centrifugation at 3000 rpm, the percentage of activity present in the supernatant represents binding to non-endotoxin substances. This percentage was 8% for *S.ab.aequi* endotoxin and 10% for *E.coli* endotoxin.

(v) The absence of changes in physicochemical parameters of endotoxins was established using light-scattering techniques as well as freeze-fracture procedures, as described by Baggerman et al. (1985).

(vi) To investigate a possible change in biochemical c.q. biological properties of the endotoxins caused by the labelling procedure, we tested one endotoxin different from those described above, but with a known dose–response curve with respect to pyrogenicity (*E.coli* 0111 : B4, Byk-Mallickrodt) after two separate labelling procedures.

Assuming the recovery of this *E.coli* endotoxin equals that of *E.coli* endotoxin described above, the Limulus Amebocyte Lysate—Chromogenic Substrate Test (Kannegieter and Baggerman, 1984) demonstrated that the labelled endotoxin contained 66% activity as compared to unlabelled endotoxin, whereas in the rabbit pyrogen test (USP XX) this percentage was 57%. Therefore, we conclude that the labelling procedure, though resulting in some loss of activity, does not affect the endotoxin nature of the particles.

Adsorption procedure

(i) To establish the adsorption of the ^{51}Cr complex and to exclude any transfer of ^{51}Cr from endotoxin to adsorbent, we diluted 1.0 ml of each dispersion (*E.coli* and *S.ab.aequi*) with 9.0 ml water for injection. To these solutions, 150 mg asbestos (Seitz) was added followed by shaking for 90 min at 4°C and centrifugation at 3000 rpm for 15 min. The radioactivity in the supernatant then amounted to less than 1% as compared to the blanks. Consequently, both supernatants as well as blanks were tested for endotoxin activity with the LAL-test (Byk-Mallinckrodt), therefore being diluted in 10-fold steps to concentrations ranging from 8×10^{-8} to 8×10^{-12} $g \cdot ml^{-1}$. Regarding the blanks, dilution to 8×10^{-11} $g \cdot ml^{-1}$ still resulted in a positive LAL-test, whereas all supernatant dilutions reacted negatively, thereby showing the adsorption of the ^{51}Cr-endotoxin complex instead of ^{51}Cr transfer.

(ii) To prove the similarity of adsorption behaviour of labelled endotoxins and unlabelled endotoxins, we investigated their adsorption in combined presence. For both endotoxin types, asbestos-adsorption profiles were obtained using the following solutions: (a) 0.8 mg ^{51}Cr endotoxin in 10 ml water for injection; (b) 0.8 mg ^{51}Cr endotoxin +0.8 mg unlabelled endotoxin in 10 ml water for injection; (c) 1.6 mg ^{51}Cr endotoxin in 10 ml water for injection. Each of these solutions was prepared in

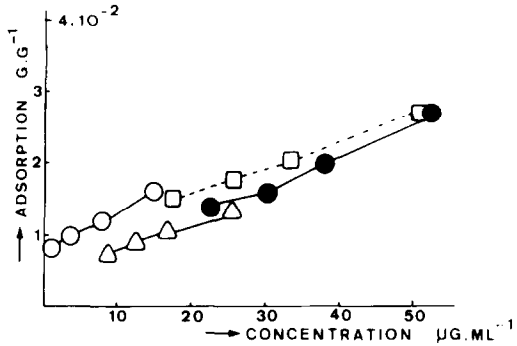

Fig. 1. Adsorption of labelled *S.ab.aequi* endotoxins in the presence of unlabelled endotoxins. ○, labelled ET 0.8 mg·ml^{-1} (a); △, labelled ET 0.8 mg·ml^{-1} + unlabelled ET 0.8 mg·ml^{-1} (b); ●, labelled ET 1.6 mg·ml^{-1} (c); □, calculated from (b), all counts duplicated. For further explanation, see text.

quadruplicate and shaken for 90 min at 4°C with increasing amounts of asbestos: 40, 60, 80 and 100 mg.

The results of these experiments for *S.ab.aequi* are shown in Fig. 1. Similar results were obtained for *E.coli* endotoxin (data not shown). Two profiles of solution b are shown, one based on actual counts and one based on duplicated counts assuming real competition between labelled and unlabelled endotoxins for binding sites. Unlabelled endotoxin appears to inhibit the adsorption of labelled endotoxins; furthermore, duplication of all values obtained from this competition-inhibited adsorption results in data very similar to those of solution c. From the above, we conclude that ^{51}Cr-labelled endotoxins are a reliable tool for studying the adsorption behaviour of endotoxins.

(iii) Adsorption profiles in various LVPs were obtained by the following procedure: 1.0 ml (0.8 mg) of the labelled endotoxin dispersion was added to 9.0 ml of the following solutions: water for injection, NaCl 0.9%, Ringer's solution, 0.1 M $CaCl_2$, dextrose 10%, Vamin-N (KABI, Sweden) and albumin 20% (CLB, Amsterdam, The Netherlands). Unless stated otherwise, these solutions were prepared sterile and

TABLE 1

COMPOSITION (IN mmol·l^{-1}) OF THE LARGE-VOLUME PARENTERALS

LVP	Na^+	K^+	Ca^{2+}	Mg^{2+}	Others
Water for injection	–	–	–	–	–
NaCl 0.9%	144	–	–	–	–
Ringer's	147	4	2.25	–	–
$CaCl_2$ 0.1 M	–	–	100	–	–
Vamin-N	25	10	1.25	0.75	Amino acids 7% (as HCl salts)
Dextrose 10%	–	–	–	–	Dextrose 10%
Albumin 20%	125	–	–	–	Albumin 20%

TABLE 2

THE ZETAPOTENTIAL (AT pH = 7.4) AND SPECIFIC SURFACE OF THE VARIOUS ADSORBENTS

Adsorbent	Zetapotential (mV)	Specific surface ($m^2 \cdot g^{-1}$)	References
Asbestos	93	4–12	Martinez and Zucker, 1960 Mark et al., 1978
Kaolin	−40	20	Hunter and Alexander, 1963
γ-Alumina	40	200–300	Mark et al., 1978
CMF-perlite	*	1.8	AMF-Cuno, 1984

* No data available.

pyrogen-free in our hospital pharmacy. The exact composition of these solutions is shown in Table 1. Each dilution was prepared in 6-fold; these 6 samples were shaken for 90 min at 4°C with increasing quantities of adsorbent: 20–100 mg for asbestos (Seitz); 300–1800 mg for γ-alumina (Merck, Darmstadt, F.R.G.); 25–250 mg for kaolin (Ph. Ned. VI) and 300–1800 mg for CMF-perlite (AMF-Cuno). After centrifugation for 15 min at 3000 rpm, the activity in 1.0 ml supernatant was counted and compared with blanks which were treated correspondingly. Some relevant physicochemical properties of the adsorbents are shown in Table 2.

(iv) The influence of various cations on the adsorption was examined using serial dilutions of the following salts: NaCl, $MgCl_2$, $CaCl_2$, $FeCl_3$, $Th(NO_3)_4$ in a concentration range 10^{-5}–1 M. Of the endotoxin dispersions, 1.0 ml was added to 9.0 ml of these dilutions and consequently shaken with 50 mg asbestos for 90 min at 4°C. The activity of the supernatant was measured as described above, followed by calculation of the percentage of adsorption compared to zero concentration of the salts.

Counting procedure

For radioactivity counting, a Wallac-500 Gammacounter was used.

Results

First of all, we established the time-course of the adsorption process using different incubation times for the same endotoxin-adsorbent system. It then appeared that adsorption is almost complete within 15 min, and slowly reaches equilibrium after 2 h. For practical reasons, we carried out all experiments using an incubation time of 90 min (Fig. 2).

Fig. 3A–E shows the results of the experiments including the Large-Volume Parenterals, demonstrating that the adsorption behaviour varies with the composition of the solution. From the asbestos series, the similarity in behaviour between *E.coli* and *S.ab.aequi* endotoxin becomes manifest, both showing a decreasing adsorption in the presence of Ca-ions, whereas the presence of substantial amounts

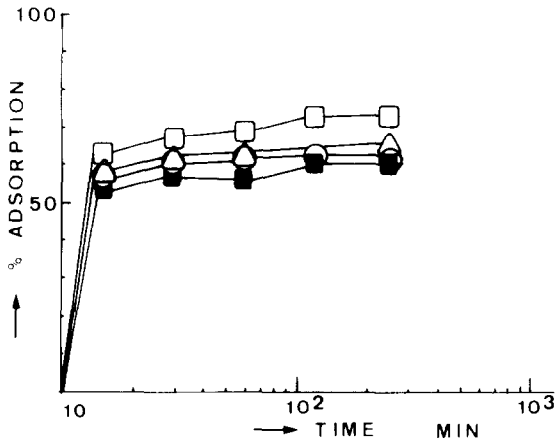

Fig. 2. Adsorption of *S.ab.aequi* endotoxins (0.8 mg in 10 ml water for injection) to various adsorbents as a function of time. □, asbestos 50 mg; △, kaolin 200 mg; ○, CMF-perlite 1200 mg; ■, alumina 900 mg.

of Na-ions (NaCl 0.9%, Vamin-N) influences in particular the slope of the adsorption curve: at lower endotoxin concentrations, adsorption is impaired; higher concentrations result in improved adsorption.

The alumina-*S.ab.aequi* series show another picture, suggesting an adsorption-favouring effect of saline and albumin, besides a far less pronounced effect of Ca-ions. Kaolin, the only negatively charged adsorbent, shows a clearly negative influence of albumin, a slightly negative influence of Ringer's, 0.1 M $CaCl_2$ and Vamin, and only a very small, if any, change in adsorption when using saline.

Finally, a very clear-cut effect can be seen while using the CMF-perlite; here, both Ringer's and 0.1 M $CaCl_2$ completely inhibit adsorption, whereas albumin slightly and Vamin or saline strongly favour adsorption. Furthermore, it is worth mentioning that for all adsorbents used the differences between water for injection and dextrose 10% are small.

Fig. 4A,B demonstrates the importance of the valence of the cations. Th^{4+} very strongly inhibits the adsorption of both *E.coli* and *S.ab.aequi* endotoxins onto asbestos. Notice also the more pronounced effect of Ca^{2+}-ions on the *S.ab.aequi* endotoxins which appeared also in the previous series (Fig. 3A,B).

Discussion

The overall picture which emerges from the LVP experiments is an adsorption-decreasing effect of Ca-ions, whereas the presence of Na-ions usually favours adsorp-

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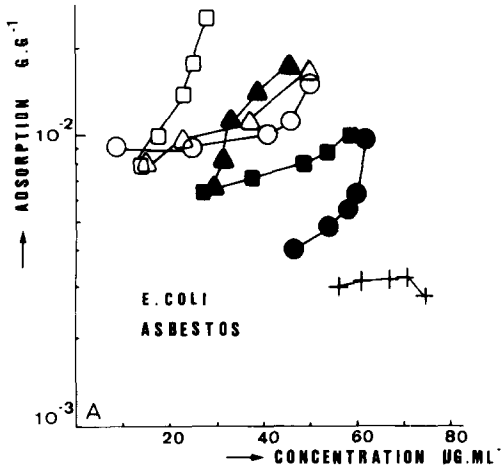
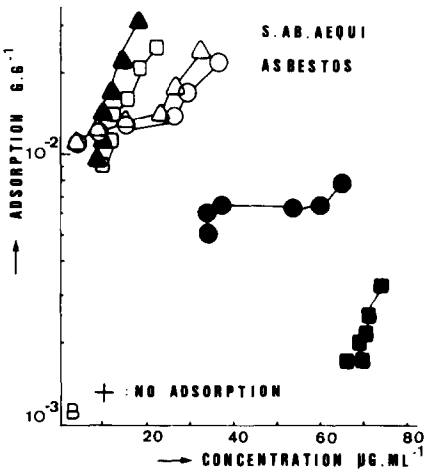
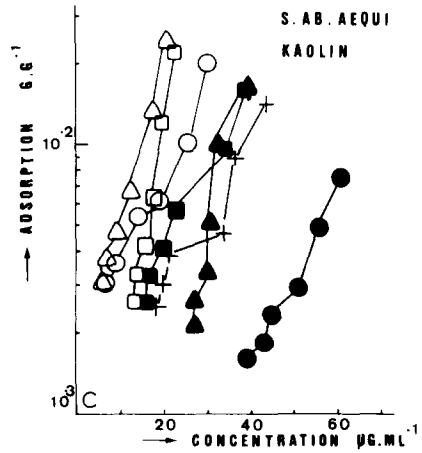
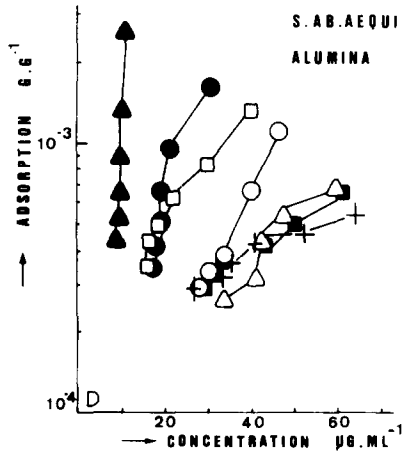
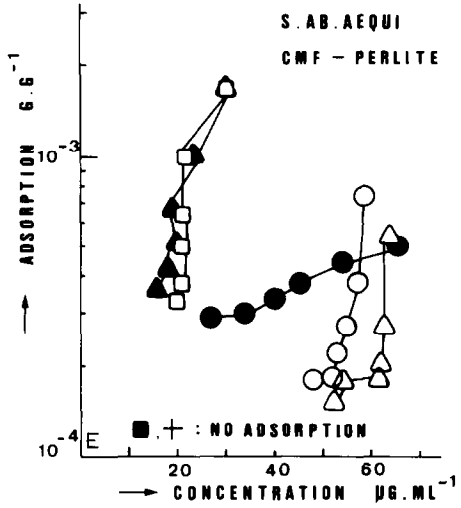
Fig. 3. Relationship between endotoxin concentration and amount of endotoxin adsorbed at equilibrium conditions. A: asbestos + *E.coli* endotoxin. B: asbestos and *S.ab.aequi* endotoxin. C: kaolin + *S.ab.aequi* endotoxin. D: alumina + *S.ab.aequi* endotoxin. E: CMF-perlite + *S.ab.aequi* endotoxin. ○, water for injection; △, dextrose 10%; □, NaCl 0.9%; ▲, vamin-N; ●, albumin 20%; ■, Ringers; +, 0.1 M $CaCl_2$.


ADSORPTION G.G⁻¹
10⁻²
10⁻³
E.COLI
ASBESTOS
A
20
40
60
80
CONCENTRATION µG.ML⁻¹


S.AB.AEQUI
ASBESTOS
ADSORPTION G.G⁻¹
10⁻²
10⁻³
+ : NO ADSORPTION
B
20
40
60
80
CONCENTRATION µG.ML⁻¹


S.AB.AEQUI
KAOLIN
ADSORPTION G.G⁻¹
10⁻²
10⁻³
C
20
40
60
CONCENTRATION µG.ML⁻¹


S.AB.AEQUI
ALUMINA
ADSORPTION G.G⁻¹
10⁻³
10⁻⁴
D
20
40
60
CONCENTRATION µG.ML⁻¹


S.AB.AEQUI
CMF – PERLITE
ADSORPTION G.G⁻¹
10⁻³
10⁻⁴
■,+ : NO ADSORPTION
E
20
40
60
CONCENTRATION µG.ML⁻¹

tion although, in the case of asbestos, for the latter the change in slope from the asbestos series indicates a shift from high-affinity adsorption to low(er)-affinity adsorption (Giles, 1981). The combined presence of Na- and Ca-ions, besides amino acids or the presence of albumin, can lead to both inhibition or stimulation.

The adsorption profiles do not follow a straightforward Langmuir relationship, which is to be expected in cases other than non-electrolyte adsorption (Jaycock and Parfitt, 1981). The resemblance in adsorption behaviour between *E.coli* and *S.ab.aequi* endotoxins do not confirm the differences observed by Palmer and Whittet (1971) using ion-exchange resins for pyrogen removal: *S.ab.aequi* endotoxin being adsorbed to basic resins and *E.coli* endotoxin to acidic resins.

The relative absence of adsorption modification by dextrose compared to the influence of ionogenic media strongly suggests that the adsorption is governed by electrokinetic forces; the electric double layers surrounding endotoxins and adsorbents are both influenced by negative c.q. positive counter-ions from the solution. Therefore, we investigated whether the Schulze–Hardy rule (i.e. the change in zetapotential being proportional to the valence of the counter-ion (Kruyt, 1952)) also applied to these adsorption systems. Indeed, an increasing inhibition was observed when using cations with increasing valency which increase the zetapotential of the negatively charged endotoxins. A similar inhibition would be expected to occur when polyvalent anions, decreasing the positive zetapotential of the asbestos, are present; indeed, we observed this phenomenon when using sodium-citrate solutions (unpublished results, Baggerman, 1984).

Endotoxins are macromolecular complexes of lipopolysaccharide molecules and may appear in different states of aggregation; however, a previous study (Baggerman et al., 1985) has shown that this is not the case for the LVPs used in this study. Furthermore, electronmicroscopic evidence was presented to hypothesize a liposome-like particle structure of the endotoxins with the lipid part of the LPS molecules pointed inward, whereas the polysaccharide chains constitute the outer layer of the particle. The phosphate groups in these polysaccharide chains are

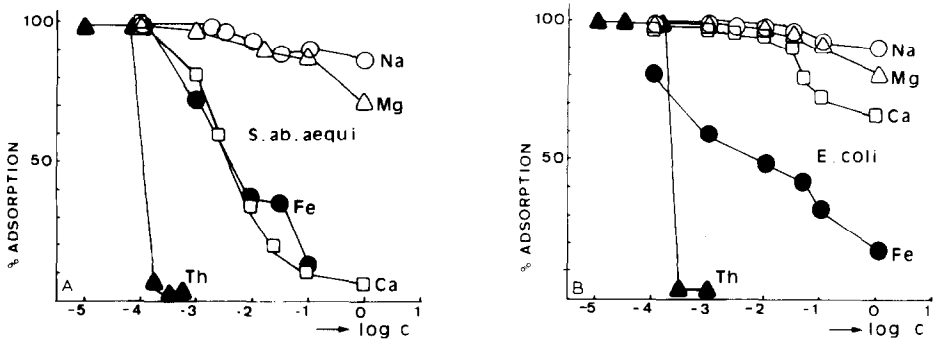


Fig. 4. The decrease in adsorption of endotoxins to asbestos as a function of valence and concentration of cations. The adsorption at zero concentrations is set at 100%. A: *S.ab.aequi* endotoxin. B: *E.coli* endotoxin.

thought to be responsible for the negative zetapotential of the particles (Schramm et al., 1952; Galanos and Lüderitz, 1975). Since these lipopolysaccharide molecules in a similar way constitute the outer layer of gram-negative bacteria, the zetapotential of the endotoxin particles might behave similarly to that of these bacteria. The electrophoretic properties of bacteria have been the subject of a great many studies and indeed show a shift towards positive zetapotentials depending on the concentration and valence of environmental cations (Davies et al., 1956; James, 1982). Charge-reversal spectra for *E.coli* bacteria show zero potentials at $[Th^{4+}] = 10^{-4}$ M, $[Ca^{2+}] = 1$ M, or $[Na^{+}] = 10$ M. Furthermore, dextrose did not affect the zetapotential of these bacteria (Davies et al., 1956). Zetapotential shifts, similar to those of bacteria, have been described for phosphatidyl-serine liposomes (Eisenberg et al., 1979) and ATPase vesicles (Schlieper et al., 1981).

These zetapotential shifts would have direct consequences for adsorption phenomena based on electrokinetic interactions. Comparable effects have been described for the adsorption behaviour of various viruses (Mix, 1974; Shields and Farrah, 1983), although hydrophobic interactions also seem to play a role in virus adsorption. We do not know yet to what extent hydrophobic interactions contribute to endotoxin adsorption; moreover, this may vary with the adsorbent concerned. The relatively low environmental influence in the case of kaolin may indicate a more substantial hydrophobic interaction than with the other materials.

The various adsorbents show different potencies for endotoxin removal on weight basis and are unmistakably superior with asbestos. This superiority, however, did not appear in dynamic filtration experiments using depth filters as described above. The zetapotential at neutral pH, being the pH of the solutions used in this study, of the adsorbents used in this study is positive with the exception of kaolin. However, this zetapotential is an overall parameter; both negative and positive surface charges have been described for asbestos particles (Schiller et al., 1980) and kaolin (Hunter and Alexander, 1963), whereas alumina and CMF-perlite appear to possess a homogenous charge distribution. Apparently, the positive surface charges on the kaolin particles are sufficient to bind the endotoxins, because this adsorbent is second in potency. The CMF-perlite, a perlite treated with polyvalent cations to change its negative zetapotential into positive, shows a disappointing performance on weight basis, which cannot easily be explained.

Probably, the specific surface of the adsorbent is of minor importance, because this parameter is usually calculated from gas-penetration studies, in which the inner porosity of the material strongly contributes to the total surface. However, the relatively large diameter of the endotoxin particles (50–200 nm) prohibit passage to these small pores. The adsorbents used in this study constitute the active components of various depth filters used for endotoxin removal during LVP-production. The results of our experiments are in good agreement with previous studies testing these depth filters in a dynamic design (Woog et al., 1977; Gerba et al., 1980; Baggerman et al., 1981), showing a decrease in filter efficiency when electrolyte-containing solutions are concerned and the absence of such a decrease in the case of carbohydrate solutions.

In conclusion, this study confirms the different behaviour of various LVPs with

respect to endotoxin removal by adsorption. Furthermore, strong evidence is presented for a predominantly electrokinetic character of this adsorption. The differences between the zetapotentials of both endotoxins and adsorbents in various LVPs will be studied in further detail.

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