

Review article

Endotoxin removal from protein solutions

Dagmar Petsch¹, Friedrich Birger Anspach^{*}

*Biochemical Engineering Division, GBF-Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1,
D-38124 Braunschweig, Germany*

Received 14 April 1999; received in revised form 17 August 1999; accepted 23 August 1999

Abstract

Endotoxins liberated by gram-negative bacteria are frequent contaminations of protein solutions derived from bioprocesses. Because of their high toxicity in vivo and in vitro, their removal is essential for a safe parenteral administration. A general method for the removal of endotoxins from protein solutions is not available. Methods used for decontamination of water, such as ultrafiltration, have little effect on endotoxin levels in protein solutions. Various techniques described in the patent literature are not broadly applicable, as they are tailored to meet specific product requirements. Besides ion-exchangers and two-phase extraction, affinity techniques are applied with varying success. Also, tailor-made endotoxin-selective adsorber matrices for the prevention of endotoxin contamination and endotoxin removal are discussed for this purpose. After giving an overview of the properties of endotoxins and the significance of endotoxin contamination, this review intends to provide an overall picture of the various methods employed for their removal. Avenues are pointed out how to optimise a method with regard to the specific properties of endotoxins in aqueous solution. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Down stream processing; Lipopolysaccharides; Endotoxin removal; Two-phase extraction; Affinity adsorption; Method optimization

Abbreviations: BFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CF, clearance factor; CIP, cleaning-in-place; DAH, diaminohexane; DEAE, diethylaminoethane; DOC, deoxycholate; EDTA, ethylenediaminetetraacetic acid; EU, endotoxin unit; FT-IR, Fourier transform-infrared; GMP, good manufacturing practice; HEPES, 4-(2-hydroxyethyl)-piperazin-1-ethanesulfonic acid; HSA, human serum albumin; IgG, immunoglobulin G; IgM, immunoglobulin M; KDO, 2-keto-3-deoxyoctonic acid; LAL, *Limulus* amoebocyte lysate; NMR, nuclear magnetic resonance; PEI, poly(ethyleneimine); PLH, poly-L-histidine; PLL, poly-L-lysine.

^{*} Corresponding author. Tel.: +49-531-6181743; fax: +49-531-6181175.

E-mail address: anspach@gbf.de (F.B. Anspach)

¹ Present address: MPB Cologne GmbH, Eupener Straße 161, D-50933 Köln, Germany.

1. Introduction

In the course of this century bacterial endotoxins turned out to be one of the most interesting and exciting molecules found in nature. Their peculiar structure, their chemical and physical diversity and their broad spectrum of biological activities has resulted in worldwide research in this field. While the knowledge about chemical composition and structure of endotoxins is well developed, many questions remain to be answered about the role of endotoxins in human health, especially its pathophysiology. Closely related to this area is a technically very important aspect, the problem of removing undesirable traces of endotoxin from aqueous solutions, especially from parenterals.

Bacterial endotoxins show strong biological effects at very low concentrations in human beings and many animals when entering the blood stream, e.g. during a bacterial infection or via intravenous application of a contaminated medicament. This requires removing even minute amounts of endotoxin from such preparations. The threshold level of endotoxin for intravenous applications is set to 5 endotoxin units (EU) per kg body weight and hour by all pharmacopoeias (European Pharmacopoeia, 1997). The term EU describes the biological activity of an endotoxin. For example, 100 pg of the standard endotoxin EC-5, 200 pg of EC-2 and 120 pg of endotoxin from *Escherichia coli* O111:B4 have an activity of 1 EU (Krüger, 1989). It is taken as a rule of thumb that 1 EU corresponds to 100 pg of endotoxin. Meeting this threshold level has always been a challenge in biological research and pharmaceutical industry (Berthold and Walter, 1994). Endotoxins are very stable molecules, their biologically active part surviving extremes of temperature and pH in comparison to proteins (Sharma, 1986). Routinely, temperatures of 180–250°C and acids or alkalis of at least 0.1 M must be chosen to destroy endotoxins in laboratory equipment. Thus, it is a challenge to remove endotoxin contaminations from sensitive substances, such as proteins.

Although common purification protocols may reduce the endotoxin content below the threshold

level, an absolute guarantee cannot be given. It may happen that one charge of the final product is accidentally contaminated and fails the quality control. This product has to be discarded; also, reprocessing is not ruled out specifically but is a costly alternative. Subjecting the product to trace decontamination is not allowed owing to GMP regulations except this is explicitly part of the validated process.

The question, how endotoxin removal can be carried out in an economical way has occupied many investigators and has been—although not published—the reason for process rearrangements in many cases. However, this item has not yet been solved satisfactorily. This article intends to discuss relevant aspects regarding endotoxin removal from protein solutions and critically review existing approaches. First, an introduction in the chemical and biological properties of endotoxins will be given as those are strongly influencing all removal techniques.

2. Properties of endotoxins

2.1. Origin of endotoxins

Since more than 100 years it is known that gram-negative bacteria carry a heat-stable toxin called endotoxin. This terminus was chosen by Richard Pfeiffer (1858–1945), a pupil of Robert Koch, to distinguish endotoxins from bacterial exotoxins, such as botulinum or tetanus toxins. Endotoxins are an integral part of the outer cell membrane of gram-negative bacteria and are responsible for the organisation and stability (Vaara and Nikaido, 1984). Approximately three-quarters of the bacterial surface consist of these molecules. As dominant surface structures they also participate in the interaction of the bacterial cell with its environment and possible hosts. Only a few gram-negative bacteria, namely some *Sphingomonas* species, are devoid of endotoxin but carry other amphiphilic molecules replacing it (Kawahara et al., 1991). Apart from these exceptions endotoxins can be regarded as a characteristic attribute of gram-negative bacteria.

Although endotoxins are firmly anchored within the bacterial cell wall (Raetz, 1990), they are continuously liberated into the surrounding medium. Endotoxin release clearly does not happen only with cell death but also during growth and division. Since bacteria can grow in nutrient-poor media such as water, saline and buffers, endotoxins are found almost everywhere. High concentrations are found where bacteria accumulate or are to be used for technical purposes, such as in bioprocessing.

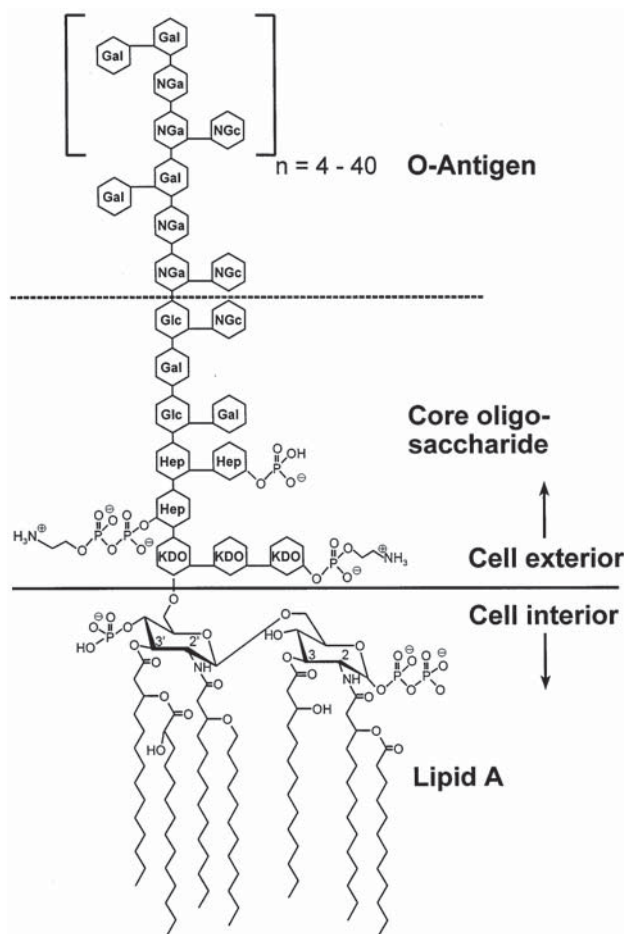


Fig. 1. Schematic view of the chemical structure of endotoxin from *E. coli* O111:B₄ according to Ohno and Morrison (1989). Hep, L-glycero-D-manno-heptose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctonic acid; NGa, N-acetyl-galactosamine; NGc, N-acetyl-glucosamine.

2.2. Chemical and supramolecular structure

Chemically endotoxins are lipopolysaccharides² that consist of three biologically, chemically, genetically and serologically different parts (Fig. 1). These are a non-polar lipid component, called lipid A, the so-called core oligosaccharide and a heteropolysaccharide representing the surface antigen (*O*-antigen).

Since the pioneering work of Westphal and Lüderitz in 1954 (Westphal and Lüderitz, 1954), numerous studies have been published about the structure and composition of each of the three endotoxin regions; a comprehensive description is provided by Rietschel (1984), and a review by Raetz (1990).

The *O*-antigen is built up of a chain of repeating oligosaccharide units (of three to eight monosaccharides each), which are strain specific and determinative for the serological identity of the respective bacterium. The bacterial *O*-antigens are an impressive example of nature's versatility along a given chemical make-up. Some deficient strains lack the *O*-antigen, such as *E. coli* K-12. This genetic defect does neither impair the viability of the microorganism nor the biological potency of endotoxin.

The core oligosaccharide has a conserved structure with an inner KDO-heptose region and an outer hexose region. In *E. coli* species five different core types are known, *Salmonella* species share only one core structure.

The most conservative part of endotoxin is lipid A, which, apart from few exceptions (Mayer and Weckesser, 1984; Vaara and Nurminen, 1999), shows very narrow structural relationship in different bacterial genera. It consists of a β -1,6 linked disaccharide of glucosamine, covalently linked to 3-hydroxy-acyl substituents with 12–16 carbon atoms via amid and ester bonds; these may be further esterified with saturated fatty acids. This hydrophobic part of endotoxin adopts an ordered hexagonal arrangement, resulting in a more rigid structure compared with the rest of the molecule. Lipid A-deficient strains show increased permeability of the outer cell membrane for

² Frequent synonyms of endotoxin are lipopolysaccharide and pyrogen.

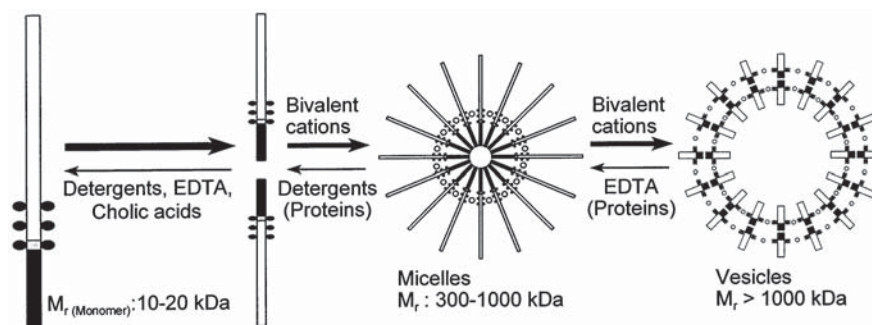


Fig. 2. Structures of endotoxin aggregates in aqueous solutions of different composition. The left symbol represents the endotoxin monomer; (open square) and (grey square) are hydrophilic sites, (black square) is lipophilic, (black circle) represent charged functional groups, (open circle) bivalent cations, such as Ca^{2+} and Mg^{2+} .

periplasmic proteins (Nurminen et al., 1997) and a decline in cell viability (Mohan et al., 1994). Strains lacking lipid A or endotoxin are not known.

The core region close to lipid A and lipid A itself are partially phosphorylated ($\text{p}K_1 = 1.3$, $\text{p}K_2 = 8.2$ of phosphate groups at lipid A, Hou and Zaniewski, 1990a); thus endotoxin molecules exhibit a net negative charge in common protein solutions.

A certain microheterogeneity results from non-stoichiometrical substitutions. For example, phosphate groups at lipid A and the core region may be substituted with arabinose, ethanolamine and phosphate in varying amounts. Also single saccharide units of the *O*-antigen may be acetylated, sialylated or glycosylated. Beyond this, *O*-antigens vary concerning the number of repeating units, causing a certain heterogeneity of the endotoxin population of each bacterium (Novotny, 1984). Active alterations of the ratio of endotoxin variants on the outer cell membrane enable bacteria to adopt to changing environmental conditions (phase variation, see Van Putten, 1993). This has the consequence that a single chemical structure of the endotoxin molecule is hardly to be found even at the same bacterium.

The molar mass of an endotoxin monomer as shown in Fig. 1 is about 10 kDa, though also 15–20 kDa is reported owing to the variability of the oligosaccharide chain. Endotoxin molecules form aggregates with high stability, therefore producing a bewildering polymorphism. In the early

days of endotoxin research the description of the supramolecular structure was rather phenomenological. Electron microscopy photographs showed endotoxins as 'snake-, donut- or rod-like filaments and flat sheets' (Shands et al., 1967; Hannecart-Pokorni et al., 1973). Modern analytical methods, such as X-ray diffraction, FT-IR spectroscopy, neutron scattering, NMR (reviewed by Seydel et al., 1993) and also molecular modelling (Kastowsky et al., 1992), revealed a more detailed image of the three-dimensional organisation of endotoxins. Although these studies did not convey a uniform picture, it is evident that endotoxins aggregate in lamellar, cubic and hexagonal inverted arrangements, such as micelles and vesicles, with diameters up to 0.1 μm (Fig. 2). The lipid A portion was identified as the major morphological determinant. It is proposed that aggregation is governed by non-polar interactions between neighbouring alkyl chains as well as to bridges generated among phosphate groups by bivalent cations (De Pamphilis, 1971; Wang and Hollingsworth, 1996).

Endotoxin micelles and vesicles are much more stable than those of simple detergents. Thus, vesicles are even found in ultrapure water. Monomers have to be explicitly created by using detergents (e.g. Triton X-114), bile acids (e.g. deoxycholic acid) and chelators (e.g. EDTA). However, also proteins may shift equilibria, releasing endotoxin monomers from aggregates (Li and Luo, 1997). The terminal state of these processes cannot be predicted; it depends on the properties of proteins

(net charge, hydrophobicity) and solutions (pH, ionic strength).

In summary it can be said that despite a common general assembly and charge there is considerable chemical and physical heterogeneity. This complicates the development of a generally applicable method for endotoxin removal from protein solutions.

2.3. Clinical aspects of endotoxin intoxication

Biomedical research revealed the toxically active part being lipid A. Endotoxins or lipid A do not act directly against cells or organs but through activation of the immune system, especially the monocytes and macrophages. These cells release mediators, such as tumour necrosis factor, several interleukins, prostaglandins, colony stimulating factor, platelet activating factor and free radicals (Pabst and Johnston, 1989; Rietschel et al., 1994), having potent biological activity and being responsible for the adverse effects seen upon endotoxin exposure. These include affecting structure and function of organs and cells, changing metabolic functions, raising body temperature, triggering the coagulation cascade, modifying haemodynamics and causing shock (Martich et al., 1993).

Many approaches have been made to prevent or treat the deleterious effects of endotoxins on immune cells. These include the use of anti-endotoxin antibodies (Ziegler et al., 1991), endotoxin partial structures for blocking endotoxin receptors (Lynn and Golenbock, 1992) and mediator receptor antagonists. However, the interaction of endotoxins with immune cells is not only mediated by specific receptors. Cell priming may also occur by non-specific intercalation of endotoxin molecules into the membranes of the target cells (Morrison, 1985).

Finally, it should not be concealed that endotoxins may also have beneficial effects. They have been used in artificial fever therapy, to destroy tumours and to improve non-specifically the immune defence. The uncertainty about its role for the human health was once described by Bennett (1963):

“Endotoxin can cause fever, but how many human fevers are endotoxic? Endotoxin can cause shock, but how often is shock in man endotoxic? Endotoxin can modify resistance to infections, but how often does endotoxin influence the susceptibility of man?”

Endotoxins' profound toxicity, however, forbade a safe use in man so far. Any superfluous endotoxin exposure must be strictly avoided to prevent complications. This is especially true for intravenously applied medicaments. Endotoxin testing has therefore become an important part of their quality assessment.

3. Significance of endotoxin contamination

Considering the data in Table 1, human beings are obviously anytime in contact with endotoxins and apparently can handle this without problems. As long as endotoxins get into contact with the skin or digestion system, they are tolerated quite well. Though, certain lung diseases are known to be linked to endotoxin inhalation, for example with cigarette smoke or in crowded animal housings (Dressel et al., 1991). In order to prevent the outbreak of endotoxin-caused reactions, limits were fixed for breath ($20 \text{ ng m}^{-3} \cong 200 \text{ EU m}^{-3}$, Hasday et al., 1996) and intravenous applications (5 EU kg^{-1} body weight and hour; European Pharmacopoeia, 1997). In a production process, the source of endotoxin contamination must be considered—is it released from within the process or is it introduced by non-sterile process conditions? Furthermore, purpose and application of the final product must be considered—for example, is the product to be used as diagnostic or therapeutic and in which form, as ointment or parenteral?

Of course, an exceptionally low endotoxin concentration is not required in every case. Since many pharmaproteins are administered in a low dose, modest endotoxin contents may comply with regulatory demands, e.g. according to the European Pharmacopoeia (1997), for insulin 10 EU mg^{-1} are allowed, for α -interferon even 100 EU mg^{-1} . However, serum albumin or monoclonal antibody preparations are administered in

amounts of several hundred or thousand milligrams per kg body weight; high endotoxin levels are out of question. With these proteins, methods are required to remove residual endotoxin traces that remain after using common purification trains.

3.1. Endotoxins in biotechnology

Modern biotechnology offers a number of methods to produce proteins. Among these are microbial bioprocesses for the expression of hu-

man proteins, such as growth hormones and interferons, and culture techniques with mammalian cells, yeast and fungi for proteins exhibiting post-translational modifications, such as monoclonal antibodies. As today bioprocesses are employed to a large extent for the production of pharmaceutical proteins, endotoxins became an important issue in manufacturing processes.

Endotoxin concentrations involved in biotechnological processes greatly depend upon the source of the product. They range from much less than 100 EU ml⁻¹ in cell culture supernatants to

Table 1

Common endotoxin concentrations in everyday life and in protein solutions of different origin

Source	Solution	Conc. (EU ml ⁻¹)
Tap and mineral water		1–20 ^a
Water in open-air swimming pool		25 600 ^a
Marjoram tea		533 ^a
Proteins from high cell density cultures of <i>E. coli</i> TG1:pλFGFB	Supernatant after homogenisation	> 2 000 000 ^b
Proteins from shaking flask cultures <i>E. coli</i>	Culture filtrate	70 000–500 000 ^b
Murine IgG ₁ from cell culture	Culture filtrate	≤ 100 ^b
Whey processed from milk of local supermarket	Supernatant after acid precipitation of milk	≈ 10 000 ^b
Commercial preparation of BSA	1 mg ml ⁻¹ of lyophilised protein	50 (Supplier 1) ^b ; 0.5 (Supplier 2) ^b

^a From Müller-Calgan (1989).

^b Measured in our laboratory.

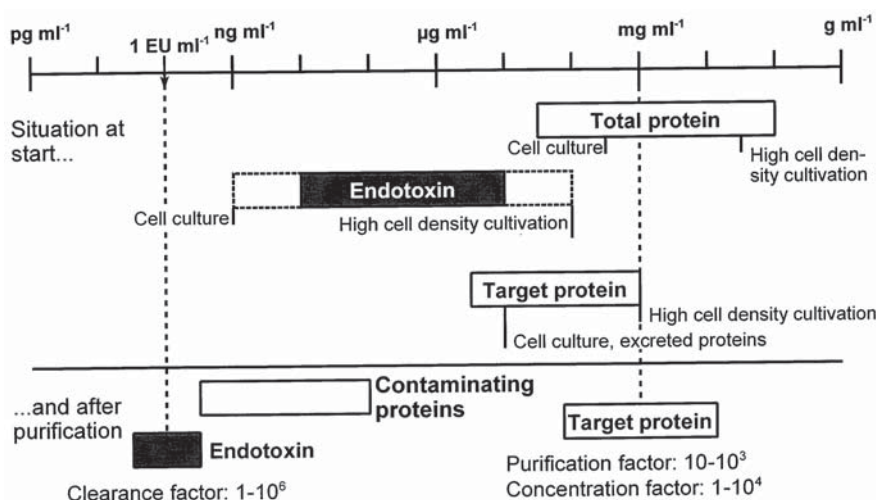


Fig. 3. Average concentrations of proteins and endotoxins from different origin before and after purification.

Table 2
Reduction of endotoxin concentration during purification of bFGF, according to Rantze (1996)

Purification procedure	BFGF (g l ⁻¹)	Endotoxins (EU ml ⁻¹)
Filtrate of cell homogenate	0.4	> 1 500 000
Fractogel EMD-SO ₃ 650 (S)	0.3	> 23 000
Heparin-Sepharose CL-6B	1.3	14
PEI-Sepharose	104	< 0.02

more than 1 000 000 EU ml⁻¹ in supernatants of high cell density bacterial cultivations (Fig. 3). Ideally, endotoxins should be absent when the product source is a cell culture supernatant and endotoxin-free media and buffers are used throughout. However, an endotoxin-free environment can hardly be realised. Especially the water quality is an often discussed problem (Bommer and Ritz, 1987). On the one hand the use of (expensive) endotoxin-free water (water for injection) is uneconomical due to the large quantities needed. On the other hand, a significant endotoxin amount can be introduced into the production stream through accumulation. Also, if the product source is human blood, endotoxin contamination can be a serious problem owing to former or acute bacterial infections of blood donors.

3.2. Endotoxin removal with product purification

Common purification protocols that include several chromatographic steps, such as ion exchange, hydrophobic interaction chromatography and gel filtration, may provide sufficient endotoxin clearance. Generally, the high endotoxin concentrations in the beginning (Fig. 3) can be reduced to about 100 EU ml⁻¹ without special treatment. Even much lower remaining endotoxin contents may be realised, e.g. Bischoff et al. (1991), purified recombinant α_1 -antitrypsin in a three-step procedure, employing ultrafiltration, anion-exchange and immobilised metal chelate affinity chromatography. The final product re-

vealed less than 0.22 EU ml⁻¹ while 1.2×10^7 EU ml⁻¹ were measured in the cell homogenate.

Other examples are known with relatively high remaining endotoxin levels despite incorporation of affinity steps. For example, Table 2 shows the progress of endotoxin removal during purification of a recombinant basic fibroblast growth factor (bFGF) from a high cell density cultivation of *E. coli*. Although protein purity was greater than 99% after serial chromatography using a cation-exchange and a heparin affinity sorbent, the final product exhibited an endotoxin content of 14 EU ml⁻¹. In another example, a microbial contamination was accidentally introduced to a purified murine IgG₁ preparation (pI = 5.5). After immediate sterile filtration, endotoxin and IgG₁ content were about 100 EU ml⁻¹ and 3 mg ml⁻¹, respectively. Reprocessing of the IgG₁ solution using IgG₁-selective methods (anion-exchange, hydrophobic interaction, and protein A affinity chromatography) was not successful. Also chromatography with endotoxin adsorbers based on polymyxin B or histidine were ineffective to reduce endotoxins to an acceptable limit when large sample volumes were applied onto columns. With small sample volumes a certain reduction of endotoxins was observed, however, at the expense of large product losses (results not published).

For both proteins the endotoxin content was finally further reduced by specifically addressing the properties of the proteins and endotoxin molecules. With bFGF the endotoxin-selective sorbent based on poly(ethyleneimine), (PEI)-Sepharose, was successful in the negative chromatographic mode, i.e. retention of bFGF was avoided while endotoxin was adsorbed (Table 2). With IgG₁ only special endotoxin adsorbers were successful (see Table 4).

In the pharmaceutical industry several alternative routes are known to come out with low-endotoxin products. However, their diversity indicates the dilemma in endotoxin removal. The procedures listed in Table 3 were developed for pharmaproteins, taking advantage of characteristics of the production process, tailored to suit specific product requirements. Though, each procedure addresses the problem in a completely different way; none of them turns out to be

broadly applicable, e.g. anion-exchange chromatography is potentially useful for the decontamination of basic proteins, such as urokinase (Green-Cross, 1986) or bFGF. However, decontamination of acidic proteins would be accompanied by a substantial loss of the product due to adsorption (Hou and Zaniewski, 1990b; Anspach and Hilbeck, 1995). For small proteins, such as myoglobin ($M_r \approx 18\,000$ Da), ultrafiltration can be useful to remove large endotoxin aggregates. With large proteins, such as immunoglobulins ($M_r \approx 150\,000$ Da) ultrafiltration would not be effective. Furthermore, ultrafiltration will fail if interactions between endotoxins and proteins cause endotoxin monomers to permeate with proteins pick-a-pack through the membrane.

In view of the large variety of products, expecting a generally applicable endotoxin clearance method is possibly utopian. On the other hand the diversity of applied techniques indicates that the special properties of endotoxin molecules are often not considered in the development stage of purification protocols.

3.3. The problem of low remaining endotoxin concentrations

At the final stages in purification trains the target protein is found at concentrations in the g l^{-1} range while endotoxins are present in $\mu\text{g l}^{-1}$

amounts (Fig. 3). The situation resembles the futile effort to search for a needle in a haystack.

A further problem is the detection of these low concentrations. Routine analysis in amounts of picograms per millilitre is not possible by direct detection techniques. Today many cases are known—although not published—in which a pharmaceutically promising new substance was regarded pyrogenic while it turned out later that the origin of pyrogenicity was not the substance but endotoxins. These escaped insensitive detection methods and thus their removal could not be properly addressed (Dinarelli et al., 1984).

Various bioassays are available to measure endotoxins, such as the rabbit test and the *Limulus* amoebocyte lysate (LAL) assay (Pearson, 1985), the chicken embryo lethality assay (Galanos et al., 1971), and the galactosamine-primed mice lethality test (Galanos and Freudenberg, 1993). Among these, the LAL assay is most sensitive and most economical.

The assay uses the blood coagulation system of the horseshoe crab *Limulus polyphemus* (or relatives, such as *Tachypleus tridentatus*, *Tachypleus gigas* or *Tachypleus rotundicauda*). In vitro, traces of endotoxin activate the isolated coagulation system either to initiate a gel formation (gel-clot variant) or to cleave a chromogen from a synthetic substrate to be read spectrophotometrically (chromogenic variant). A sensitivity of 0.02 EU

Table 3

Examples of patented procedures for removal of endotoxins from protein solutions

Product	Procedure	Reference
Superoxid dismutase	Ultrafiltration	Koyama et al., 1983
Myoglobin		Toyo-Soda, 1989
Cu-Zn-Superoxid dismutase	Anion-exchange chromatography	Green-Cross, 1986
Urokinase		Nippon-Kayaku, 1991a
TNF, IL-1	Adsorption on quaternised chitosan	Dainippon-Pharmaceuticals, 1987
Cu-Zn-Superoxid dismutase	Adsorption on non-polar polymers, such as Amberlit XAD	Nippon-Kayaku, 1991b
Catalase		
Hepatitis B surface antigen	Hydrophobic interaction chromatography	Merck-USA, 1987
Pertussis vaccine	Sucrose gradient centrifugation	Takeda Chemicals, 1988
Immunoglobulins	Extraction with bile salts	Centocor, 1988
Lipocortine	Extraction with detergents and HIC	Behringwerke, 1990
Immunoglobulins	Pepsin digestion removes endotoxin-binding Fc-fragment	Zimmermann, 1982

ml⁻¹ can be accomplished with this assay (end-point method); the kinetic method is even more sensitive. Although the LAL assay has proved to be a sensitive and reliable method for endotoxin monitoring, many substances interfere with the coagulation cascade, causing false negative or positive results. Such interferences are usually concentration dependent and may be avoided by dilution of the sample; however, this causes a loss of sensitivity. Also, it may fail occasionally, for example in complex biological fluids, such as blood, solutions of cationic proteins and with liposome-encapsulated endotoxin (Dijkstra et al., 1987; Petsch et al., 1998a; Pool et al., 1998). Owing to this, research carries on looking for alternative, sensitive bioassays. A whole blood stimulation assay is a recent development (Hartung and Wendel, 1996), which is currently in prevalidation stage at the Paul–Ehrlich Institute in Langen, Germany.

3.4. Interactions of endotoxins and proteins

Endotoxins show a remarkable capability to interact with other substances, including proteins. Molecular recognition can be assumed for interactions with anti-endotoxin antibodies and proteinaceous endotoxin receptors (e.g. CD14, CD16, CD18; Morrison et al., 1994). Many other interacting proteins, such as lysozyme (Ohno and Morrison, 1989), lactoferrin (Elass-Rochard et al., 1995), and transferrin (Berger and Berger, 1988), are basic proteins ($pI > 7$). Electrostatic interactions can be assumed as the main driving force.

However, other mechanisms must additionally exist as interactions with neutral (haemoglobin, Kaca et al., 1994) and even acidic proteins ($pI < 7$) are known, taking place also at low ionic strength. Still it is controversially discussed how these interactions take place. With serum albumins fatty acid binding domains might be involved (David et al., 1995). Generally hydrophobic interactions with proteins are conceivable, however, strong evidence that these govern the interaction mechanism is missing. It is more probable that competition of protein-bound carboxylic groups and endotoxin-bound phosphoric acid groups about Ca^{2+} may result in dynamically stable calcium bridges between proteins and endotoxins.

Regardless the mechanism that proves most significant, these interactions result in a masking of endotoxin molecules and consequently to a partial escape from removal procedures. A typical example is described by Karplus et al. (1987). They failed to reach the endotoxin limit if decontamination of bovine catalase was done with the affinity sorbent Polymyxin B–Sephadex using the standard protocol. In another study, Petsch et al. (1998a) found that the removal of small amounts of endotoxins can be more difficult from basic proteins than from acidic proteins.

Owing to protein–endotoxin interactions, endotoxin removal from protein solutions requires techniques that can generate strong interactions with endotoxins, such as affinity chromatography. Alternatively, a specific dissociation of protein–endotoxin complexes may improve the availability of endotoxin molecules, e.g. Karplus et al. (1987) employed the surfactant octyl- β -glucopyranoside to dissociate human IgG-endotoxin complexes and to support endotoxin adsorption to Polymyxin B–Sephadex. However, this practice implies the next problem, the removal of residual surfactant.

4. Selective removal of endotoxins

The most secure way to avoid any microbial contamination and with it the release of endotoxin is absolute sterility during the production and downstream processes. Yet, if a decontamination method is to be employed, it must ensure a high recovery of the target product. Data collected in our laboratory suggest that it must be strictly distinguished between the removal of endotoxin from protein-free and protein-containing solutions (Anspach and Hilbeck, 1995; Petsch et al., 1998b). In a protein-free solution, methods can be employed that take advantage of the different size of endotoxin and water as well as salt and other small molecules.

4.1. Ultrafiltration

Gel filtration chromatography reveals that more than 80% of the endotoxin activity of a

protein-free solution elutes as aggregates with the void volume (Morrison and Leive, 1975). Corresponding radii of gyration and molar masses can be estimated between 50 and 100 nm, respectively, 4–8 MDa from light scattering.

Ultrafiltration using membranes with about 10 kDa nominal-molecular-weight cut-off is routinely employed to get ultrapure water in laboratory systems and it is found in operation wherever endotoxins are not allowed to enter sterile equipment, such as in haemodialysis. Additional security is provided since mainly endotoxin aggregates exist in this environment. However, caution is advisable: although a slow process, partial decomposition of endotoxin molecules may occur at high temperature ($\approx 100^\circ\text{C}$) or acidic pH (pH 3.5–4.5; Caroff et al., 1988; Jiao et al., 1989). This may lead to the permeation of lipid A (M_r 2–4 kDa), the true toxic part of the molecule.

Ultrafiltration can also be employed to remove endotoxins from product solutions if the products are of low molecular weight. This is the case for glucose, salts, many chemotherapeutics and radiopaques, most antibiotics, etc. Membranes with 10 kDa nominal-molecular-weight cut-off are commonly employed (Sweadner et al., 1977); the product permeates through the membrane, endotoxins are retained. To allow permeation of proteins, membranes with cut-off greater than 100 kDa must be employed. Yet, in the presence of proteins dissociation of endotoxin aggregates occurs in favour of smaller units, similar as in the presence of EDTA or cholic acids (Fig. 2). Since endotoxin monomers and proteins are comparable in size, both permeate through the ultrafiltration membrane.

A way to improve separation from proteins by ultrafiltration was recently shown by Li and Luo (1998). By adding 45 mM Ca^{2+} to a solution of $85 \mu\text{g ml}^{-1}$ haemoglobin and $5 \mu\text{g ml}^{-1}$ endotoxin, they shifted the solution structure of endotoxins towards large aggregates and could reduce the endotoxin content to less than 6 pg ml^{-1} (6 orders of magnitude difference). Using an ultrafiltration membrane of 300 kDa nominal-molecular-weight cut-off assured permeation of haemoglobin. Although this approach is rational

in view of the solution behaviour of endotoxins (Fig. 2), it is limited to those proteins whose solubility is not largely affected by the presence of high concentrations of calcium ions.

4.2. Two-phase extraction

Through addition of detergents, an improvement of chromatographic protocols is possible, which show interferences due to protein–endotoxin interactions (Karplus et al., 1987). Yet detergents can also be employed in two-phase extraction. Above the critical micelle concentration of detergents, endotoxins are accommodated in the micellar structure by non-polar interactions of alkyl chains of lipid A and the detergent and are consequently separated from the water phase. Detergents of the Triton series show a miscibility gap in aqueous solutions. Above a critical temperature, the so-called cloud point, micelles aggregate to droplets with very low water content, by that forming a new phase. Endotoxins remain in the detergent-rich phase. Through centrifugation or further increase in temperature the two phases separate with the detergent-rich phase being the bottom phase (Bordier, 1981; Aida and Pabst, 1990). If necessary, this process is repeated until the remaining endotoxin concentration is below the threshold limit (Fig. 4). The cloud point of Triton X-114 is at 22°C , which is advantageous when purifying proteins. It requires mixing of the endotoxin-containing protein solution in the cold (usually at 4°C) and allows separation of the two phases at $T > 22^\circ\text{C}$. In contrast, the cloud point for Triton X-100 is at 75°C , which is not acceptable for most proteins.

The separation of endotoxin and exopolysaccharides from *Klebsiella* sp. I-714 is difficult to achieve with other techniques than two-phase extraction. Using Triton X-114, Adam et al. (1995) showed a 100-fold endotoxin reduction in two steps with a final endotoxin content of 30 EU mg^{-1} at 50% loss in bioactivity of the exopolysaccharide. Also about 100-fold endotoxin reduction was shown by Cotten et al. (1994), from plasmid DNA with a final endotoxin content of 0.1 EU in $6 \mu\text{g}$ DNA; though, they got a slightly better removal efficiency with a polymyxin B sorbent

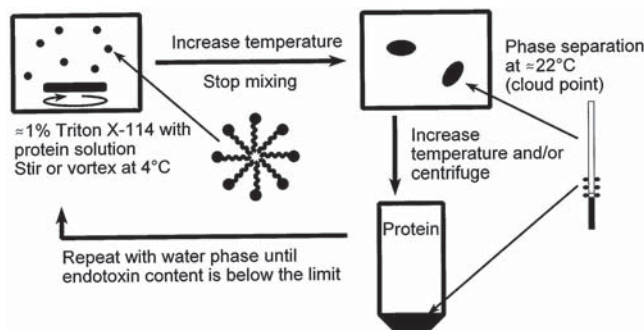


Fig. 4. Removal of endotoxins by two-phase extraction with Triton X-114.

(Section 4.3.2.1). A comparison of affinity adsorption and Triton X-114 two-phase extraction for the decontamination of the recombinant proteins cardiac troponin I, myoglobin and creatine kinase isoenzymes is described by Liu et al. (1997). They concluded that phase separation was the most effective method, reducing the endotoxin content to 98–99% with remaining amounts of 2.5–25 EU mg^{-1} , depending on the protein.

Since always a certain amount of detergent remains in the protein solution, this needs to be removed by adsorption or gel filtration—additional processes, leading to 10–20% product loss (Aida and Pabst, 1990). Also, it is time consuming, as it usually requires multiple repetitions. Adopting to a large-scale process may be difficult owing to the temperature shifts. Furthermore, it is restricted to those biopolymers that will partition exclusively into the water phase, i.e. mainly for hydrophilic proteins and DNA; the process was originally developed to separate integral membrane proteins from hydrophilic proteins (Bordier, 1981).

4.3. Adsorption techniques

Most frequently, adsorption techniques are employed for the removal of endotoxins from protein solutions. In principle, non-selective adsorption on activated carbon or other adsorber materials is possible, as described for the decontamination of plasma (Nagaki et al., 1991). However, using non-selective adsorbers also for the decontamination of protein solutions must be re-

jected owing to irreversible adsorption of proteins to activated carbon. It should be said that 50% endotoxin reduction from whole blood is to be considered as a success in medical application, as a significant number of patients will survive, who otherwise would die. In contrast, a 50% reduction is insignificant in biotechnological processes, where endotoxins may need to be reduced over several orders of magnitude to meet threshold limits.

4.3.1. Anion-exchange chromatography

Since endotoxins are negatively charged, anion exchangers are employed for their adsorption from protein-free solutions, such as DEAE chromatographic matrices or DEAE membranes or matrices functionalised with quaternary amino groups (Gerba and Hou, 1985; Hou and Zaniewski, 1990a,b; Neidhardt et al., 1992). Clearance factors of more than five orders of magnitude can be obtained; however, these exceptional efficiencies are restricted to high endotoxin feed concentrations ($> 1 \mu\text{g ml}^{-1}$). At feed concentrations of less than 10 ng ml^{-1} , which is a common endotoxin contamination level, about three to four orders of magnitude are feasible. A prerequisite for maximal adsorption is a low ionic strength in the feed, corresponding to less than 50 mM NaCl.

If solutions with acidic proteins are to be decontaminated, protein co-adsorption is a problem. This has the consequence of competing interactions at binding sites. Therefore, endotoxin concentrations often cross the limits in column

effluents after exhaustion of the binding capacity for the protein, as is shown exemplarily in Fig. 5(a), using a DEAE sorbent and BSA as model protein. From this point of view, only proteins with net positive charge, i.e. basic proteins, ought to be treated with anion-exchangers. These proteins are repelled from the anion-exchange matrix; a technique usually designated negative chromatographic mode. However, competition of the ion-exchanger and net-positively charged proteins about endotoxin may take place, causing the proteins to drag endotoxin along the column (Section 3.4).

Manipulation of the pH to meet or to get beneath the isoelectric point of an acidic protein is a possible way for optimisation. Neutralisation or charge inversion causes suppression of protein adsorption, by that preventing competition. However, this is restricted to proteins with sufficient solubility and stability at the isoelectric point. Furthermore, optimisation usually passes a maximum with lowering the pH beneath the isoelectric point. If a net positive charge is established, endotoxins may be dragged along as with basic proteins.

4.3.2. Affinity adsorption

According to the concept of affinity interac-

tions, clearance by an endotoxin-selective affinity sorbent should be possible and should guarantee a protein recovery of almost 100%. To meet the various chemical structures of endotoxins from different bacteria and strains, group-selective affinity ligands recognising conservative structure elements should be most successful for the removal of unknown endotoxin variants.

4.3.2.1. Polymyxin B. The bactericidal activity of the antibiotic polymyxin B against gram-negative bacteria is based on the ability to deorganise the bacterial wall after insertion (Newton, 1956). The surface active cyclic peptide (Fig. 6) can break down endotoxin aggregates (Lopes and Inniss, 1969). Owing to the interaction of polymyxin B with lipid A, it should be a group-selective ligand with potential to recognise endotoxins from different origin.

Its use as ligand in affinity sorbents displays clearance factors of greater than 10^5 from heavily contaminated culture filtrates ($1\text{--}10\text{ }\mu\text{g ml}^{-1}$) of different gram-negative bacteria (Issekutz, 1983). Talmadge and Siebert (1989) showed at input concentrations of $6000\text{--}6700\text{ EU ml}^{-1}$ ($\approx 0.6\text{--}0.7\text{ }\mu\text{g ml}^{-1}$ endotoxin) that CF of about 10^3 did only slightly change in the presence of up to 10 mg ml^{-1} BSA or human IgG in batch experi-

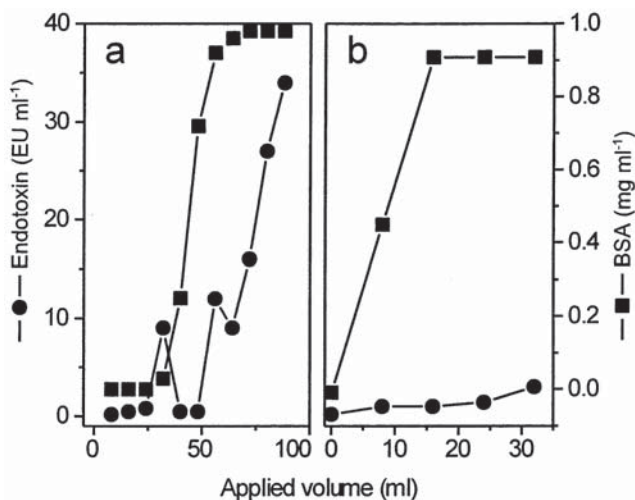


Fig. 5. Dynamics of adsorption of endotoxin (●) and BSA (■) on an anion exchanger (DEAE-Sepharose CL-6B, a) and an endotoxin-selective sorbent (PLL-Sepharose 4B, b) during decontamination of about 1 mg ml^{-1} BSA and 2500 EU ml^{-1} under identical chromatographic conditions (adapted from Petsch et al., 1998b).

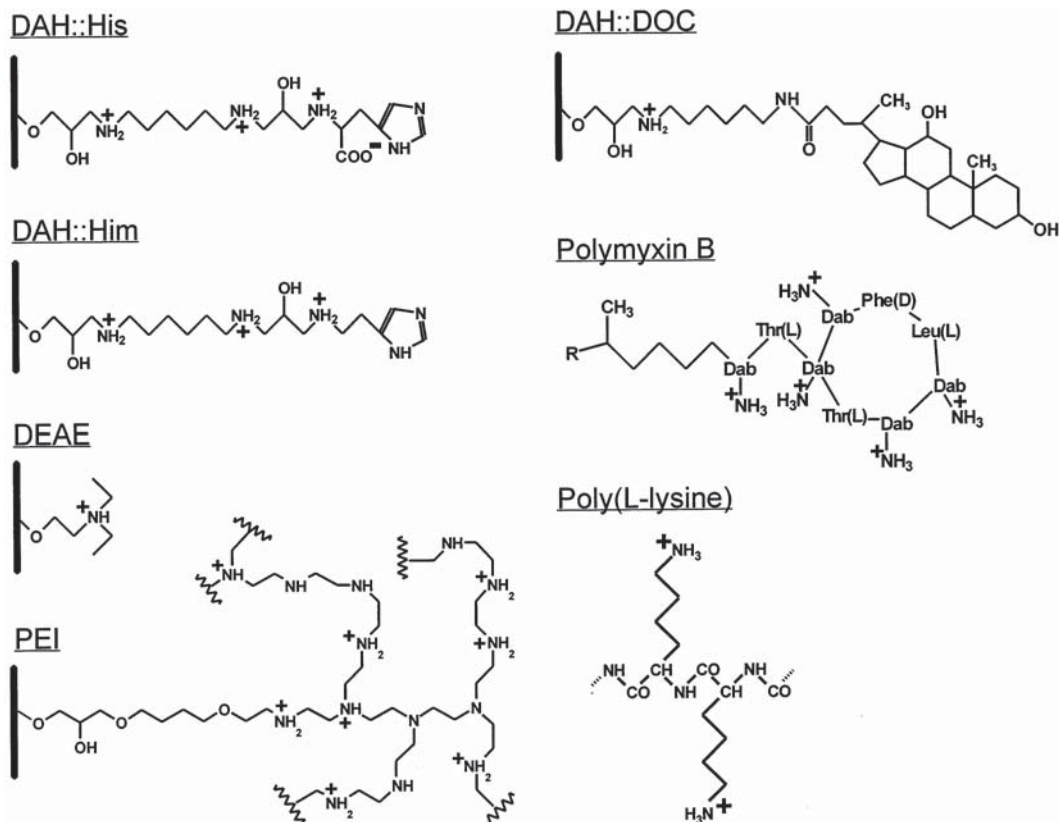


Fig. 6. Structure and charge distribution of endotoxin-selective ligands. The spacer DAH has a favourable effect on the function of some ligands.

ments with contact times of 16 h. However, clearance factors from solutions of different monoclonal antibodies may differ significantly as was shown for an anti-horseradish peroxidase antibody (2.1 mg ml⁻¹ with 20 EU ml⁻¹) and an anti-human chorionic gonadotropin antibody (0.5 mg ml⁻¹ with 61 EU ml⁻¹) which were reduced to 0.3 EU ml⁻¹ (CF ≈ 100) and 6 EU ml⁻¹ (CF ≈ 10) in 20 mM phosphate buffer, pH 6.5, respectively. Data about protein recoveries were not provided.

Clearance factors of more than 1000 were possible from bovine catalase (Karplus et al., 1987). Even blood plasma of endotoxemic patients could be treated, however, with less than 50% endotoxin removal (Kodama et al., 1997).

The major drawbacks, if released from the sorbent, are the neuro- and nephrotoxicity of

polymyxin B (Newton, 1956) and stimulation of monocytes to release interleukin-1 (Damais et al., 1987). Furthermore, protein losses during passage through polymyxin B columns were reported (bovine catalase 24% loss, Karplus et al., 1987; BSA 20% loss, Anspach and Hilbeck, 1995). These are due to the cationic properties of this ligand (Fig. 6), leading to electrostatic interactions with net-negatively charged proteins at the low ionic strengths commonly chosen. This is also the reason why, in spite of 200–10 000-fold reduction of endotoxins from plasmid DNA preparations, DNA recovery is only about 50% (Wicks et al., 1995; Montbriand and Malone, 1996).

4.3.2.2. Histamine and histidine. The introduction of these ligands goes back to Kanoh et al. (1968), who discovered interaction between ribonucleic

acids and endotoxins. Later Minobe et al. (1982) showed that besides the bases adenine, cytosine and others, histidine and histamine were equally successful in the removal of endotoxins from culture filtrates of various microorganisms. Although the histamine sorbent was favoured (Minobe et al., 1983), they later switched to histidine owing to the biological activity of histamine (Minobe et al., 1988). Both ligands were as effective as polymyxin B for culture filtrates and were able to decontaminate solutions of various proteins, including serum albumins, insulin, lysozyme, myoglobin and others with clearance factors ranging from 5 to 200, depending on protein concentrations and environmental conditions. In a consequent paper of Matsumae et al. (1990), only data from independent measurements of product recovery and removal efficiencies are presented. As with polymyxin B, best protein recoveries and removal efficiencies cannot be achieved independently. Data from other laboratories (Anspach and Hilbeck, 1995; Legallais et al., 1997) indicate that the presence of proteins strongly affects the removal of endotoxins, leading to a more than ten-fold reduction of endotoxin clearance factors in the presence of BSA and ineffectiveness in the presence of murine IgG₁ ($pI = 5.5$), if the process is carried out at neutral pH. Also the type of buffer salt seems to influence the removal efficiency (Legallais et al., 1997).

Despite similar removal efficiencies of the various ligands employed by Minobe et al. (1982), their chemical structures are quite different. According to Minobe et al. (1988), the mechanism of endotoxin binding is attributed to synergistic hydrophobic and electrostatic interactions, originating from the spacer DAH and imidazole, respectively (Fig. 6). Histidine or histamine may not be essential for the function of this adsorber. The DAH spacer itself is also effective, as was shown for the decontamination of a 10% cytochrome C solution with 1130 EU ml⁻¹ and a 20% HSA solution with 85 EU ml⁻¹ (5000- and 100-fold endotoxin reduction, respectively) using a PVA-based membrane support (Nagamatsu et al., 1991). Also Hou and Zaniewski (1991) proved that DAH and other diaminoalkanes are effective for endotoxin adsorption owing to a synergistic

effect of ionic and hydrophobic interaction; though, they did not provide data from protein solutions. The function of the histidine ligand is linked to the presence of a positive charge at the imidazole ring, as was shown by Petsch et al. (1997). With incorporation of a non-charged bisoxirane-based spacer, significant removal efficiencies were only found at $pH \leq 5$, where the imidazole ring is charged ($pK_{\text{imidazole}} = 6.0$).

4.3.2.3. Polycationic ligands. According to molecular dynamics, the three-dimensional structure of endotoxin is rather flexible compared to proteins (Kastowsky et al., 1992), which may play a decisive role during adsorption. This is supported by the observation that only a fraction of adsorbed endotoxins can be desorbed at a high salt concentration (> 1 M NaCl) although adsorption can be effectively suppressed in the presence of 0.5 M NaCl (Montbriand and Malone, 1996; Rantze, 1996). Most probably, this is attributed to short-range interactions, evolving after an approximation of ligand and endotoxin due to long-range electrostatic interactions. Secondary binding leads to additional van-der-Waals and hydrogen bonds after a structural adaptation to the microstructures at the surface of sorbents. Only harsh conditions (30% ethanol with up to 1 M NaOH) are successful to clean sorbents used for endotoxin adsorption.

This points out to the fact that an exact structural match between affinity ligands and endotoxins is not necessary. From this point of view an endotoxin-selective ligand should meet the characteristics of a polyanionic molecule with hydrophobic moieties.

Indeed, several cationic polymers were successfully employed as ligands (Fig. 6). Mitzner et al. (1993), used poly(ethyleneimine) (PEI) immobilised on cellulose beads for the extracorporeal removal of endotoxin from plasma and obtained a similar efficacy to polymyxin B at superior biocompatibility. Immobilisation of PEI to cellulose fibres revealed greater endotoxin removal from BSA solutions than corresponding histidine-immobilised fibres with less dependence on the ionic strength (Morimoto et al., 1995); solutions of myoglobin, γ -globulin and cytochrome C were

almost completely cleared of endotoxins ($> 98\%$ removal efficiency with $< 0.05 \text{ ng ml}^{-1}$ remaining) at greater 98% protein recovery in a batch process.

Also PLL displayed a slightly better clearance of low amounts of endotoxins from BSA solution compared with the ligands histamine, histidine, polymyxin B and DEAE at, however, a higher protein recovery (Anspach and Hilbeck, 1995). In contrast to a DEAE ion exchanger, the PLL sorbent is still applicable after exhaustion of the protein binding capacity (Fig. 5b). Pre-coating of microplates with high-molecular-weight PLL ($M_r = 150\,000\text{--}300\,000$) was more effective to improve endotoxin binding to the wells in comparison to PLH and polymyxin B (Takahashi et al., 1992). Recently, Hirayama et al. (1999) have extended their concept of charged polymeric matrices by including PLL in a polymer matrix. Karl et al. (1991) showed that zirconia-immobilised PLH improves endotoxin removal from a BSA solution in comparison to the bare zirconia surface. PLH, however, is a quite expensive ligand that is unstable under alkaline conditions (0.1 M NaOH; Wäsche, 1997).

The underlying principle of these polycation-endotoxin interactions is possibly the same as for the flocculation of cells and cell debris. Also in flocculation a polycation-polyanion-complex is initially formed. Then replacement of water molecules follows and finally flocs form, a process which is also called complex coacervation (Xia and Dubin, 1994). If this process takes place also with immobilised polycations and endotoxins, it would continuously withdraw complexes from solution. This would explain the high affinity binding site observed in thermodynamic investigations of these sorbents and also the selectivity of polycationic ligands in the presence of proteins (Petsch et al., 1998b).

4.3.2.4. Polymeric matrices with cationic functional groups. Through amination of spherical porous poly(γ -methyl-L-glutamate) beads Hirayama et al. (1990, 1992), yielded adsorbents, which showed superior endotoxin binding capacity than commercial endotoxin adsorbents based on histidine and chitosan. Furthermore, less dependence on

the ionic strength (the working range corresponds up to 0.4 M NaCl) and a higher selectivity towards BSA are claimed. Additionally, penetration of proteins into the pore system is prevented by adjusting reaction conditions to yield beads with small pore sizes. This allows for high recoveries of net-negatively charged proteins and strong endotoxin adsorption at the same time. The authors conclude that the high efficiency is attributed to the adsorption of mainly endotoxin aggregates to the adsorber surface, while the BSA binding capacity is rather low.

A great disadvantage is the low chemical stability of an ester bond by which the charge-carrying functional groups are immobilised in the side chain; thus, CIP at high or low pH is ruled out. In a more recent publication, *N,N*-dimethyl-aminopropylacrylamide/*N*-allylacrylamide copolymers were therefore introduced (Hirayama et al., 1994). The charge density is manipulated by adjusting the ratio of the two monomers, and also this concept allows to adjust the pore size of the beads. Removal efficiencies were 96–99% (pH 7, $\mu = 0.05$) with remaining endotoxin amounts of less than 1 EU ml^{-1} at 0.5 mg ml^{-1} of BSA, myoglobin, γ -globulin or cytochrome C and protein recoveries greater than 99%.

Immobilisation of ligands to microfiltration membranes may also yield polymers with cationic functional groups. The inner surface of these membranes (this is mainly the wall of the flow through pores) is first covered by a hydrophilic polymer, such as dextran, hydroxyethyl cellulose or poly(vinylalcohol). Then either small ligands, such as histidine, deoxycholate, polymyxin B or DEAE, or polycationic ligands, such as PLL or PEI are immobilised inside the hydrophilic polymer network (Petsch et al., 1997). Differences between high and low molecular weight ligands are not as noticeable any more; the whole network acts like a cationic polymer (Petsch et al., 1998b). Although these membrane adsorbents show cationic properties and therefore adsorb net-negatively charged proteins, displacement of endotoxins is not observed with the ligands PEI, PLL and even not with DEAE after exhaustion of the protein binding capacity. Corresponding adsorption isotherms show distinct binding sites for

endotoxins and proteins. Under optimised environmental conditions, protein recoveries can be close to 100% (Table 4) owing to the low protein binding capacity. With the ligand DOC endotoxin removal is less dependent on pH and ionic strength; for example, serum albumin solutions yields better results at neutral pH despite the proteins being negatively charged at this pH.

4.3.3. Immunoaffinity chromatography

With regard to competing interactions at the surface of sorbents and with proteins in solution as described above, utilisation of immunoaffinity ligands recognizing endotoxins at the molecular level should be most promising at first sight. However, relevant information about the usefulness of immuno sorbents for endotoxin removal is rare (see for example Goldbaum et al., 1994); also the patent literature does not provide additional data. Generally, immuno sorbents are used reluctantly at the end of a purification train, as ligand leakage may lead to further contamination of an almost pure product. However, the concept is doubtful also in another respect.

Strong efforts were undertaken in the past 10–15 years to develop therapeutic proteins based on IgG and IgM antibodies to take steps against the problem of endotoxin intoxication, a common problem with patients suffering from haemodialysis and acute bacterial infections. In spite of promising attempts (Teng et al., 1985; Ziegler et al., 1991; Di Padova et al., 1993), all clinical trials failed. A reason for this failure is the large chemical variety of endotoxin structures built from microorganisms (Baumgartner and Glauser,

1993). On the other hand, antibodies raised against the non-polar lipid A, such as HA-1A (Centoxin), displayed mainly non-specific hydrophobic interactions, recognizing hydrophobic moieties of proteins as well (Helmerhorst et al., 1998). These ligands were not employed as ligands in immuno sorbents until now and, therefore, a success is not out of question. However, both the specific recognition of only one antigen as well as non-specific interactions are not favourable in the respect of endotoxin removal from protein solutions.

4.3.4. Kinetics of endotoxin adsorption

Data from Minobe et al. (1991), and also results compiled in our laboratory indicate a kinetic control of endotoxin adsorption, which is most distinct at low concentrations (Petsch et al., 1998b). This is caused by the size of micelles and vesicles and their stability. Steric restrictions hinder the penetration of aggregates into the pore system of adsorbers (Hirayama et al., 1992). Therefore, the adsorption of endotoxin takes place mainly at the outer surface of adsorber particles, which, however, only amounts to a fraction of the binding capacity of adsorbers (for example $\ll 1\%$ with Sepharose beads). Adsorption inside the pore system requires the dissociation of aggregates—a very slow process that would explain the flat uptake curves observed in batch adsorption experiments. Due to the slow diffusion of large endotoxin structures they have hardly a chance to interact with the adsorber (a residence time problem). The latter is the most probable explanation of the generally lower clear-

Table 4

Decontamination of protein solutions with membrane adsorbers; 15 ml feed with protein and endotoxin contaminations as stated in column tops was applied to one membrane disc (13.4 cm² membrane area) at optimal pH (in parentheses; Petsch et al., 1997)

Ligand	Protein recovery (left), remaining endotoxin concentration (right, EU ml ⁻¹)					
	Lysozyme (pH 7)		BSA (pH 4.7)		Murine IgG ₁ (pH 5.5)	
	1 mg ml ⁻¹	134 EU ml ⁻¹	1 mg ml ⁻¹	65 EU ml ⁻¹	3 mg ml ⁻¹	63 EU ml ⁻¹
PEI	88%	0.1	97%	0.1	94%	0.05
DEAE	95%	3.4	97%	0.2	92%	0.2
DAH::DOC	90%	2.7	89%	0.2	89%	0.05

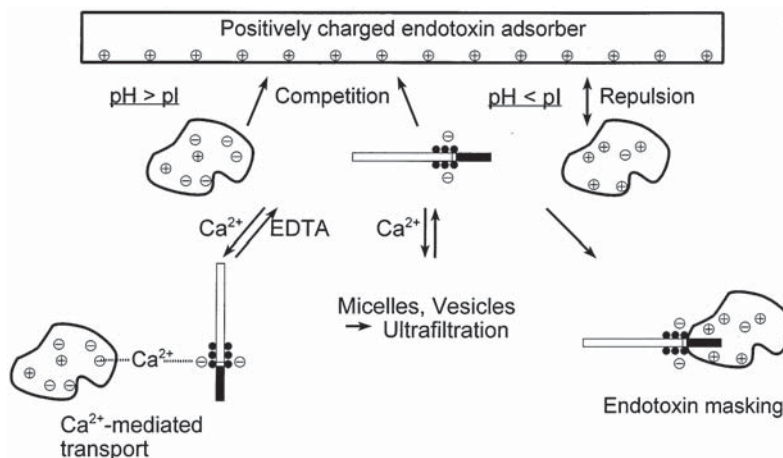


Fig. 7. Interactions between endotoxins and proteins at adsorber surfaces and in solution; see Fig. 2 for explanation of endotoxin symbol.

ance factors found in dynamic operations (in the column) than to be expected from thermodynamic measurements (at equilibrium in a batch).

To overcome transport limitations, the pore diameters of adsorbers should exceed the diameter of adsorbates at least by a factor of five to ten (Smith, 1986); thus, to accommodate endotoxin aggregates pore diameters should be larger than 1 μm . Alternatively, the adsorption may take place exclusively at the surface of small particles with corresponding higher external surface area, which are for example HPLC sorbents with $d_p \leq 5 \mu\text{m}$ or non-porous particles. However, endotoxin-selective sorbents of these sorts are not offered commercially.

Another alternative are membrane adsorbers based on microfiltration membranes. Such membrane adsorbers show low-mass transport restriction and allow a high throughput at minimum expenditure. Anion-exchange membranes of this type are described by Hou and Zaniewski (1990a,b) and by Belanich et al. (1996); other anion-exchange membranes may be employed accordingly. The immobilisation of endotoxin-selective ligands to polymer-coated microfiltration membranes shows endotoxin clearance factors greater than 10^5 from protein-free solutions at residence times in the membranes of about 6 s (Petsch et al., 1997). The latter selectively adsorb endotoxins from solutions of net-negatively and

net-positively charged proteins and allow for almost 100% protein recoveries (Table 4).

4.3.5. Optimisation of adsorption conditions

The principle of endotoxin adsorption from protein solutions is simplified in Fig. 7. Anion-exchange ligands as well as all endotoxin-selective ligands carry a net-positive charge under operating conditions (Fig. 6). Thus, net-negatively charged proteins and endotoxins adsorb at low ionic strengths, whereby the recovery for proteins and endotoxins is almost zero in the beginning. After exhaustion of the sorbent's capacity (independent of whether it is classified as endotoxin-specific or not) the protein recovery will approach 100%. Depending on the ligand employed a certain amount of binding sites are the same for proteins and endotoxin. Thus competition about these sites affects endotoxin removal and also some adsorbed endotoxins are displaced owing to the six orders of magnitude higher concentration of proteins than endotoxin.

On the other hand, complexes are formed between endotoxins and net-positively charged proteins so that affinity ligands and proteins compete for endotoxin. If the charge density at the adsorber surface is high or a highly selective ligand is used, equilibrium will shift to the adsorber, which allows an endotoxin reduction. As net-positively charged proteins are repelled from

anion exchangers, almost 100% protein recovery can be achieved.

Until now, none of the above-mentioned adsorber concepts has proved generally applicable for endotoxin removal from protein solutions. However, ionic forces play an important factor particularly in the primary adsorption process. Generally low ionic strengths, corresponding to less than 50 mM NaCl, are recommended for anion-exchangers. Slightly higher ionic strengths may be employed for affinity sorbents whose ligands combine electrostatic and hydrophobic interactions, such as polymyxin B and DAH-based ligands; also polycationic ligands are less dependent on the ionic strength. Under optimised conditions, association constants exceed 10^9 M^{-1} (based on endotoxin monomers; Minobe et al., 1988; Petsch et al., 1998b).

Recommendations for environmental conditions follow the isoelectric point of the protein to be decontaminated and its pH stability. Generally, best endotoxin reduction can be reached at a pH close to the isoelectric point of the protein. Since the native structure of strongly basic or acidic proteins may then be irreversibly lost and also precipitation may be a problem, compromises must be accepted. With acidic proteins the pH in the environment should be adjusted as close to the *pI* as possible and the use of polymeric or slightly hydrophobic ligands should be considered. Proteins with an isoelectric point close to 7 should be decontaminated at $\text{pH} \approx \text{pI}$. Anion exchangers can be employed, although better results might be obtained with endotoxin-selective ligands. It is quite probable that anion exchangers generally show good results for the decontamination of basic proteins. Especially ligands with quaternary amino groups display a high charge density under alkaline conditions and so are well suited for the adsorption of endotoxins at elevated pH.

It must be considered that not only the net charge of proteins is manipulated through pH changes but also the charge density of all cationic ligands, except those with quaternary amino groups. Therefore, increasing the pH causes a decline of the charge density according to the *pK* or *pI* of the ligands.

In the case of Ca^{2+} -mediated interactions between endotoxins and net-negatively charged proteins the addition of 1–5 mM EDTA is recommended. EDTA is a strong chelator, shifting the equilibrium of the protein- Ca^{2+} -endotoxin complex to the free molecules, by that improving the removal efficiency of an adsorber. Furthermore, the addition of octyl- β -glucopyranoside or other non-charged detergents should be screened, as was shown by Karplus et al. (1987). However, additives are generally disadvantageous, as problems may arise in their subsequent removal.

It is strongly recommended to evaluate the development of the endotoxin concentration in the column effluent of every adsorber after exhaustion of its protein binding capacity. The same is true in batch adsorption as changes in protein or endotoxin concentration will effect both the removal efficiency and protein recovery.

4.4. Other techniques

Preparative fractionation of proteins by isoelectric focussing can be envisaged by using a multi-compartment electrolyser fitted with membranes of predetermined *pI*s. Similarly endotoxins can be removed from protein solutions, as was shown by Lucas et al. (1990). By continuous circulation of a myoglobin solution (*pI* 7.3, 8 mg ml⁻¹) between membranes of pH 6.98 and pH 8.04 in 1 mM HEPES, pH 5.1, the endotoxin content was reduced from 6000 to 6 pg ml⁻¹ in 3 h. Using membranes with other immobilised pH allows removal conditions to be adjusted to the *pI* of the other protein. It is characteristic for this process that it requires a very low ionic strength to keep a low electrical current.

Because of the high affinity of phosphate and phosphate esters for zirconia surfaces, endotoxins can be well adsorbed to colloidal zirconia (Karl et al., 1991). However, the efficiency of these adsorbents drops significantly in the presence of BSA and most probably also of other proteins owing to non-specific interactions with the zirconia surface.

Another endotoxin-selective sorbent is based on cross-linked chitosan beads, whose chemical structure is β -1,4-poly-D-glucosamine (Dainippon-

Pharmaceuticals, 1987). Below pH 7 chitosan is positively charged and can be used like other positively charged ligands described above. To improve the efficiency under alkaline conditions, quaternised chitosan was introduced (Kurita Water, 1995).

As suggested by Matsumae et al. (1990), selective endotoxin adsorption from protein solutions can also be achieved at greater than 3 M salt concentration using histidine-immobilised sorbents. This is mainly due to a hydrophobic contribution of the ligand; therefore, similar behaviour can be expected from other endotoxin-selective ligands as well as in hydrophobic interaction chromatography (Hou and Zaniewski, 1991) with working in the negative chromatographic mode. However, the protein remains in a high salt concentration that consequently has to be removed, a process, which is economically questionable.

5. Discussion

In most of earlier studies on endotoxin removal it is assumed that endotoxins are present in aggregated form ($M_r \approx 1$ MDa) and are thus best separated by ultrafiltration. This view is kept in many places so far; it is supported by the high efficiency of ultrafiltration for the decontamination of water and solutions containing low-molecular weight substances.

Yet, it is evident that large endotoxin aggregates are repressed in favour of smaller units and monomers in the presence of proteins—in fact independent of their net charge. The ratio of small to large units is determined by the concentration of endotoxin, however, also the concentration of calcium ions, chelators and proteins are of great influence (Fig. 2). It can be assumed that at the low, but problematic endotoxin concentration (< 100 EU ml⁻¹), monomers are present to a large extent. These are associated with proteins, more or less strongly bound either directly by electrostatic interactions or indirectly through calcium bridges (Fig. 6). With those low endotoxin concentrations ultrafiltration is ineffective.

Two-phase extraction is a valuable tool for the decontamination of those pharmaproteins that

were purified on a small scale and are subjected to first in vivo tests. If later high product quantities need to be produced, it may need to be substituted by other techniques.

Usually, when standard purification methods fail the scientific community turns to naturally evolved recognition mechanisms. However, nature does not offer a cure-all against endotoxins; in the millions of years of bacterial existence they have managed to evade defence mechanisms of their hosts by a high flexibility of the cell wall. Therefore, it is unlikely that in future a group-selective antibody will be within reach, which exclusively addresses a conservative part of endotoxin without showing non-specific binding of proteins.

What remains, are affinity ligands whose recognition for endotoxin is best circumscribed as diffuse. In other words, this can be expressed as synergism of electrostatic and hydrophobic interactions. These are most probably supplemented by secondary interactions, which become when active endotoxin and ligand approached a certain distance (short-range interactions).

All endotoxin-selective ligands described above fall into this category. Depending on the contribution of non-polar and charged groups of ligands, hydrophobic or electrostatic interactions are more developed. Consequently, also the range of usable ionic strengths varies with the ligands employed and also differences of the efficiency between ligands are noticed. However, electrostatic forces are decisive with all ligands to achieve significant adsorption. Thus, in any case, competition with negatively charged proteins must be expected, its extent, however, depending on the particular ligand and underlying matrix. The pH is a key parameter in endotoxin clearance, especially for ligands with a relatively high density of charged groups, such as ion-exchangers. It is indicated that removal using more hydrophobic ligands is less dependent on the pH and ionic strength. To what extent polymeric ligands or polymeric supports lead to better removal efficiencies and whether this is indeed attributed to the flexibility of polymer chains and endotoxin will be proved in future; the more recent investigations are concentrating on this subject.

Optimisations of adsorption conditions are possible with all ligands. Only a few are restricted to acidic conditions, such as chitosan or histidine and histamine. Quaternary amino groups are generally best applicable at alkaline pH. Neutralisation or reversal of charges at the target protein is recommended to face the carrier effect observed with positively charged proteins. The addition of EDTA is recommended if this carrier effect is mediated by calcium bridges.

In any case one should disregard the idea that product-selective affinity sorbents will generally solve both the purification and the endotoxin problem. More likely the opposite is true: using affinity sorbents with high selectivity for the product and so reducing the number of purification steps may result in notable amounts of endotoxin remaining in the final product.

Nonetheless, a change of the purification strategy is recommended for endotoxin-contaminated products. Instead of only concentrating on methods to further purify an almost pure product, methods should be chosen that allow capture of the contaminant.

In view of the slow diffusion of endotoxins and the low concentrations to be removed, membrane adsorbers show great potential. The common argument of having a too low binding capacity is not valid here. Generally, draw-backs against particular sorbents in terms of selectivity are not to be expected. In contrast, through binding ligands to flexible hydrophilic chains, better results can be obtained than with particulate matrices.

By consideration of equilibria leading to endotoxin aggregates and monomers (Fig. 2) and competing interactions about binding sites and in solution (Fig. 6) as well as a certain persistence (and time) to experiment with different adsorbents (including membrane adsorbers) or phase systems, efficient endotoxin removal should be possible in every case and should be valuable. Knowledge of the connections simplified in Figs. 2 and 6 is the key to success. However also in future, we will most probably have to live with single solutions.

References

- Adam, O., Vercellone, A., Paul, F., Monsan, P.F., Puzo, G., 1995. A non-degradative route for the removal of endotoxin from exopolysaccharides. *Anal. Biochem.* 225, 321–327.
- Aida, Y., Pabst, M.J., 1990. Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods* 132, 191–195.
- Anspach, F.B., Hilbeck, O., 1995. Removal of endotoxins by affinity sorbents. *J. Chromatogr. A* 711, 81–92.
- Baumgartner, J.-D., Glauser, M.-P., 1993. Immunotherapy of endotoxemia and septicemia. *Immunobiology* 187, 464–477.
- Behringwerke, 1990. Separation of toxins from protein solutions, especially lipo-cortins. German patent, DE 3911629.
- Belanich, M., Cummings, B., Grob, D., Klein, J., O'Connor, A., Yarosh, D., 1996. Reduction of endotoxin in a protein mixture using strong anion-exchange membrane adsorption. *Pharm. Technol.* 3, 142–150.
- Bennett, I.L., 1963. Introduction. In: Landy, M., Braun, W. (Eds.), *Bacterial Endotoxins*. Proc. Symp. Rutgers Univ. Rutgers University Press, New Brunswick.
- Berger, D., Berger, H.G., 1988. Comparison of the endotoxin-binding capacity of human transferrin and a human applicable immunoglobulin preparation. *Drug Res.* 38, 817–820.
- Berthold, W., Walter, J., 1994. Protein purification: aspects of processes for pharmaceutical products. *Biologicals* 22, 135–150.
- Bischoff, R., Speck, D., Lepage, P., Delatre, L., Leoux, K., Brown, S.W., Roitsch, C., 1991. Purification and characterization of recombinant α_1 -antitrypsin variants expressed in *E. coli*. *Biochemistry* 30, 3464–3472.
- Bommer, J., Ritz, E., 1987. Water quality—a neglected problem in haemodialysis. *Nephron* 46, 1–6.
- Bordier, C., 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256, 1604–1607.
- Caroff, M., Tacken, A., Szabo, L., 1988. Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the 'isolated lipid A' fragment of the *Bordetella pertussis* endotoxin. *Carbohydr. Res.* 175, 273–282.
- Centocor, 1988. Removing endotoxin contaminates from biological material. US patent, U 03773.
- Cotten, M., Baker, A., Saltik, M., Wagner, E., Buschle, M., 1994. Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus. *Gene Therapy* 1, 239–246.
- Dainippon-Pharmaceuticals, 1987. Removing nucleic acid and endotoxin from solutions using chitosan treatment. Japanese patent, J 302870 and European patent, EP 240348.
- Damais, C., Jupin, C., Parant, M., Chedid, L., 1987. Induction of human interleukin-1 production by polymyxin B. *J. Immunol. Methods* 101, 51–56.

- David, S.A., Balam, P., Mathan, V.I., 1995. Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: implications for an endotoxin carrier function for albumin. *J. Endotoxin Res.* 2, 99–106.
- De Pamphilis, M.L., 1971. Dissociation and reassembly of *Escherichia coli* outer membrane and of lipopolysaccharide and their reassembly onto flagellar basal bodies. *J. Bacteriol.* 105, 1184–1199.
- Dijkstra, J., Mellors, J., Ryan, J.L., Szoka, F.C., 1987. Modulation of the biological activity of bacterial endotoxin by incorporation into liposomes. *J. Immunol.* 128, 2663–2670.
- Dinarello, C.A., O'Connor, J.V., LaPreste, G., Swift, R.L., 1984. Human leukocyte pyrogen test for detection of pyrogenic material in growth hormone produced by recombinant *E. coli*. *J. Clin. Microbiol.* 20, 323–329.
- Di Padova, F.E., Brade, H., Barclay, G.R., Poxton, I.R., Liehl, E., Schuetze, E., Kocher, H.P., Ramsay, G., Schreier, M.H., McClelland, D.B.L., Rietschel, E.T., 1993. A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharides. *Infect. Immun.* 61, 3863–3872.
- Dressel, H., Stein, M., Eckardt, B., Hoffmann, B., Odebrecht, W., Mehlhorn, G., 1991. Zum endotoxingehalt von sedimentationsstaub. *Pharmazie* 46, 606–607.
- Elass-Rochard, E., Roseanu, A., Legrand, D., Trif, M., Salmon, V., Motas, C., Montreuil, J., Spik, G., 1995. Lactoferrin-lipopolysaccharide interaction. *Biochem. J.* 312, 829–845.
- European Pharmacopoeia, 1997. 3rd ed.
- Galanos, C., Freudenberg, M.A., 1993. Mechanisms of endotoxin shock and endotoxin hypersensitivity. *Immunobiology* 187, 346–356.
- Galanos, C., Rietschel, T.E., Lüderitz, O., Westphal, O., 1971. Interaction of lipopolysaccharide and lipid A with complement. *Eur. J. Biochem.* 19, 143.
- Gerba, C.P., Hou, K., 1985. Endotoxin removal by charge-modified filters. *Appl. Environ. Microbiol.* 50, 1375–1377.
- Goldbaum, F.A., Rubbi, C.P., Fossati, C.A., 1994. Removal of LPS from a *Brucella* cytoplasmic fraction by affinity chromatography with an anti-LPS monoclonal antibody as immunosorbent. *J. Med. Microbiol.* 40, 174–178.
- Green-Cross, 1986. Purification of urokinase and its precursor. Japanese patent, J 61227782.
- Hannecart-Pokorni, E., Dekegel, D., Depuydt, F., 1973. Macromolecular structure of lipopolysaccharides from gram-negative bacteria. *Eur. J. Biochem.* 38, 6–13.
- Hartung, T., Wendel, A., 1996. Detection of pyrogens using human whole blood. *In Vitro Toxicol.* 9, 353–359.
- Hasday, J., Dubin, W., Fitzgerald, T., Bascom, R., 1996. Cigarettes are a rich source of bacterial endotoxin. *CHEST* 109, 63S–64S.
- Helmerhorst, E.J., Maaskant, J.J., Appelmelk, B.J., 1998. Anti-lipid A monoclonal antibody centoxin (HA-1A) binds to a wide variety of hydrophobic ligands. *Infect. Immun.* 66, 870–873.
- Hirayama, C., Ihara, H., Li, X., 1990. Removal of endotoxins using macroporous spherical poly(γ -methyl L-glutamate) beads and their derivatives. *J. Chromatogr.* 530, 148–153.
- Hirayama, C., Sakata, M., Ihara, H., Ohkuma, K., Iwatsuki, M., 1992. Effect of the pore size of an aminated poly(3-methyl L-glutamate) adsorbent on the selective removal of endotoxin. *Anal. Sci.* 8, 805–810.
- Hirayama, C., Sakata, M., Yugawa, Y., Ihara, H., 1994. Cross-linked *N,N*-dimethylaminopropylacrylamide spherical particles for selective removal of endotoxin. *J. Chromatogr. A* 676, 267–275.
- Hirayama, C., Sakata, M., Nakamura, M., Ihara, H., Kunitake, M., Todokoro, M., 1999. Preparation of poly(Epsilon-lysine) adsorbents and application to selective removal of lipopolysaccharides. *J. Chromatogr. B* 721, 187–195.
- Hou, K.C., Zaniewski, R., 1990a. Depyrogenation by endotoxin removal with positively charged depth filter cartridge. *J. Parenter. Sci. Technol.* 44, 204–209.
- Hou, K.C., Zaniewski, R., 1990b. Endotoxin removal by anion-exchange polymeric matrix. *Biotechnol. Bioeng.* 12, 315–324.
- Hou, K.C., Zaniewski, R., 1991. The effect of hydrophobic interaction on endotoxin adsorption by polymeric affinity matrix. *Biochim. Biophys. Acta* 1073, 149–154.
- Issekutz, A.C., 1983. Removal of gram-negative endotoxin from solutions by affinity chromatography. *J. Immunol. Methods* 61, 275–281.
- Jiao, B.H., Freudenberg, M., Galanos, C., 1989. Characterization of the lipid A component of genuine smooth-form lipopolysaccharide. *Eur. J. Biochem.* 180, 515–518.
- Kaca, W., Roth, R.I., Levin, J., 1994. Hemoglobin, a newly recognized lipopolysaccharide (LPS)-binding protein that enhances LPS biological activity. *J. Biol. Chem.* 269, 25078–25084.
- Kanoh, S., Kohlhaage, H., Siegert, R., 1968. Pyrogenic principle of the ribonucleic acid from the yeast *Candida utilis*. *J. Bacteriol.* 96, 738–743.
- Karl, D.W., Magnusson, J.C., Carr, P.W., Flickinger, M.C., 1991. Preliminary assessment of removal of pyrogenic lipopolysaccharides with colloidal zirconia adsorbents. *Enzyme Microb. Technol.* 13, 708–715.
- Karplus, T.E., Ulevitch, R.J., Wilson, C.B., 1987. A new method for reduction of endotoxin contaminations from protein solutions. *J. Immunol. Methods* 105, 211–220.
- Kastowsky, M., Gutberlet, T., Bradaczek, H., 1992. Molecular modelling of the three-dimensional structure and conformational flexibility of bacterial lipopolysaccharide. *J. Bacteriol.* 174, 4798–4806.
- Kawahara, K., Seydel, U., Matsuura, M., Danbara, H., Rietschel, E.Th., Zähringer, U., 1991. Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. *FEBS* 292, 107–110.
- Kodama, M., Tani, T., Hanasawa, K., Hirata, K., Hirasawa, H., Oda, S., Otsuka, T., Yamamoto, Y., Kanesaka, S., Takahashi, Y., Maekawa, K., Wakabayashi, Y., Tamakuma, Y., Sugimoto, T., 1997. Treatment of sepsis

- by plasma endotoxin removal: hemoperfusion using a polymyxin-B immobilized column. *J. Endotoxin Res.* 4, 293–300.
- Koyama, K., Ohno, S., Murayama, K., 1983. Removal of endotoxin from protein solutions by means of ultrafiltration. *Hakko Kogaku Kaishi* 61, 421–423.
- Krüger, D., 1989. Assessing the quality of medicinal products containing ingredients obtained by gene technology. *Drugs Made Germany* 32, 64–67.
- Kurita Water, 1995. Removing nucleic acid and endotoxin from solution using chitosan treatment. Japanese patent, JP 07289238.
- Legallais, C., Anspach, F.B., Bueno, S.M.A., Haupt, K., Vijayalakshmi, M.A., 1997. Strategies for the depyrogenation of contaminated IgG solutions by histidine-immobilized hollow fiber membrane. *J. Chromatogr. B* 691, 33–41.
- Li, L., Luo, R.G., 1997. Protein concentration effect on protein–lipopolysaccharide (LPS) binding and endotoxin removal. *Biotechnol. Lett.* 19, 135–138.
- Li, L., Luo, R.G., 1998. Use of Ca^{2+} to re-aggregate lipopolysaccharide (LPS) in hemoglobin solutions and the subsequent removal of endotoxin by ultrafiltration. *Biotechnol. Tech.* 12, 119–122.
- Liu, S., Tobias, R., McClure, S., Styba, G., Shi, Q., Jackowski, G., 1997. Removal of endotoxin from recombinant protein preparations. *Clin. Biochem.* 30, 455–463.
- Lopes, J., Inniss, W.E., 1969. Electron microscopy of effect of polymyxin on *Escherichia coli* lipopolysaccharide. *J. Bacteriol.* 100, 1128–1130.
- Lucas, J., Faupel, M., Goecking, C., 1990. Preparative separation of pyrogens from proteins by isoelectric focussing using a multicompartment electrolyser with immobiline membranes. *Electrophoresis* 11, 981–982.
- Lynn, W.A., Golenbock, D.T., 1992. Lipopolysaccharide antagonists. *Immunol. Today* 13, 271–276.
- Martich, G.D., Boujoukos, A.J., Suffredini, A.F., 1993. Response of man to endotoxin. *Immunobiology* 187, 403–416.
- Matsumae, H., Minobe, S., Kindan, K., Watanabe, T., Sato, T., Tosa, T., 1990. Specific removal of endotoxin from protein solutions by immobilized histidine. *Biotechnol. Appl. Biochem.* 12, 129–140.
- Mayer, H., Weckesser, J., 1984. 'Unusual' lipid A's: structures, taxonomical relevance and potential value for endotoxin research. In: Rietschel, E.Th. (Ed.), *Handbook of Endotoxin*, vol. 1. Elsevier, Amsterdam, pp. 221–247.
- Merck-USA, 1987. Removal of pyrogens and yeast proteins from hepatitis B virus surface antigen. US patent, US 4707542.
- Minobe, S., Watanabe, T., Sato, T., Tosa, T., Chibata, I., 1982. Preparation of adsorbents for pyrogen adsorption. *J. Chromatogr.* 248, 401–408.
- Minobe, S., Sato, T., Tosa, T., Chibata, I., 1983. Characteristics of immobilized histamine for pyrogen adsorption. *J. Chromatogr.* 262, 193–198.
- Minobe, S., Watanabe, T., Sato, T., Tosa, T., 1988. Characteristics and applications of adsorbents for pyrogen removal. *Biotechnol. Appl. Biochem.* 10, 143–153.
- Minobe, S., Nawata, M., Watanabe, T., Sato, T., Tosa, T., 1991. Specific assay for endotoxin using immobilized histidine and limulus amoebocyte lysate. *Anal. Biochem.* 198, 292–297.
- Mitzner, S., Schneidewind, J., Falkenhagen, D., Loth, F., Klinkmann, H., 1993. Extracorporeal endotoxin removal by immobilized polyethylenimine. *Artificial Organs* 17, 775–781.
- Mohan, S., Kelly, T.M., Eveland, S.S., Raetz, C.R.H., Anderson, M.S., 1994. An *Escherichia coli* gene (FabZ) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to fabA and suppression of mutations in lipid A biosynthesis. *J. Biol. Chem.* 269, 32896–32903.
- Montbriand, P.M., Malone, R.W., 1996. Improved method for the removal of endotoxin from DNA. *J. Biotechnol.* 44, 43–46.
- Morimoto, S., Sakata, M., Iwata, T., Esaki, A., Hirayama, C., 1995. Preparations and applications of polyethyleneimine-immobilized cellulose fibres for endotoxin removal. *Polymer J.* 27, 831–839.
- Morrison, D.C., 1985. Non-specific interactions of bacterial lipopolysaccharides with membranes and membrane components. In: Berry, L.J. (Ed.), *Handbook of Endotoxin*, vol. 3. Elsevier, Amsterdam, pp. 25–55.
- Morrison, D.C., Leive, L., 1975. Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. *J. Biol. Chem.* 250, 2911–2919.
- Morrison, D.C., Kirikae, T., Kirikae, F., Lei, M.-G., Chen, T., Vukajlovich, S.W., 1994. The receptor(s) for endotoxin on mammalian cells. *Prog. Clin. Biol. Res.* 388, 3–15.
- Müller-Calgan, H., 1989. Der limulustest in der pharmazeutischen praxis. In: Scheer, R. (Ed.), *Der Limulustest*. Wissenschaftliche Verlagsgesellschaft, Stuttgart, p. 51.
- Nagaki, M., Hughes, R.D., Lau, J.Y., Williams, R., 1991. Removal of endotoxin and cytokines by adsorbents and the effect of plasma protein binding. *Int. J. Artif. Organs* 14, 43–50.
- Nagamatsu, S., Tanaka, Y., Shibata, T., 1991. Separating membrane and separation method. European patent application, EP 0418517 A2.
- Neidhardt, E.A., Luther, M.A., Recny, M.A., 1992. Rapid, two-step purification process for the preparation of pyrogen-free murine immunoglobulin G1 monoclonal antibodies. *J. Chromatogr.* 590, 255–261.
- Newton, B.A., 1956. The properties and mode of action of the polymyxins. *Bac. Rev.* 20, 14–27.
- Nippon-Kayaku, 1991a. *Escherichia coli*-derived pyrogen removal. Japanese patent, J 03244390.
- Nippon-Kayaku, 1991b. Method for removing substances derived from *Escherichia coli*. Japanese patent, J 03277274.
- Novotny, A., 1984. Heterogeneity of endotoxins. In: Rietschel, E.T. (Ed.), *Handbook of Endotoxin*, vol. 1. Elsevier, Amsterdam, pp. 308–332.

- Nurminen, M., Hirvas, L., Vaara, M., 1997. The outer membrane of lipid A-deficient *Escherichia coli* mutant LH530 has reduced levels of OmpF and leaks periplasmic enzymes. *Microbiology* 143, 1533–1537.
- Ohno, N., Morrison, D.C., 1989. Lipopolysaccharide interaction with lysozyme. *J. Biol. Chem.* 264, 4434–4441.
- Pabst, M.J., Johnston, R.B., 1989. Bacterial LPS: a mediator of inflammation. In: Henson, P.M., Murphy, R.C. (Eds.), *Handbook of Inflammation*, vol. 6. Elsevier, Amsterdam, pp. 361–393.
- Pearson, F.C., 1985. *Pyrogens*. Marcel Dekker, New York, pp. 119–220 (Chapters 9–11).
- Petsch, D., Beeskow, T.C., Anspach, F.B., Deckwer, W.-D., 1997. Membrane adsorbers for selective removal of bacterial endotoxin. *J. Chromatogr. B* 693, 79–91.
- Petsch, D., Deckwer, W.-D., Anspach, F.B., 1998a. Proteinase K digestion improves detection of bacterial endotoxins by the Limulus amoebocyte lysate assay: application for endotoxin removal from cationic proteins. *Anal. Biochem.* 259, 42–47.
- Petsch, D., Rantze, E., Anspach, F.B., 1998b. Selective adsorption of endotoxin inside a polycationic network of flat-sheet microfiltration membranes. *J. Mol. Recognit.* 11, 222–230.
- Pool, E.J., Johaar, G., James, S., Petersen, J., Bonic, P., 1998. The detection of pyrogens in blood products using an ex vivo whole blood culture assay. *J. Immunoassay* 19, 95–111.
- Raetz, C.R.H., 1990. Biochemistry of endotoxins. *Ann. Rev. Biochem.* 59, 129–170.
- Rantze, E., 1996. Neue Pyrogen-spezifische Liganden auf der Basis von immobilisierten Polymeren, Diploma thesis, Technical University of Hannover, Germany.
- Rietschel, E.T. (Ed.), 1984. *Handbook of Endotoxin*, Vol. 1, Chemistry of Endotoxin. Elsevier, Amsterdam.
- Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Lippnow, H., Ulmer, A.J., Zähringer, U., Seydel, U., di Padova, F., Schreier, M., Brade, H., 1994. Bacterial endotoxin: molecular relationship of structure to function. *FASEB J.* 8, 217–225.
- Seydel, U., Labischinski, H., Kastowsky, M., Brandenburg, K., 1993. Phase behavior, supramolecular structure and molecular conformation of lipopolysaccharide. *Immunobiology* 187, 191–211.
- Shands, J.W., Graham, J.A., Nath, K., 1967. The morphologic structure of isolated bacterial lipopolysaccharide. *J. Mol. Biol.* 25, 15–21.
- Sharma, S.K., 1986. Endotoxin detection and elimination in biotechnology. *Biotechnol. Appl. Biochem.* 8, 5–22.
- Smith, S.M., 1986. Restricted diffusion through pores with periodic constrictions. *AIChE J.* 32, 1039–1042.
- Sweadner, K.J., Forte, M., Nelson, L.L., 1977. Filtration removal of endotoxin (pyrogens) in solution in different states of aggregation. *Appl. Environ. Microbiol.* 34, 382–385.
- Takahashi, K., Fukada, M., Kawai, M., Yokochi, T., 1992. Detection of lipopolysaccharide (LPS) and identification of its serotype by an enzyme-linked immunosorbent assay (ELISA) using poly-L-lysine. *J. Immunol. Methods* 153, 67–71.
- Takeda Chemicals, 1988. Pertussis endotoxin removal in toxoid production. Japanese patent, J 126394 and European patent, EP211968.
- Talmadge, K.W., Siebert, C.J., 1989. Efficient endotoxin removal with a new sanitizable affinity column: affi-prep polymyxin. *J. Chromatogr.* 476, 175–185.
- Teng, N.N.H., Kaplan, H.S., Hebert, J.M., Moore, C., Douglas, H., Wunderlich, A., Braude, A.I., 1985. Protection against gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. *Proc. Natl. Acad. Sci. USA* 82, 1790–1794.
- Toyo-Soda, 1989. Removing method of pyrogen from super-oxid-dismutase. Japanese patent, J 1196294.
- Vaara, M., Nikaido, H., 1984. Outer membrane organization. In: Rietschel, E.T. (Ed.), *Handbook of Endotoxin*, vol. 1. Elsevier, Amsterdam, pp. 1–45.
- Vaara, M., Nurminen, M., 1999. Outer membrane permeability barrier in *Escherichia coli* mutants that are defective in the late acyltransferases of lipid A biosynthesis. *Antimicrob. Agents Chemother.* 43, 1459–1462.
- Van Putten, J.P.M., 1993. Phase variation of lipopolysaccharide directs interaction of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. *EMBO J.* 12, 4043–4051.
- Wang, Y., Hollingsworth, R.I., 1996. An NMR spectroscopy and molecular mechanics study of the molecular basis for the supramolecular structure of lipopolysaccharides. *Biochemistry* 35, 5647–5654.
- Wäsche, S., 1997. Membranadsorber zur Entfernung von DNA aus Proteinlösungen, Diploma thesis, Technical University of Braunschweig, Germany.
- Westphal, O., Lüderitz, O., 1954. Chemische erforschung von lipopolysacchariden gramnegativer bakterien. *Angew. Chem.* 66, 407–417.
- Wicks, I.P., Howell, M.L., Hancock, T., Kohsaka, H., Olee, T., Carson, D.A., 1995. Bacterial lipopolysaccharide copurifies with plasmid DNA: Implications for animal models and human gene therapy. *Hum Gene Therap* 6, 317–323.
- Xia, J., Dubin, P.L., 1994. Protein-polyelectrolyte complexes. In: Dubin, P., Bock, J., Davies, R.M., Schulz, D.N., Thies, C. (Eds.), *Macromolecular Complexes in Chemistry and Biology*. Springer, Berlin, pp. 247–271.
- Ziegler, E.J., Fisher, C.J., Sprung, C.L., Straube, R.C., Sadoff, J.C., Foulke, G.E., Wortel, C.H., Fink, M.P., Dellinger, R.P., Teng, N.N.H., Allen, I.E., Berger, H.J., Knatterud, G.L., LoBuglio, A.F., Smith, C.R., 1991. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. *New Engl. J. Med.* 324, 429–436.
- Zimmermann, G., 1982. Pyrogen removal from immunoglobulins. German patent DD 157417.