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Purification of a Polyelectrolyte Sample from Complex-Forming Charged Trace Impurities by Continuous Convective Electrophoresis: Depyrogenization of Human Albumin

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ABSTRACT

Using the separation of endotoxin (pyrogens) from albumin as an example, it has been verified that continuous convective electrophoresis can be applied for the purification of a polyelectrolyte sample from complex-forming charged impurities. In a one-stage process, 40% of endotoxin-contaminated albumin could be depyrogenized (<1 μg/L). A multistage process would further increase the yield of depyrogenized albumin, but a complete recovery of depyrogenized albumin is not possible because endotoxins tend to form complexes with albumin. However, the presented method demonstrates a simple way to purify a considerable amount of contaminated albumin without introducing, e.g., detergents or complex inactivation reagents.

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INTRODUCTION

Continuous convective electrophoresis (CCE) based on countercurrent electrolysis in a porous membrane has been extensively studied for separating small cations (1–7). These studies have clearly shown that CCE is an efficient way to separate ions. The technique is based on the simultaneous effects of diffusion, migration, and convective flow through a porous membrane on the transport of ions. The convective flow opposes electrical migration and diffusion. The selectivity in CCE is exponentially dependent on convection, and therefore the increase in convection very rapidly increases separation (1). However, as a result of increased convection, the fluxes are decreased because of depletion of the permeate stream. In order to increase the flux, its migrational contribution is raised by an electric current. This is possible only for suitably charged species and that is why CCE can be so efficiently applied in the purification of proteins by controlling, with the pH, the charge of the protein in the mixture. For example, it is possible, due to countercurrent convection, to prevent very efficiently the transport of an uncharged molecule through the membrane while a charged one is transferred across the membrane to the permeate stream (8). In this arrangement, the electrophoretic movement of a charged particle against a moving frame of reference (forced convection through the porous membrane) results in a separation according to the charge and viscous drag. While similar concepts have been used in biochemistry for a long time for the separation and molecular weight determination of proteins by gel electrophoresis, CCE provides a continuous electrophoresis.

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In Ref. 8 we showed that polyelectrolytes, like proteins, can be separated by using CCE provided the isoelectric points deviate and no special interaction such as complex formation between the molecules to be separated exists. For the present paper we have studied a typical separation problem where the complexation takes place, i.e., the separation of endotoxin from albumin.

Endotoxins are high-molecular-weight lipopolysaccharides associated with the outer membrane of gram-negative bacteria. Because of their fever-producing nature, they are also called pyrogens. Even less than 1 μg/L endotoxin in solutions injected into humans or animals is known to induce temperature elevations (9). Because endotoxins are associated with gram-negative bacteria, they are ubiquitous, and, like bacteria, are found in air, water, and food. Due to their ubiquity, they are the most significant contaminants of pharmaceutical products. Depyrogenation can be

achieved in two ways: by inactivation or by removal of endotoxin. Inactivation can be accomplished by detoxifying the lipopolysaccharide molecule using various chemical treatments. However, these methods are not suitable for materials which cannot tolerate the conditions required by the method. That is why depyrogenation of, for instance, protein solutions is always carried out by removal of endotoxins.

Endotoxin removal can be achieved by a variety of methods, based either on the physical characteristics of the endotoxin, such as size, molecular weight, and electrostatic charge, or on the affinity of endotoxin to bind to various substances. Such methods are gel chromatography (10), filtration (11), affinity chromatography (12), adsorption (13, 14), phase separation with detergents (15), and isoelectric focusing (16, 17). A good separation method should fulfill the following demands: the process is continuous, the protein loss is not too high, the required endotoxin limit is reached, and the separation technique itself should not introduce to the protein solution unwanted substances (e.g., detergents, erosion products, etc.) difficult to eliminate. Naturally, the cost of the separation must be in line with the price of the product. None of the methods quoted meets these demands.

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When applying CCE to the case where complexation evidently takes place, the following procedure was followed: First, to find the most favorable conditions for separation, the process was carried out with the pure components; second, the process was realized using the above-obtained running parameters for the mixture of the components to be separated in order to see the effect of complexation; third, the effect of complex-decomposing agents, such as detergents, was studied; and finally, the possibility of transferring the product instead of the impurity through the membrane was considered, even though this alternative is the most expensive one.

Furthermore, due to the many adjustable parameters involved in CCE, mathematical modeling is of great importance because the experimental parameters have to be set according to the simulations of the process to get reasonable results. Therefore, the diffusion coefficient and effective charge number of the molecules to be separated have to be known. Since there are no reliable data available about the diffusion coefficients of the endotoxin molecule (or the state of its aggregation) or its effective charge number under the conditions used in the separation process, we measured the diffusion coefficient and effective charge number of endotoxin using a method based on convective diffusion which is closely related to the separation method used in this work (18–20).

EXPERIMENTAL

Materials

All the experiments were carried out by using human albumin which was artificially contaminated with endotoxin. A 20% human albumin solution (purity 96.9%), containing 6.65 g/L sodium caprylate as stabilizer, was supplied by the Finnish Red Cross Blood Transfusion Service. Two kinds of endotoxin from *Escherichia coli*, serotype 055:B5, was used: 1) from Sigma (<3% proteins) and 2) endotoxin standard, 2.5 mg/vial (Whittaker Bioproducts, Inc., Walkersville, Maryland, USA) for use in the LAL test.

The base electrolytes and buffers, CH_3COOH, CH_3COONa, and NaCl (Merck, pro analysi), and NaH_2PO_4 and K_2HPO_4 (Baker Analyzed Reagents), were used as received.

All solutions were sterilized using Sartorius Minisart NML disposal filter units (pore size 0.2 μm), and all glassware was sterilized and depyrogenized at 250°C for 3 hours.

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Analysis

Albumin and endotoxin concentrations were determined by UV spectrometry in the case where endotoxin concentration was >10 mg/L. The absorption maximum of albumin is at 280 nm and that of endotoxin at 254 nm, at which wavelength albumin has an absorption minimum. By measuring the absorbances both at 280 and 254 nm, the concentration of albumin and endotoxin could be calculated. Endotoxin concentrations less than 10 mg/L were determined by a manual gel-clot Limulus amebocyte lysate (LAL) test (21, 22) (Whittaker Bioproducts Inc., Walkersville, Maryland, USA), which was standardized using endotoxin standard BRP, Lot. 1, from European Pharmacopoeia Commission (Strasbourg, France).

The concentrations of the base electrolyte were determined by analyzing for the Na^+ ion concentration by AAS to determine the concentration of sodium acetate and by measuring pH to obtain the concentration of acetic acid.

The fraction of dimers and polymers in the albumin sample were determined according to Suomela et al. (23).

Separation Measurements

A schematic drawing of the cell where CCE is carried out is presented in Fig. 1 and the practical realization in Fig. 2. The cell was made of PVC, and the volume of each compartment was 0.8 cm^3. The porous membrane (PM) between the α- and β-compartments was Millipore BS with a 2-μm pore size, a thickness of 0.15 mm, and an exposed surface area of 1 cm^3.

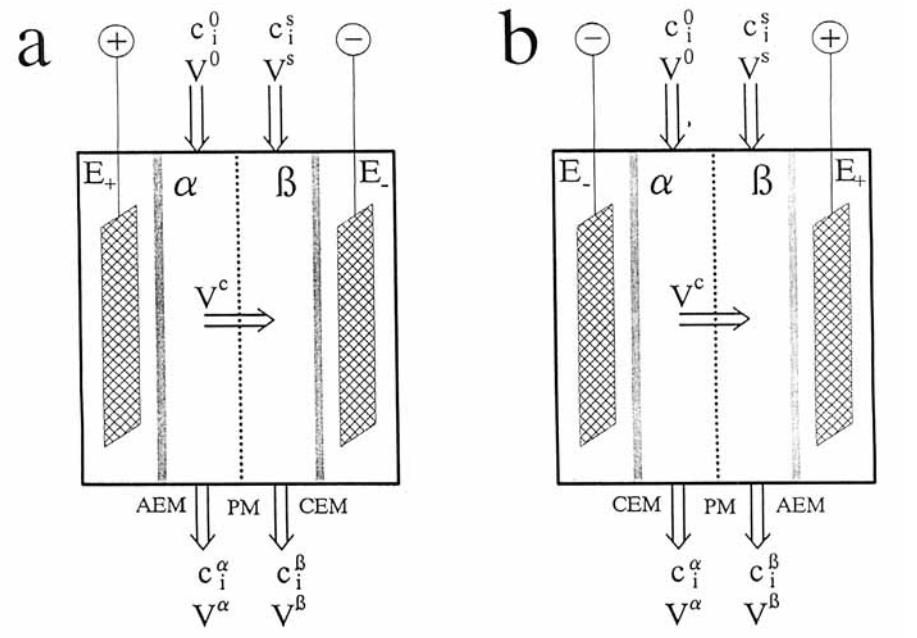

FIG. 1 Schematic drawing of the cell configurations for convective electrophoresis. The cell consists of four compartments (α, β, E_-, and E_+) separated by two ion-exchange membranes (AEM and CEM) and a porous membrane (PM). The feed solution is pumped into the β-compartment at the flow rate V^s. A convective flow rate V^c through the porous membrane is controlled by the adjustment of the flow rates of the stripping stream V^0 and the permeate stream V^α so that $V^c = V^0 - V^\alpha$. Different velocities of proteins and endotoxin in the porous membrane result in separation to permeate and retentate (V^β) streams.

The electrodes were made of platinum, and the ion-exchange membranes were from Ionics, 103PZL389 as an anion-exchange membrane (AEM) and 61AZL389 as a cation-exchange membrane (CEM). Temperature was controlled by mounting the cell in a thermostatic bath.

All separation experiments were carried out at pH 4.6 ± 0.2, where albumin is positively charged and endotoxin is negatively charged. The pH was adjusted with an HAc/NaAc buffer solution. 0.01 M phosphate buffer solution (pH 6.8) was used in the electrode compartments in order to prevent the H^+ and OH^- ions generated in the electrode reactions from entering the α- and β-compartments. Steady-state was considered to be reached when the concentrations of albumin and endotoxin in the output solutions (c_A^α, c_A^β and c_E^α, c_E^β) remained constant; each experiment was carried out for a period of several days, although steady-state was usually reached in a few hours. Great care was taken to ensure the sterility

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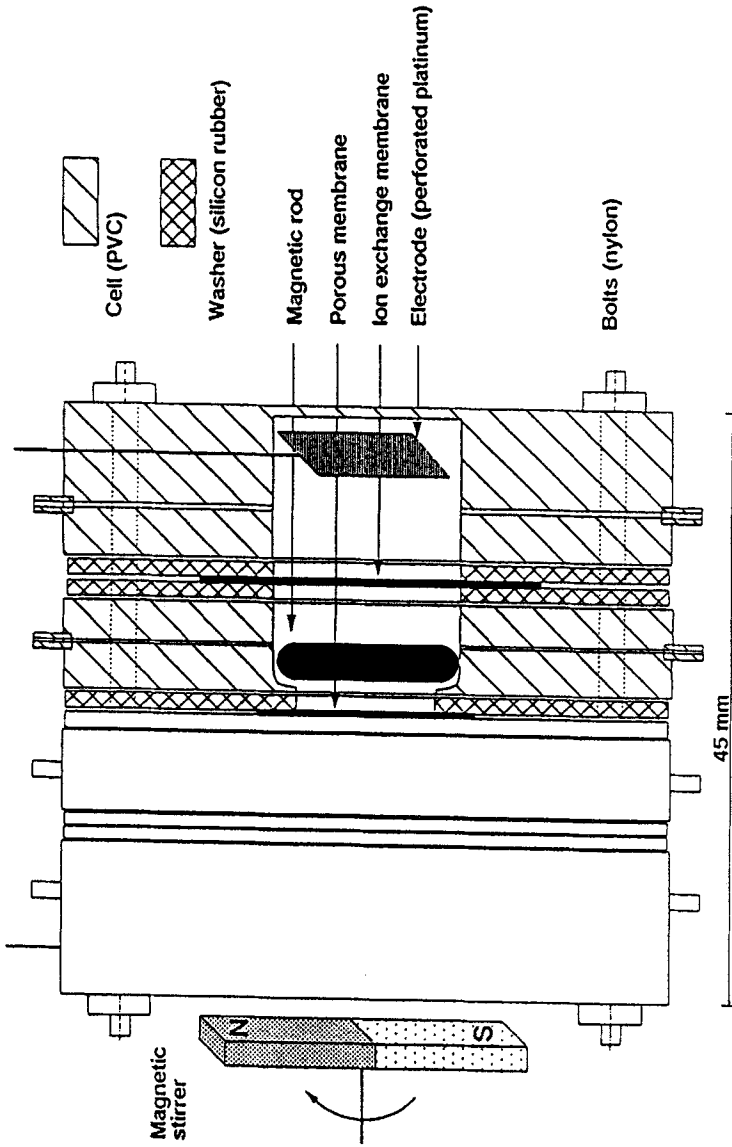

FIG. 2 A side view of the experimental cell used.

of the solutions and the absence of leaks. The cell and the tubes were treated between every measurement by pumping 0.2 M NaOH solution through the cell for 30 minutes.

Flow rates were measured by weighing, and densities were measured with a densitometer (Anton Paar 40). The convective flow was calculated from the material balances and electroosmotic fluxes as described before (8).

In order to have a base electrolyte concentration of 0.15 mol/L in the cell at steady-state, the feed concentrations of the buffer solution (c_B^0 and c_B^s) can be approximately calculated from the mass balance equation of NaAc

$$V^0 c^0 + V^s c^s = V^\alpha c^\alpha + V^\beta c^\beta + (i/F) \tag{1}$$

where i is the electric current and the volume flows V^0, V^s, V^α, and V^β have the meaning indicated in Fig. 1. The pH of the cell was adjusted by trial and error: changing the concentration ratio HAc/NaAc in the feed solutions and measuring the pH of the output solutions. Every measurement was started with pure albumin solution and after steady-state was reached, endotoxin was added to the feed solution, except in the case where endotoxin alone was studied.

The following experimental conditions were employed: V^0 = 1.00–1.42 mL/h, V^s = 0.72–0.76 mL/h, V^α = 0.28–0.73 mL/h, i = 20 mA, $c^0_{NaAc} = c^s_{NaAc}$ = 0.5–0.7 M, $c^\alpha_{NaAc} = c^\beta_{NaAc}$ = 0.15 M, $c^0_A = c^0_E = 0$, c^s_A = 10 g/L, c^s_E = 0.01–900 mg/L, T = 13°C. Subscript A denotes albumin and subscript E denotes endotoxin.

Four sets of experiments were carried out:

1) Experiments with pure albumin and pure endotoxin varying the electric current density. Albumin or endotoxin was fed to the cathodic side of the porous membrane (= β-compartment in Fig. 1a).

2) The albumin–endotoxin solution was fed to the cathodic side of the porous membrane (= β-compartment in Fig. 1a) which means that endotoxin should be transferred through the membrane toward the anode and albumin should remain in the β-compartment. The concentration of endotoxin was varied in the feed solution while the concentration of albumin as well as the flow rates and electric current were kept constant.

3) The same experiments as in 2) but adding selected complex-decomposing materials (urea and Triton X100) to the feed solutions.

4) The albumin–endotoxin solution was fed to the anodic side of the porous membrane (= β-compartment in Fig. 1b), which means that albumin should be transferred through the membrane toward the cathode, and endotoxin in complex form should stay in the β-compartment. The

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concentration of albumin in the feed solution and the electric current were kept constant.

Measurement of the Diffusion Coefficient and the Effective Charge Number

The diffusion coefficient and the effective charge number of endotoxin were measured using a method based on a convective diffusion process across a porous membrane as described before (18–20). The following experimental conditions were employed: $V^s = 0.30$ mL/h, $V^0 = 0.39$ mL/h, $V^\alpha = 0.31$ mL/h, $V^c = 0.08$ mL/h, $c^0_{NaCl} = 0.15$ M, $c^s_{NaCl} = 0.15$ M, $c^0_E = 0$, $c^s_E = 200$ mg/L, pH = 4.60 (10^{-3} M HAc/NaAc buffer), $T = 13$°C. The flow rates (V^0, V^s, V^α) were chosen so that the output concentration of endotoxin in the steady-state (c^α_E) was not less than 25 mg/L in order to have proper analysis accuracy. The pH (4.6) was chosen because the pI value of human albumin is 4.9, and the most favorable condition for the separation is that the ions to be separated have opposite signs. Albumin is positively charged when pH < 4.9, and endotoxin is then negatively charged.

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RESULTS AND DISCUSSION

The Diffusion Coefficient and the Effective Charge Number of Endotoxin

For the diffusion coefficient of endotoxin in 0.15 M NaCl solution at pH 4.6 and temperature 13°C, we got a value of 2.5×10^{-6} cm^2/s, which is a surprisingly high value. This means that endotoxin molecules are in the above-mentioned conditions, completely disaggregated into small monomeric units. Using Stokes' law, the molecular weight of endotoxin can be estimated to be 10,000, which is considerably less than that of albumin (67,000).

The effective charge number of endotoxin under the above-mentioned conditions was found to be -2. By the effective charge number we mean the net charge of the polyelectrolyte subtracted from the charge of the double layer which moves with the macromolecule (so-called ion binding). Hydrogen ion titration of endotoxin, the molecular weight of which is 134,000, gives the following values for charge numbers and intrinsic dissociation constants K_{int} (24): 130 carboxyl groups with $pK_{int} = 2.5$, 89 amino groups with $pK_{int} = 6.5$, and 89 phosphate groups with $pK_{int} = 1.3$. Thus, at pH 4.6 a molecule of molecular weight 10,000 should have a net charge (or stoichiometric charge) of about -9. The degree of ion binding of linear coiled macromolecules is usually about 70%, which means that

the effective charge number is about 30% of the stoichiometric charge number (25). Thus, the charge number -2 can be regarded as a reasonable value.

Separation Measurements

The separation factor G_i, which describes the separation efficiency, is defined as

$$G_i = \frac{c_i^{\alpha} V^{\alpha}}{c_i^{\alpha} V^{\alpha} + c_i^{\beta} V^{\beta}} = \frac{c_i^{\alpha} V^{\alpha}}{c_i^{s} V^{s}} \tag{2}$$

where G_i assumes values from zero to unity. From the separation factors it is possible to calculate the separation ratio as

$$s = G_2/G_1 \tag{3}$$

where the species number 2 is the one which should transfer through the membrane and species number 1 is the one which should remain in the β-compartment ($s > 1$). Thus, the larger the value of s, the more efficient is the separation process.

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Experiments with Pure Albumin and Pure Endotoxin

In order to find the most favorable conditions for separation (i.e., to get high G for endotoxin and low G for albumin), the experiments were first carried out separately for pure albumin and pure endotoxin. The results of these experiments where only one component is fed into the cell (schematically represented in Fig. 1a) are shown in Fig. 3. It can be seen that an electric current value of 20 mA gives a large separation factor (80%) for endotoxin while the separation factor of albumin is only 6%. This means that if we use 20 mA for the electric current value, and that albumin and endotoxin have no complex formation, about a 6% albumin loss should occur while 80% of the endotoxin is removed. This result encouraged us to perform subsequent experiments with 20 mA.

Contaminated Albumin Feed without Additives into the Cathodic Compartment of the Cell

This series of experiments was carried out while keeping the feed concentration of albumin as well as the flow rates and electric current constant and changing the concentration of endotoxin in the feed. In this situation the negatively charged endotoxin should transfer through the membrane to the α-compartment while the positively charged albumin should stay in the β-compartment.

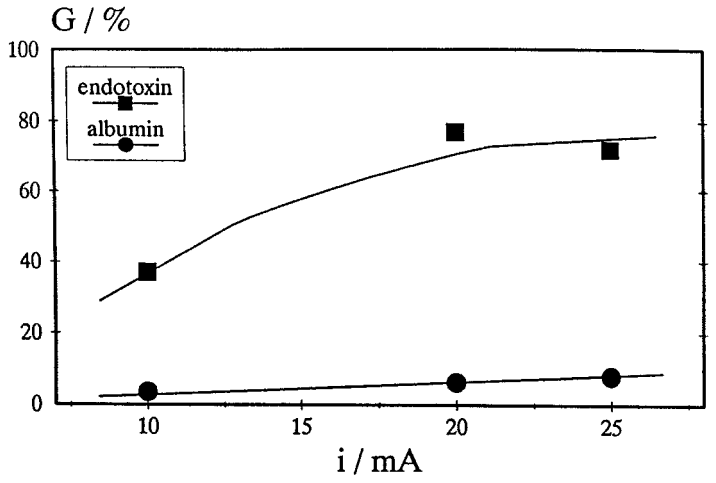

FIG. 3 The separation factors for pure albumin and pure endotoxin as a function of electric current when measured in the cell in Fig. 1(a).

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The results are presented in Table 1. For the sake of comparison the result of the experiment with pure endotoxin with an electric current of 20 mA is included (Experiment 1). It can be seen that the presence of albumin considerably decreases the separation factor of endotoxin. Thus, it is evident that endotoxin is at least partly bound to albumin, and that

TABLE 1
The Feed Concentration c_E^s, the Output Concentration c_E^a, and the Separation Factor G_E of Endotoxin in Experiments Where Contaminated Albumin Was Fed into the Cathodic Compartment of the Cell. The Feed Concentration and the Separation Factor of Albumin (c_A^s and G_A) Were 10 g/L and 11%, Respectively, Except Experiment 1 Where No Albumin Was Used

Experiment	c_E^s (mg/L)	c_E^a (mg/L)	G_E (%)	s
1	915	572	63	—
2	229	57	22	1.8
3	104	13	13	0.9
4	14	0.7	5	0.45
5	1.5	0.09	6	0.56
6	0.2	$<10^{-3}$	<0.1	<0.01
7	0.01	$<10^{-3}$	<0.1	<0.01

this albumin–endotoxin complex is not transferred through the membrane as easily as free endotoxin.

It is also evident from Table 1 that a decrease of endotoxin feed concentration reduces the separation factor of endotoxin as well as the selectivity of the separation, indicating strong complexation. When the feed concentration of endotoxin is <0.2 mg/L (i.e., the feed ratio $c_A^s/c_E^s > 5 \times 10^4$), the separation factor and the selectivity of the separation are both extremely low (Experiments 6 and 7). In these experiments the endotoxin level of albumin in the α-compartment is less than 1 μg/L, which means that practically all the endotoxin is bound to albumin. Accordingly, it seems obvious that the complex formed is negatively charged.

Complex-Decomposing Additives

Triton (1%) and urea in various concentrations were used in order to facilitate the dissociation of albumin-bound endotoxin. The results, which are presented in Table 2, clearly show that the use of urea does not increase the separation factor of endotoxin. In fact, the selectivity of the separation is increased but only because the separation factor of albumin decreases with urea. This is due to the fact that the pI value of albumin is higher in concentrated urea solutions (26), and thus it is more positively charged at pH 4.6. Therefore, it is evident that urea does not significantly facilitate the dissociation of the albumin–endotoxin complex.

The use of 1% triton increases the separation factor of endotoxin from 22 to 44% while the separation factor of albumin is not affected. That is why the selectivity of the separation increases from 1.8 to 4.3. However, the influence of triton on separation is not enough to compensate for the disadvantage of the formation of additives.

TABLE 2
The Feed Concentrations c^s, Output Concentrations c^α, and Separation Factors G of Endotoxin and Albumin in Experiments Where Contaminated Albumin Together with a Complex-Decomposing Additive Was Fed into the Cathodic Compartment of the Cell

Additive	c_E^s (mg/L)	c_E^α (mg/L)	c_A^s (g/L)	c_A^α (g/L)	G_E (%)	G_A (%)	s
No additive	229	57	9.9	1.4	22	12.5	1.8
2 M Urea	200	23	10.0	0.2	9.3	1.4	6.6
4 M Urea	215	21	9.6	0.1	8.8	0.9	9.8
6 M Urea	193	17	9.9	0.1	8.7	0.9	9.7
1% Triton	175	81	10.2	1.1	44	10.1	4.3

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Contaminated Albumin Fed into the Anodic Compartment of the Cell

When the contaminated albumin was fed to the cathodic side of the cell, it decreased the endotoxin/albumin ratio and hence markedly decreased the separation factor of endotoxin. Because endotoxin did not transfer through the membrane, we decided to carry out the experiment inversely by forcing albumin to transfer through the membrane while endotoxin remains in the β-compartment. The cell arrangement for this procedure is according to Fig. 1(b). The albumin concentration was kept constant in the feed, and the flow rates were adjusted so that the separation factor of albumin varied from 0.1 to 60%.

The results in Table 3 show that albumin which has transferred through the membrane is practically free of endotoxin. The endotoxin concentration does not rise above 1 μg/L until the separation factor of albumin is about 60%. Thus it is quite evident that the albumin–endotoxin complex must be negatively charged (AE_n), and therefore it is transferred through the membrane only when the convective flow is low enough. Thus, it is possible to "prepare" practically endotoxin-free albumin with at least 40% yield using continuous convective electrophoresis.

In order to check the quality of the purified albumin, the fraction of monomers, dimers, and polymers was determined for albumin purified in two different experiments. The results are shown in Table 4. As can be seen, the amount of dimers and polymers is higher at pH 4.4 than at pH 4.6. Therefore, it is necessary to carry out the separation process within the pH limit: $4.6 < \text{pH} < 4.8$, when albumin still is positively charged and aggregate formation is minimized.

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TABLE 3
The Output Concentrations c^a and the Separation Factors G of Endotoxin and Albumin in Experiments Where Contaminated Albumin Was Fed into the Anodic Compartment of the Cell. The Feed Concentrations of Endotoxin and Albumin Were $c_E^s = 1.5$ mg/L and $c_A^s = 10$ g/L in Each Experiment

Experiment	c_E^a (mg/L)	G_E (%)	c_A^a (mg/L)	G_A (%)	s
1	$<10^{-3}$	<0.07	0.01	0.1	>1.5
2	$<10^{-3}$	<0.07	0.2	2	>30
3	$<10^{-3}$	<0.07	1.1	8.4	>120
4	$<10^{-3}$	<0.07	4.5	39.5	>560
5	0.17	11	7.9	60.0	5.5

TABLE 4
The Fraction of Monomers, Dimers, and Polymers in the Albumin Samples from Two Experiments with Different pHs

pH	4.4	4.6
Monomers (%)	66.5	90.5
Dimers (%)	29.0	7.0
Polymers (%)	5.0	3.0

CONCLUSIONS

It has been experimentally verified that with CCE at least 40% of endotoxin-contaminated albumin can be recovered in a one-step process. No doubt a multistage process would increase the efficiency of the method even better. The attractive features of the method are its continuous character and the fact that there is no need to use additives, such as detergents or amphophilic buffers. Furthermore, the running cost of this technique is low compared with the price of biotechnical products, and the process can be mathematically modeled with good accuracy. Also, the scale-up of CCE can be realized analogously to existing membrane processes such as electrodialysis.

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