A Gel-Chromatographic and Light Scattering Study of the *Salmonella typhi* Endotoxin

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The endotoxin of *Salmonella typhi,* strain O-901, was isolated by extraction with hypertonic (1 M) sodium chloride solutions and studied by gel-chromatography and light scattering methods. The gel-chromatographic separation was performed on Sepharose 2B, Sepharose 4B and Sephadex G-200 gels, and the fractionated material was monitored by ultraviolet and phenol-sulfuric acid colorimetry as well as by a photometric latex agglutination test. The extracted material consisted of two components: one was the high molecular weight endotoxin and the other a protein-polysaccharide complex of a molecular weight lower than 66,000 dalton. The light scattering experiments of endotoxin extracts showed the average molecular weights from 1.9 to 4.9 million dalton. The separation of the low molecular weight proteinic component was attempted by thermal denaturation, but this had to be abandoned owing to the denaturation and degradation of endotoxin. A high molecular weight endotoxin component was isolated by elution on a Sephadex G-200 column and had the molecular weight of 5.6 million dalton, which was in good accord with the value previously determined for a Boivin extraction sample. The high molecular weight endotoxin sample proved to be a highly polydispersed material. From the estimates of various averages of gyration radii it has been concluded that the particles of this sample have a more compact structure than those of the Boivin extraction sample, possibly due to the tertiary structuring of polypeptide chains in the protein-lipopolysaccharide complex of the endotoxin particle.

INTRODUCTION

Although a vastly growing literature on bacterial endotoxins in the last decades has rendered much information on the chemical and biological properties of these complex substances, our knowledge of their physical properties has remained insufficient. In a most comprehensive treatise[1] a rather short chapter[2] is devoted to the physical characteristics of bacterial lipopolysaccharides as revealed by the method of electron microscopy and ultracentrifugation. As seen from this treatise and from other publications, the standard methods for studying the size and shape of macromolecules in solution, such as light scattering and viscometry, have been adopted only occasionally. Several years ago some of us[3] reported on light scattering and viscometric experiments per-

formed with *Salmonella typhi* (strain O-901) endotoxin samples isolated by the Boivin trichloroacetic acid method. Tripodi and Nowotny undertook[4] a light scattering study of endotoxin samples of *Serratia marcescens* prepared by the same method. More recently Cross and Milner[5] reported on light scattering, osmometric and viscometric experiments on a lipopolysaccharide extracted from *Salmonella enteritidis* with the aqueous ether method. Since these data could not give much insight into the size and shape of molecular species in endotoxin samples, we decided to undertake the present study as a continuation of our previous efforts to elucidate the physical properties of endotoxin in solution.

An endotoxin material exhibits its biological properties when embedded in an aqueous medium, regardless of whether it is situated on the bacterial surface or moves freely through the medium. If we want to correlate physical parameters with biological activity, we should study endotoxin particles in their hydrated state. The easiest way to achieve this is to study endotoxin solutions. Therefore, the results of the structural studies of endotoxin materials obtained by methods in which dry molecules are observed (*e. g.* electron microscopy) should be accepted with reserve. The removal of water from a hydrophilic material may change its internal structure and lead to a sort of denaturation as encountered with protein molecules. Our objective was to determine by light scattering the size of molecular species in endotoxin samples prepared by methods which mostly preserve their native structure and to collect information on other properties which might give an insight into their structure.

MATERIALS AND METHODS

Preparation of endotoxin and endotoxin solutions

S. typhi (strain O-901) bacterial cells, grown by the methods of both stationary and submerged cultivation, were separated from the culture medium by centrifugation. After rinsing with physiological saline and subsequent centrifuging the cells were extracted with 1 M solution of sodium chloride at room temperature for 30 minutes. After centrifuging, the extraction with 1 M solution chloride, followed by centrifugation, was repeated. Both supernatants were combined and the dissolved substances were precipitated twice with 70% ethanol.

It has been found that drying of endotoxin samples (*e. g.* by freeze-drying) produces substances which are hard to redissolve to homogeneous solutions. The dry endotoxin powder resuspends in the aqueous medium in the form of a heavily aggregated material and cannot be redissolved again in satisfactory yields. Even wet ethanol precipitates were redispersed with difficulty. The following procedure was therefore adopted: a dense paste of a wet precipitate was suspended in a small volume of 3—5 M sodium chloride, and after keeping it for several hours at + 4 °C, the suspension was dialyzed against 0.15 or 0.1 M sodium chloride at the same temperature until the equilibrium was reached. Such equilibrated solutions were used in all experiments.

Determination of endotoxin concentration

The concentration of endotoxin in solution was determined gravimetrically and expressed in grams of solute per cm^3 or gram of solution. The solvent was eliminated either by freeze-drying or by evaporation over a water bath. The residual water was eliminated by drying *in vacuo* over P_2O_5 at room temperature for 24 hours. When working with solutions containing electrolytes, the gravimetric determination was performed with the equilibrated solvent, and the concentration of macromolecular material was determined from the difference of the total dry matter in the solution and the dry matter in the solvent.

Chemical analyses

Total hexoses were determined colorimetrically by the anthrone method according to Roe[6], with glucose taken as the standard.

Rhamnose was determined by the colorimetric method of Dische and Shettles[7], and L-(—)-rhamnose was the standard.

Proteins were determined by means of the biuret reaction according to the usual procedure[8]. A crystalline sample of human serum albumin was used as the standard.

Lipid determination was made by the method of Duron and Nowotny[9] consisting of the methylation of carboxylic acids by boron trifluoride and of the colorimetric determination of methylated lipids by the method of Snyder and Stephens[10]. Palmitic acid was used as the standard.

In gel-chromatographic experiments the saccharide component was detected colorimetrically by the phenol-sulfuric acid method[11].

Detection of immunochemical activity

The immunochemical activity of endotoxin solutions was detected by a photometric latex test[12] specially developed for gel-chromatographic experiments. A suspension of monodisperse polystyrene latex particles of about 400 nm in diameter (latex sample LS-97T prepared in our laboratory as previously described[13] and extensively dialyzed against distilled water) was added to the solution together with certain amounts of human serum albumin to stabilize the latex particles and sodium azide to prevent microbial growth in the suspensions. After the macromolecular component from the solution was adsorbed on the latex surface, a diluted hyperimmune rabbit serum (obtained by immunization of rabbits with whole *S. typhi* O-901 bacteria) was added. The mixture was allowed to stand overnight at room temperature and the turbidity of the suspension was determined photometrically by the transmission method at the 436 nm light wavelength. The details of the procedure are described elsewhere[14]. A constant high turbidity value showed the absence of immunochemical interaction, whereas a decrease in turbidity, resulting from the sedimentation of agglutinated latex particles, indicated such an interaction. The final concentrations of ingredients were as follows: latex particles 80 μg/cm³, albumin 4 μg/cm³, hyperimmune serum dilution 1 : 200, and sodium azide 1 mg/cm³. The ionic strength of the suspensions was about 0.05 mol/l, and TRIS buffer, pH = 8.0, was used to dilute suspensions, solutions and the serum.

Gel chromatography

Fractionation by gel chromatography was carried out on Sepharose gels 2B and 4B and Sephadex gel G-200 with the equipment manufactured by LKB, Stockholm, Sweden, including the »Uvicord« attachment for monitoring uv-absorbing substances. The eluant was a 0.15 M sodium chloride solution containing 0.1% sodium azide as a preservative.

Optical rotation

Optical rotation was measured by using a Keston Standard Model D polarimetric attachment in combination with a Model DU Beckman spectrophotometer. The calibration of this device was performed with standard sucrose solutions at wavelengths of 589, 546 and 436 nm at room temperature.

Refractometry

Refractive index increments were measured by using a Rayleigh-Haber-Löwe interferometer manufactured by Zeiss, Jena, Germany, in cells with an optical path length of 2 cm at constant temperature regulated to ± 0.02⁰. The measurement was performed as described in detail previously[15], at the 546 nm wavelength and at a temperature close to the room temperature at which the light scattering experiments were performed (about 23 ⁰C).

Light scattering

The light scattering experiments were performed by using Oster-Aminco and Brice-Phoenix Dual light scattering photometers. The measurements with the Oster--Aminco instrument were performed by the standard scatterer method[16] as described earlier[17]. The photometer calibration was performed with benzene by taking the Rayleigh ratio value[18] at 546 nm and 25 °C as R_u (90) $= 16.1 \times 10^{-6}$ cm^{-1}. Zimm type conical cells immersed into the water contained in an outer cylindrical cell were used. The outer back side of the cylindrical cell was painted black. A reflexion correction[19] was applied. The measurements with the Brice-Phoenix instrument were performed both by the working standard method with standard Phoenix light scattering cylindrical cells and by the standard scatterer method with Zimm cells. For other details in the photometer set-up see a previous paper[20]. The clarification of solutions from dust and aggregated material was performed by centrifuging at 24,000 RCF for 2 hours. All glassware was cleaned from dust by routine procedures.

<div align="center">RESULTS</div>

Our previous results of the gel-chromatographic separation of endotoxin samples, isolated by the rinsing of bacteria with electrolyte solutions of high ionic strength, showed a typical appearance of two components[21]. Figure 1 shows such fractionation curves for an endotoxin sample (sample II) obtained on a Sepharose 4B column. Two pairs of curves correspond to ultraviolet (uv) and phenol-sulfuric acid colorimetry (PSA), and each pair consists of the curves obtained with the material after the first and second rinsing respectively. The first component was eluted almost completely with the void volume and showed almost no absorption in the uv spectral region. From the colorimetric detection with the phenol-sulfuric method from the analytical data and from uv spectra (being of the same appearance as the spectra shown in Ref. 3, Figure 1) this component proved to be a practically pure endotoxin without nucleic acids impurities. It was of a high molecular weight, since Sepharose 4B
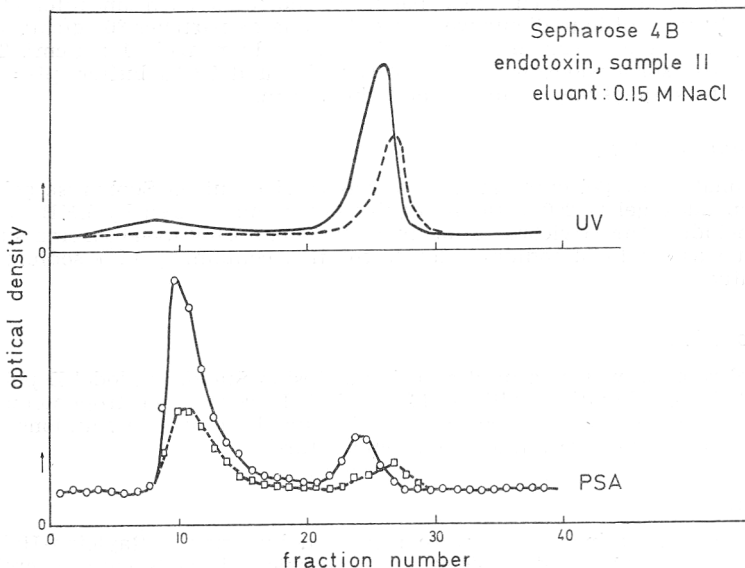

Figure 1. Fractionation of an endotoxin sample, sample II, on Sepharose 4B gel with **0.15 M** sodium chloride as eluant. The curves correspond to samples obtained after two subsequent extractions of the endotoxin with a 1 M sodium chloride solution: first extraction ○———○, second extraction □----□

separated it only partially (according to the manufacturer's claim Sepharose 4B can separate polysaccharides with molecules up to 5 million dalton in size — this component should have the molecular weight around this maximal value). The second component, having a lower molecular weight, was recognized as a protein-polysaccharide complex of a predominantly proteinic nature. Immuno-chemical activity was observed only in the first component, while the second was not active.

The elimination of the proteinic component was attempted at first by thermal denaturation. After heating in a water bath for 1 hour the solution, being at the beginning faintly opalescent, became more turbid. After centrifugation the solution was again eluted on the Sepharose 4B column. The curves in Figure 2 (the relative scale of optical densities is the same as in Figure 1) show that the proteinic maximum was greatly reduced, but the first endotoxinic maximum shifted somewhat to lower molecular weights suggesting a partial degradation of endotoxin molecules. The third curve (LA) indicates the latex agglutination with hyperimmune rabbit serum. Except for the remaining maximal agglutination at fraction 9 (minimum of turbidity on the agglutination curve), which corresponds to the maxima on fractionation curves of the native sample in Figure 1 (including the LA curve left unpresented), no immunochemical interaction was noted. Thus the thermal denaturation of endotoxin is followed by a decrease in the immunochemical activity suggesting a denaturation of the native structure. For this reason and the points discussed later in detail, thermal denaturation could not be adopted as a suitable method for the elimination of the proteinic component from endotoxin.

An estimation of the molecular weight of the proteinic component was obtained by separating a mixture of endotoxin extract and human serum
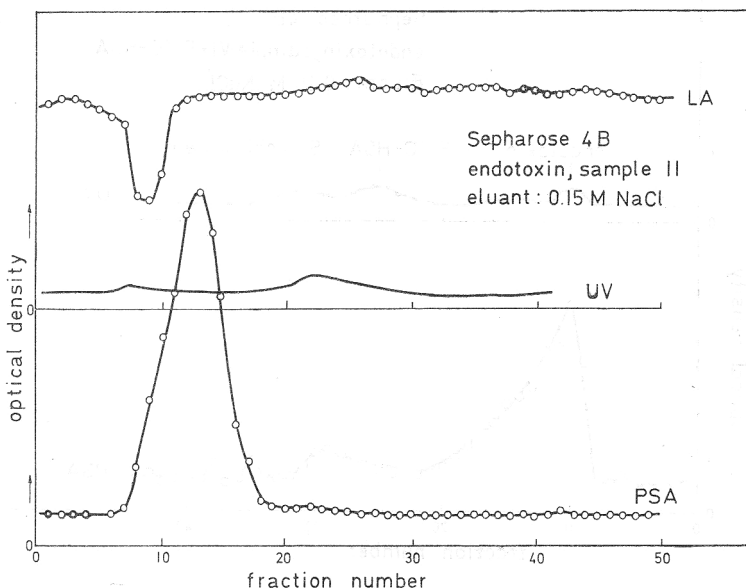

Figure 2. Fractionation of an endotoxin sample, sample II, heated for 1 hour at 100° C on Sepharose 4B gel with 0.15 M sodium chloride as eluant

albumin labelled with fluorescein-isothiocyanate (FITC-HSA). From the uv curve in Figure 3 and by visual examination of fluorescence the S. typhi protein has a molecular weight lower than 66,000 dalton. The PSA curve shows the usual picture with two maxima (the small maxima at fractions 35 and 38 could be explained by the presence of occasional impurities).

By fractionation of the endotoxin on Sepharose 2B gel (capable of separating polysaccharides up to 20 million dalton in molecular weight) a good separation with two maxima was achieved (Figure 4). The endotoxinic maximum appeared to be very broad, indicating a high polydispersity of the endotoxin component.

Finally, for the separation of endotoxin from its proteinic contamination the separation on a Sephadex G-200 column was chosen, as this gel separates only globular protein molecules below 800,000 dalton and polysaccharides below 200,000 dalton. The curves in Figure 4 again show two maxima, but the endotoxic material, characterized by the analytical data (Table II) and uv--spectra, was collected in fractions 12—18 corresponding to the void volume and it was sharply separated from proteinic contamination. Thus, separation on Sephadex G-200 is a successful method for the preparation of native endo-toxin.

Light scattering experiments were performed in equilibrated endotoxin solutions containing the proteinic component, in thermally denatured samples, and in the endotoxin preparation collected from the Sephadex G-200 column. A summary of data is shown in Table I. As noted in connection with the fractionation experiments, it is obvious that thermal denaturation should have led to a lowering of the weight average molecular weight. The exceptionally high value found for sample III could be ascribed to the presence of DNA,
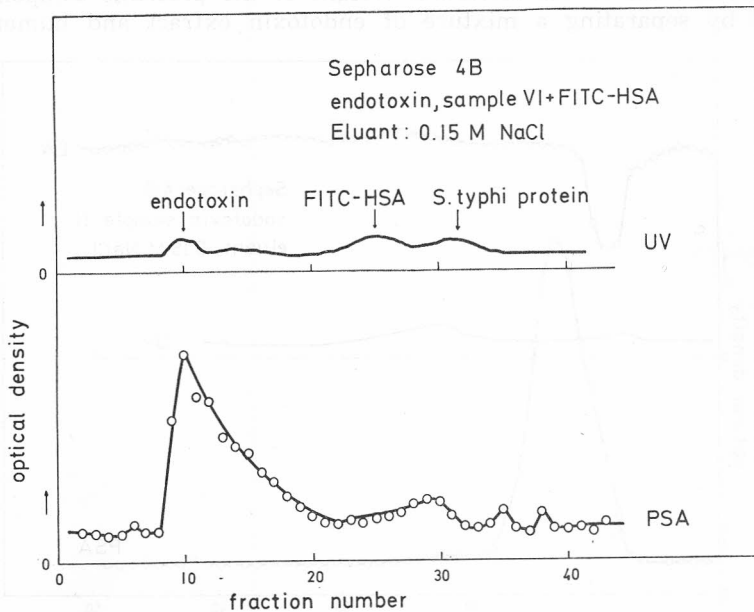

Figure 3. Fractionation of a mixture of an endotoxin sample, sample VI, and human serum albumin labelled with fluorescein -isothiocyanate (FITC-HSA) on Sepharose 4B gel with 0.15 M sodium chloride as eluant
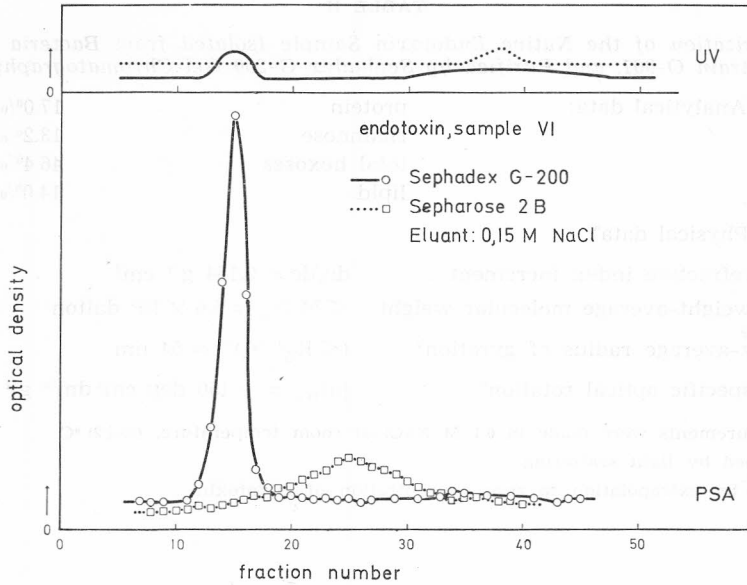
Figure 4. Fractionation of an endotoxin sample, sample VI, on Sepharose 2B and Sephadex G-200 gels with 0.15 M sodium chloride as eluant

TABLE I

Weight-Average Molecular Weight, $< M >_w$, Refractive Index Increment at 546 nm, dn/dc, and the Rhamnose and Protein Content of Endotoxin Samples Isolated from Bacteria S. typhi, Strain O-901, Measured in the Native (N) and Thermally Denaturated (TD) State; Denaturation was Performed by Heating at 100 °C for 1 or 2 Hours

Sample		Rhamnose[a] (%)	Protein[a] (%)	Refractive index increment dn/dc $(g^{-1} \ cm^3)$	Weight-average molecular weight $< M >_w \times 10^{-6}$ (dalton)
I	(TD, 1 hour at 100 °C)	14.2	17.5	0.146	1.92
II	(N)	—	36	0.154	3.42
II	(TD, 1 hour at 100 °C)	14.7	15.6	0.133	3.44
III	(N)	5.4	16.9	0.142	30.2[b]
III	(TD, 1 hour at 100 °C)	7.4	7.8	0.142	3.86
III	(TD, 2 hours at 100 °C)	7.0	8.0	0.162	3.02
IV	(N)	3.6	13.1	0.172	4.87
IV	(TD, 2 hours at 100 °C)	6.0	9.3	0.158	2.47
V	(N)	—	12.3	0.149	4.15
VI		10.4	31	0.151	2.76

[a] Data from Ref. 21
[b] High value resulting from heavy DNA contamination — see the text

TABLE II

Characterization of the Native Endotoxin Sample Isolated from Bacteria S. typhi, *Strain O-901, and Purified by Sephadex G-200 Gel-Chromatography*

Analytical data:	protein	17.0%
	rhamnose	13.2%
	total hexoses	46.4%
	lipid	14.0%

Physical data[a]:

refractive index increment	$dn/dc = 0.164 \ g^{-1} \ cm^3$
weight-average molecular weight[b]	$<M>_w = 5.6 \times 10^6$ dalton
z-average radius of gyration[b]	$(<R_G^2>_z)^{1/2} = 54$ nm
specific optical rotation[c]	$[\alpha]_{D,o} = + 150 \ deg \ cm^3 \ dm^{-1} \ g^{-1}$

[a] All measurements were made in 0.1 M NaCl at room temperature, (25 ± 2) °C
[b] Determined by light scattering
[c] Obtained by extrapolation to zero concentration of endotoxin
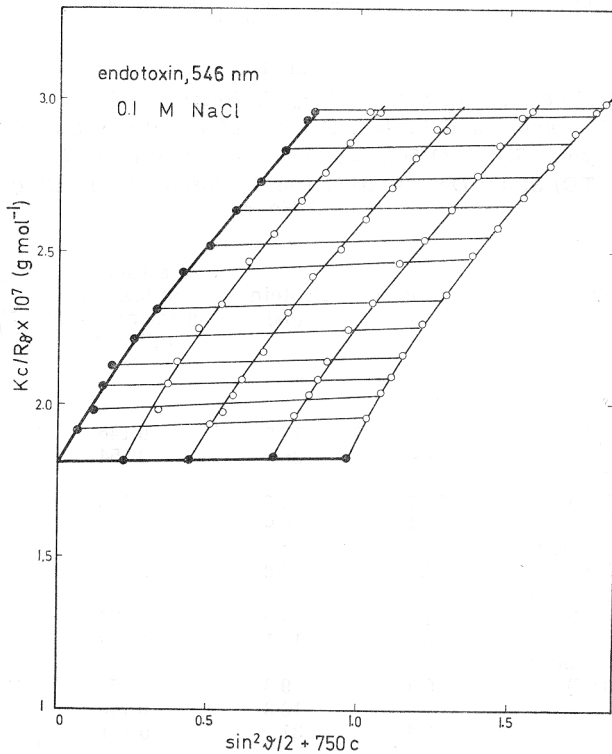

Figure 5. The Zimm plot of an endotoxin sample purified by gel-chromatography measured in 0.1 M sodium chloride solution at the 546 nm wavelength of light

TABLE III

Molecular weight, M, and Gyration Radius, R_G, Averages of the Endotoxin Sample Purified by Gel-Chromatography and of the Boivin Extraction Method Sample; n — Number Average, w — Weight Average, z — z-Average; Measured in 0.1 M NaCl

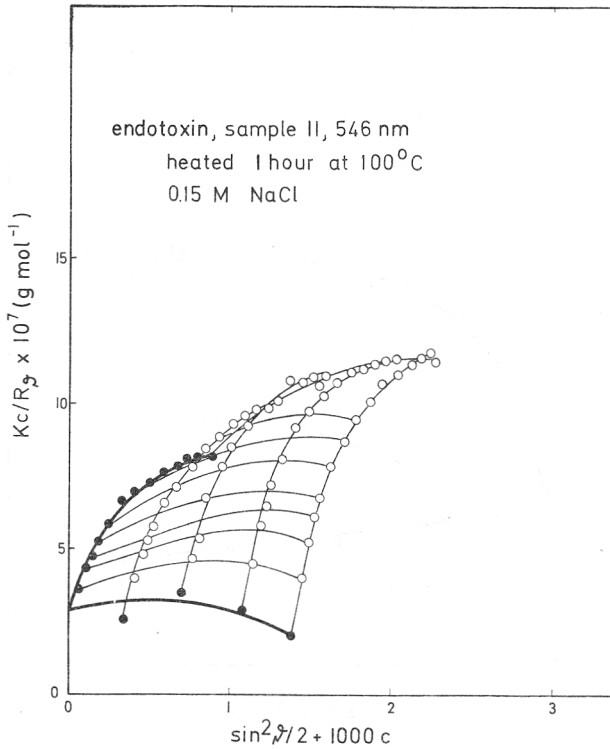| Sample	Molecular weight (dalton) $\times 10^{-6}$			Radius of gyration (nm)		
	z-average $<M>_z$	weight average $<M>_w$	number average $<M>_n$	z-average $(<R_G^2>_z)^{1/2}$	weight average $(<R_G^2>_w)^{1/2}$	number average $(<R_G^2>_n)^{1/2}$
Native endotoxin	19.7	5.6	2.3	54	30	20
Boivin's extraction	14.3	6.2[a]	2.4	83[a]	57	35

[a] Data from Ref. 3

Figure 6. The Zimm plot of an endotoxin sample, sample II, heated for 1 hour at 100 °C, measured in 0.15 M sodium chloride solution at the 546 nm wavelength of light

as proven by the existence of a 260 nm peak in the ultraviolet spectrum otherwise missing in pure endotoxic preparations[3]. This was the result of an unplanned treatment of bacteria with 1 M sodium chloride at 60 °C which caused the rupture of bacterial cell walls and the contamination of endotoxin solution with DNA.

Finally, the endotoxin preparation purified by Sephadex G-200 gel-chromatography was characterized by various analytical and physical methods. A summary of data is given in Table II.

All $<M>_w$ data given in Tables I and II were obtained from Zimm plots. Normal plots with a slight curvature of the line obtained by extrapolation to zero concentration (Figure 5) were found. The thermal denaturation of solutions provoked a distortion of Zimm plots (Figure 6). Changes in both extrapolated $(c \rightarrow 0, \vartheta \rightarrow 0)$ curves are noted (Figure 7) suggesting a lowering of molecular weight and an increase in polydispersity, thus indicating a degradation of endotoxin molecules provoked by heating.

Optical rotation measurements showed an extraordinarily strong sensitivity of the specific optical rotation to changes in concentration. Thus, a value obtained by extrapolation to zero concentration is included in Table II.
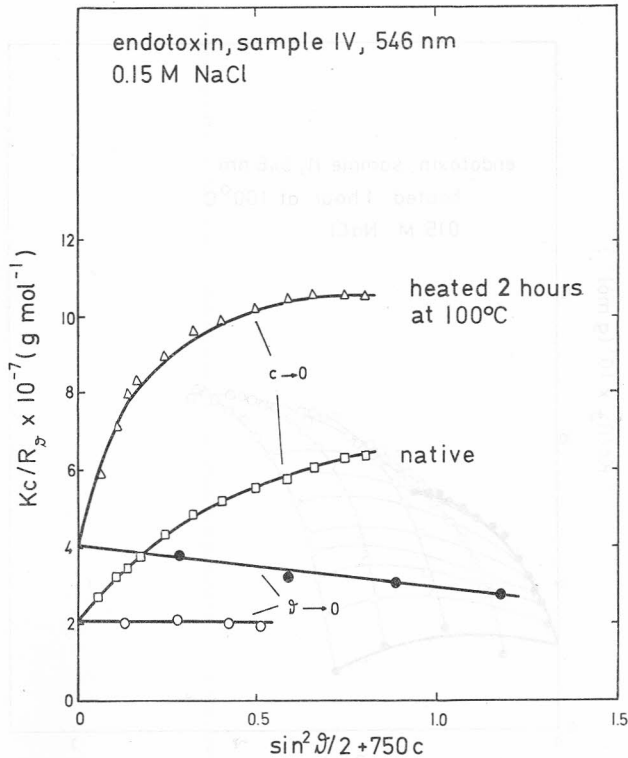

Figure 7. The Zimm extrapolation lines to the zero scattering angle and zero concentration of an endotoxin sample, sample IV, in the native and denaturated (heated 2 hours at 100° C) state, measured in 0.15 M sodium chloride solution at the 546 nm wavelength of light

DISCUSSION

Different methods are employed[22] to extract endotoxic materials from bacterial cell walls. Some of them lead to the isolation of lipopolysaccharide--protein complexes, commonly denoted as endotoxins, or of lipopolysaccharides freed from the protein component. Among the former, the method with trichloroacetic acid (Boivin's method), and among the latter the phenol-water method (Westphal's method) are the best known. Since both methods are capable of disturbing the native structure of polypeptide chains, the phenol--water method even provoking a dissociation of these chains from the endotoxin complex, one has to find a milder method for native endotoxin extraction. Simple rinsing of bacteria with monovalent electrolyte solutions of high ionic strength seemed to be most promising in this respect. The method of extraction with 1 M sodium chloride is very similar to the older method of Raynaud and Digeon[23] (in which the extracting solution consists of 1 M sodium chloride and 0.1 sodium citrate). The material obtained with these methods is very similar in its behavior to the materials isolated by incubation with EDTA[24]. It always consists of a high molecular weight endotoxic component and a lower molecular weight proteinic component common to all enterobacteria[25,26].

In some papers in which the endotoxic material was studied by electron microscopy, its morphological features found to be similar to cell wall cross--sections are described. It is reported on »bimolecular leaflets«[27,28,29] or similar lipide-like structures. Most of the papers, however, are based on the observation of samples prepared by the phenol-water method. Since this method renders a heavily aggregated material, and for the reasons mentioned in the Introduction, one has to be very careful in drawing conclusions about the endotoxin structure from such experiments.

Our results on the molecular weights for several samples are in the order of magnitude of the value obtained with the sample prepared by Boivin's method[3] amounting to 6×10^6 dalton. If corrected for the presence of low molecular weight protein, the results appear to come even nearer to this value.

The slight curvature in the extrapolated Zimm lines obtained by extrapolation to zero concentration suggests polydispersity of samples, as revealed also by the gel-chromatography experiments. The analysis of these lines by the usual methods applied in the light scattering work[30] leads to values for various molecular weight, M, and gyration radius, R_G, averages collected in Table III for the endotoxin studied in this work and compared with the data for the Boivin method sample[3]. For reasons explained by Kratochvíl, the size of molecules still allows estimation of $\langle M \rangle_n$, $\langle M \rangle_z$, $\langle R_G^2 \rangle_w$ and $\langle R_G^2 \rangle_n$, but these values are less reliable than the directly determined $\langle M \rangle_w$ and $\langle R_G^2 \rangle_z$ values obtained by extrapolation to the zero scattering angle. The data suggest, however, that the polydispersity ratio for both samples about $\langle M \rangle_w : \langle M \rangle_n = 2.5$, typical of broad macromolecular distributions. It is interesting to note that the $\langle M \rangle_w$ values for both samples can be regarded as being the same within the limits of the experimental error, whereas the $\langle R_G^2 \rangle_z$ values, proportional for theoretical reasons to $\langle M \rangle_w$, are markedly different, the values for the Boivin extraction sample being more than twice as high as those for the native endotoxin sample (the total experimental error of both parameters, $\langle M \rangle_w$ and $\langle R_G^2 \rangle_z$, can be estimated to be between 10 and 20%). This suggests a more compact structure of the presently measured

sample, probably due to the tertiary structuring of polypeptide chains. The molecular weight of the lipopolysaccharide component in this sample should be lower than that in the Boivin extraction sample (due to a somewhat higher protein content), but possibly no less than some $20^0/_0$ of the value 5.6×10^6 dalton, which is still not enough to meet the marked difference in $<R_G{}^2>_z$ values.

Unfortunately, owing to the high polydispersity and unknown molecular size distributions, an analysis of the particle shape from particle scattering functions $P(\vartheta)$ is not possible. According to one of our previous findings, the viscosity of endotoxin solutions appeared to be very sensitive to changes in the ionic strength of solutions[3]. We concluded at that time that these substances »behave as typical flexible coils«. Now, we have to correct somewhat this view. As endotoxin complexes consist of three main components (polysaccharide, protein, lipid), highly hydrophilic and hydrophobic regions alternate throughout the whole particle. Although there may exist highly ordered structures, as those found in polypeptide chains, and even bimolecular leaflet lipid structures are not excluded, the polysaccharide moiety behaves as a strongly hydrophilic flexible structure. Depending on the method of extraction, it is possible to obtain a particle with a more or less compact structure, the more compact structure being probably nearer to the native state.

In several reports about experiments on lipopolysaccharide solutions by using ultracentrifugation mention has been made of the rod-like shapes of molecules or molecular subunits, as obtained from a combination of sedimentation and viscometric data[21]. A careful examination of the experimental conditions quoted in these papers has revealed that in most cases no allowance was made for the hydration of molecules or particles, which could have significantly influenced the evaluation of sedimentation data. In view of this negligence and the complications arising from the polydispersity of samples, one has to look at such reports with a critical eye, at least so far as the native or nearly native endotoxin particles are concerned.

The results of the present study are in accord with the previous findings[3-5] that endotoxin preparations are highly polydisperse samples with $<M>_w$ ranging from 4×10^6 to 6×10^6 dalton. The data of Cross and Milner[5] about the endotoxin solution in N,N-dimethylformamide are consistent with ours ($<M>_w = 7 \times 10^6$ dalton and $<R_G>_z = 52$ nm) but in water a greater aggregation of the endotoxin has been found ($<M>_w = 56 \times 10^6$ dalton). It is highly probable that endotoxin preparations consist of complex particles in which lipopolysaccharide subunits are held together by weak forces and can be easily dissociated, *e. g.* by surface active agents[32]. Thus, the definition of endotoxin as macromolecular aggregates united into a quaternary structure[33] seems to be the most appropriate to describe its structure. The sensitivity of the endotoxin preparation to aggregation by some chemical extraction methods, to dissociation and to thermal denaturation calls for caution when preparing materials of certain biological and antigenic properties as well as when trying to reach some conclusions on the native structure of these highly complex substances.

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SAŽETAK

Istraživanje endotoksina iz bakterija *Salmonella typhi* **kromatografijom na gelu i rasipanjem svjetlosti**

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Endotoksin iz bakterija *Salmonella typhi*, soj O-901, izoliran je ekstrakcijom hipertoničnom (1 M) otopinom natrijeva klorida i ispitivan metodama kromatografije na gelu i rasipanja svjetlosti. Kromatografsko frakcioniranje izvođeno je na gelovima Sepharose 2B, Sepharose 4B i Sephadex G-200, a detekcija pojedinih komponenata u frakcijama izvođena je pomoću ultraljubičaste apsorbometrije, kolorimetrijske metode s fenol-sumpornom kiselinom i fotometrijskog lateks-aglutinacijskog testa. Ekstrahirana tvar sastojala se iz dvije komponente: jedna je bila endotoksin visoke molekulske mase, a druga proteinsko-polisaharidni kompleks relativne molekulske mase niže od 66 000. Eksperimentima rasipanja svjetlosti na endotoksinskim ekstraktima određene su prosječne molekulske mase od 1.9 do 4.9 milijuna. Pokušaji da se proteinska komponenta manje molekulske mase odijeli od endotoksina termalnom denaturacijom nisu bili uspješni zbog denaturacije i degradacije endotoksina. Endotoksinska komponenta velike molekulske mase izolirana je elucijom na stupcu gela Sephadex G-200 i imala je relativnu molekulsku masu od 5.6 milijuna, što se dobro slagalo s prijašnjim vrijednostima dobivenim na uzorku priređenom ekstrakcijom prema Boivinu. Ta je komponenta bila vrlo polidisperzna tvar. Iz procjene raznih prosjeka polumjera vrtnje zaključeno je da su čestice uzorka opisanog u ovom radu mnogo kompaktnije strukture od čestica uzorka dobivenog Boivinovom ekstrakcijom, što može biti posljedica tercijarnog strukturiranja polipeptidnih lanaca u kompleksu protein-lipopolisaharid čestice endotoksina.

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