Action of Polymyxin B on Bacterial Membranes, I

Binding to the O-Antigenic Lipopolysaccharide of *Salmonella typhimurium*

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Salmonella typhimurium, lipopolysaccharide, cell envelope, polymyxin B, antibiotic

Polymyxin B (PX) forms complexes with the O-antigenic lipopolysaccharide (LPS) of *Salmonella typhimurium* which are stable in aqueous 0.14 M sodium chloride at neutral pH. PX can be quantitatively recovered from the complex by changing the pH from 7 to 1, or by treatment with the cationic detergent cetylpyridinium chloride. This proves that complex formation does not involve covalent linkages, but is due to electrostatic, and possibly hydrophobic interactions. Quantitative investigations of the binding properties of mutant LPS of the chemotype Rc and Re, of degraded polysaccharide and isolated lipid A point to the negatively charged ketodeoxyoctonate-lipid A region of LPS as binding site for PX. These biochemical results are confirmed by a blueshift of the fluorescence emission maximum of LPS-bound mono-*N*-dimethylaminonaphthalenesulfonyl PX from 550 to 515 nm which corresponds to a location of the dansyl-PX at a polar-apolar interface [34]. Using a published molecular weight of the Rc LPS of 10 000 [29], it is calculated that one LPS molecule can bind 2.7 molecules PX. It is discussed that the PX-LPS interaction may be necessary to alter the permeability of the outer membrane which is a prerequisite for the antibiotic to gain access to it's final target, the cytoplasmic membrane.

Introduction

In addition to a breakdown of the permeability barrier of the cytoplasmic membrane [1] the polypeptide antibiotic polymyxin B also induces permeability changes in the outer membrane or plastic layer of Gram-negative bacteria [2–4]. This mechanism of action has been attributed to electrostatic interactions of the cationic antibiotic molecules with anionic phospholipids of the cell envelope [1, 5]. However, extraction of these lipids from isolated envelopes of *Salmonella typhimurium* did not completely abolish the binding capacity for radioactive polymyxin B [6] suggesting additional binding components. A clue to the identity of the latter polymyxin receptors emerged from reports on polymyxin-induced alterations of lipopolysaccharide, *i. e.* alteration of phage receptor properties [7, 8], neutralisation of endotoxin toxicity [9] and Schwartzman reactions [10], inhibition of enterobacterial hemagglutination and hemolysis [11], and elimination of the typical vesicular appearance of isolated LPS under the electron microscope [12].

Chemically defined LPS-polymyxin complexes have not yet been described. We attempted the demonstration of the physical existence of such complexes. Moreover, the binding site of LPS for polymyxin and the mechanism of binding were of interest since the study of these properties could provide a more detailed understanding of the polymyxin action against the surface of susceptible bacteria. A preliminary report has been presented [13].

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Material and Methods

Salmonella typhimurium SL 1135 (smooth wild-type) and SL 1102 (chemotype Re) were provided by R. Schlecht, Max-Planck-Institut für Immunbiologie, Freiburg. *S. typhimurium* G 30 lacking UDP-galactose-4-epimerase (rough mutant of chemotype Rc when grown in the absence of D-galactose) was obtained from M. J. Osborn, University of Connecticut at Farmington.

For the preparation of LPS, cells were grown at 37 °C with rapid shaking into logarithmic phase ($9 \cdot 10^8$ cells/ml) in a glycerol-salts-medium [14] which was supplemented with 0.3% casein hydrolysate and 0.01% L-tryptophane in the case of strain SL 1102. Cells were harvested by centrifugation and washed once with 0.14 M sodium chloride. Cell envelopes were prepared by disintegrating the cell suspension with glass beads in a Vibrogen disintegrator (Bühler, Tübingen) after addition of ribonuclease and deoxyribonuclease (30 μg/ml). LPS was extracted from the washed envelopes as described by Osborn [15]. The G 30 and SL 1102 LPS were accordingly precipitated with magnesium ions while

the SL 1135 – LPS was precipitated with a five-fold volume of cold ethanol. The latter procedure removed contaminating phospholipids. For this purpose, the G 30 LPS had to be extracted with 20 volumes of chloroform-methanol (2 : 1, v : v) [16] whereas the SL 1102 LPS was purified by application of chloroform-methanol (1 : 2, v : v) [17]. The LPS preparations used for the presented experiments were free of nucleic acids and phospholipids as checked by UV-spectroscopy and thinlayer chromatography in solvent system II, respectively.

Lipid A was prepared by hydrolysis of G 30 LPS with acetic acid at pH 3.5 for 1 hour at 100 °C. The precipitated lipid A was collected by centrifugation at 6000 × *g*, washed with distilled water and extracted for 2 hours with a small amount of pyridine at 37 °C. The slightly turbid pyridine layer was mixed with rapid stirring with a tenfold volume of distilled water [18]. The mixture was further homogenised by sonication. To remove the pyridine, the sample was dialised against distilled water for several days at 4 °C. Some precipitated material was removed by centrifugation to yield a clear, colloidal solution of lipid A. Thin-layer chromatography of this fraction with solvent system II showed the typical patterns [19]. Lipid A from the wildtype LPS was obtained following the same procedure with the exception that hydrolysis was performed with 1 N acetic acid for 1 hour at 100 °C. The used lipid A fractions did not contain L-glycero-D-mannoheptose as determined by the cystein-sulfuric acid reaction [20]. The degraded polysaccharide was prepared from the neutralised supernatant of the LPS hydrolysates by dialysis against distilled water.

Polymyxin B was determined by dinitrophenylation using a modified method of Ghuysen [21]: To 50 µl samples containing 5 to 50 µg of polymyxin B were added 0.2 ml of a 1% solution of $Na_2B_4O_7 \cdot 10\,H_2O$ in distilled water and 25 µl of a 100 mM solution of 1-fluoro-2,4-dinitrobenzene in ethanol. The mixture was incubated at 37 °C for 1 hour. After addition of 1 ml of 2 N HCl, the water-insoluble dinitrophenol-polymyxin B was extracted from the turbid fluid with 1 ml of *n*-butyl alcohol. The phases were separated by centrifugation and the *n*-butyl alcohol layer measured at 420 nm in a Zeiss PMQ II spectrometer. 1 µMole polymyxin B gave a OD_{420}^{1cm} 19.0 corresponding to an OD of 3.8 per µ equivalent aminogroup.

Total organic phosphate [22], 2-keto-3-deoxyoctonate [20], and glucosamin [23] were determined as described in the literature. Heptose was measured according to Osborn [20]. The use of synthetic L-glycero-D-mannoheptose [24] as a standard yielded routinely a $\Delta E_{505} - E_{545}$ of 0.7 for 1 µmole of heptose in contrast to the originally published value of 1.0 [20].

Column chromatography was performed with a 29 × 1 cm column of Sephadex G 25 (Pharmacia, Uppsala) at room temperature with distilled water as solvent.

For thinlayer chromatography on cellulose sheets (Merck, Darmstadt) the solvent system I (*n*-butyl alcohol-pyridine-water-acetic acid = 60-40-30-3, v: v: v: v) was used. Chromatography on Kieselgel G plates (Merck) was with solvent system II (chloroform-methanol-water = 65-25-4, v: v: v).

Radioactive and biologically active mono-*N*-acetyl-^{14}C-derivatives of polymyxin B were isolated as previously described [25]. Polymyxin B sulfate (sterile powder) was a generous gift of Pfizer GmbH, Karlsruhe. Mono-*N*-dimethylnaphthalene sulfonyl polymyxin B was prepared by P. Schindler of this laboratory by condensation of polymyxin B with dimethylnaphthalene sulfonylchloride (Serva, Heidelberg) yielding a preparation exerting about 50% of the original antibiotic activity [26].

Radioactivity was determined in toluene based scintillation liquid in a Beckman LS 100 spectrometer. The proton magnetic resonance spectra were recorded at 27 °C in the Varian 100 spectrometer of the Gesellschaft für Strahlen- und Umweltforschung mbH München. The apparatus was locked in at $\delta =$ 4.8 ppm by the addition of H_2O to the D_2O solution of polymyxin B and LPS. The fluorescence emission spectra were recorded with a modified Zeiss fluorescence spectrometer.

Results

Fig. 1 shows a scheme of the covalent structure of the *Salmonella typhimurium* LPS as adopted and modified from Lüderitz *et al.* [27]. This figure serves to demonstrate that the LPS molecule is composed of a polar, but neutral carbohydrate chain (region I) linked to a highly negatively charged site (region II) which in turn is attached to a negatively charged phosphoryl-glucosaminylglucosamin backbone being *O*- and *N*-acylated with saturated fatty acids.

Addition of polymyxin B sulfate to a colloidal solution of purified, phospholipid and nucleic acid free wildtype LPS in distilled water did not result in a visible reaction. However, if the mixture was allowed to pass over a column of Sephadex G 25, the LPS fractions contained elevated amounts of aminogroups as measured by dinitrophenylation (Figs. 2 a and b). Thinlayer chromatography of these fractions

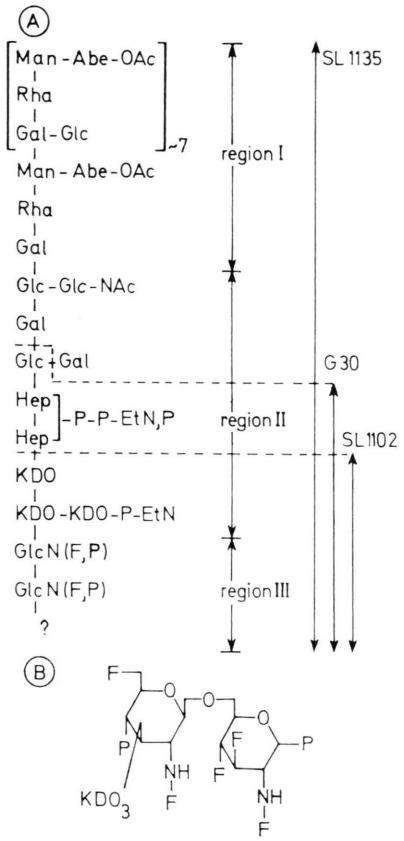

Fig. 1. Scheme of the covalent structure of the *Salmonella typhimurium* lipopolysaccharide according to Lüderitz *et al.* [27]. A: Region I = O-antigen specific chain; region II = basal core polysaccharide; region III = lipid A. The LPS structures of the strains used in this publication are indicated at the right hand: SL 1135 = smooth wildtype; G 30 = rough mutant of chemotype Rc; SL 1102 = rough mutant of chemotype Re. B: Detailed structure of a proposed lipid A unit according to Gmeiner *et al.* [35]. Abe = Abequose; Gal = D-galactose; Glc = glucose; Glc N = glucosamine; Hep = L-glycero-D-mannoheptose; KDO = 2-keto-3-deoxyoctonate; EtN = ethanolamine; F = fatty acid (lauric, palmitic, myristic or β-hydroxymyristic acid); P = esterified phosphate.

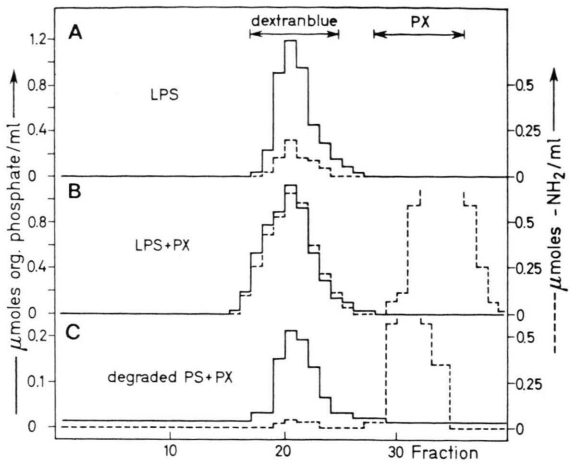

Fig. 2. Demonstration of LPS-polymyxin aggregation by column chromatography on Sephadex G 25 in distilled water. LPS and degraded polysaccharide are expressed as organic phosphate, polymyxin B as $-NH_2$ whereby 1 μmole polymyxin B corresponds to 5 μmoles $-NH_2$. The LPS and degraded polysaccharide were isolated from the smooth wildtype strain SL 1135 of *Salmonella typhimurium*. A. Position of the unmixed components showing that LPS also contained some $-NH_2$. B. Separation of 1 ml of a mixture of LPS (2.1 mg) and polymyxin B sulfate (1.8 mg). C. Separation of 1 ml of a mixture of degraded polysaccharide (0.52 μmoles organic phosphate) and 1.8 mg of polymyxin B sulfate.

with solvent system I on cellulose revealed only one ninhydrin positive spot moving away from the origin where the LPS is located. The R_F value of this spot was 0.26 in accordance with a reference probe of polymyxin B [25]. The capacity of LPS to combine with polymyxin B in aqueous medium with a stability so as to allow the isolation of the complex by column chromatography raised two questions: 1. Which parts of the LPS molecule react with the antibiotic? 2. What is the nature of this interaction?

1. The lipid A as receptor site of LPS for polymyxin B

LPS can be hydrolysed into its carbohydrate chain (= degraded polysaccharide) and it's lipid portion under relatively mild, acid conditions although some further degradations of both residues probably take place [18, 27]. Fig. 2 c demonstrates that the degraded polysaccharide was unable to combine with polymyxin B. Isolated and solubilised lipid A, in contrast, was immediately precipitated together with the antibiotic (Fig. 3). Thinlayer chromato-

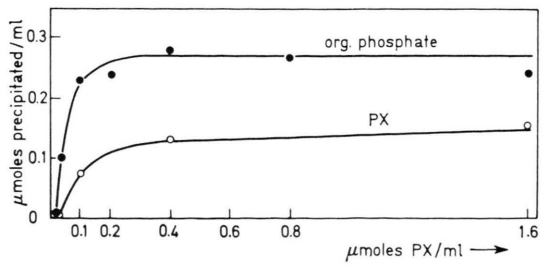

Fig. 3. Polymyxin-dependent coprecipitation of solubilised lipid A (0.26 μmoles organic phosphate/ml) from strain G 30 and polymyxin B in distilled water after 10 min at 37 °C. The precipitate was removed by centrifugation. PX was determined in the supernatant.

Table I. Binding capacities for polymyxin B of different LPS and lipid A fractions from *Salmonella typhyimurium*. The data were obtained for the SL 1135 LPS as described in Fig. 2, for the G 30 and SL 1102 LPS as described in Fig. 4 and for the SL 1135 and G 30 lipid A as described in Fig. 3.

Fraction	Chemotype	Strain	Nature of complex	Chemical characterisation of LPS			Polymyxin-binding capacity	
				phosphate μmoles/mg dry wt.	heptose	glucosamine μmoles/μmole phosphate	μmoles/μmole phosphate	μmoles/mg dry wt
LPS	S	SL 1135	sol.	0.27	0.76	0.54	0.11	0.03
LPS	Rc	G 30	ppt.	1.0	0.97	0.51	0.27	0.27
LPS	Re	SL 1102	ppt.	0.71	0.0	0.68	0.44	0.31
lipid A	—	G 30	ppt.	0.24	0.0	1.43	0.48	0.115 *
lipid A	—	SL 1135	ppt.	n.d.	0.0	n.d.	0.49	n.d.

sol. = soluble; n.d. = not determined; ppt. = precipitate; * this low value is due to loss of phosphate during the hydrolytic preparation of lipid A.

graphy on cellulose with solvent system I again showed the presence of unaltered polymyxin B in the lipid A precipitate, whereas chromatography with solvent system II on Kieselgel G substantiated that all the phosphorus containing fractions of lipid A had been precipitated. Because of the presumptively degraded state of the hydrolysed LPS fractions, their described behaviour with the antibiotic can only be taken as circumstantial evidence for lipid A as the polymyxin receptor. Yet, examination of mutant LPS lacking known regions of the carbohydrate chain (see Fig. 1) should indicate the position of the polymyxin binding site.

The data compiled in Table I unequivocally point to the KDO-lipid A portion (region III) of LPS as polymyxin receptor, excluding the heptose-bound phosphate and the O-antigenic side chain (most of region II and region I). This is obvious from the finding that the amount of polymyxin bound per mg dry weight LPS is much higher in mutant LPS with short carbohydrate chains (Rc and Re chemotype) than in the complete wildtype LPS. Also, the ratio polymyxin to LPS-phosphorus was maximal in the most rudimentary LPS (glycolipid [27]) of the Re chemotype. It should be noted that the polymyxin binding capacity of LPS varied somewhat with the used preparation of LPS, however, within one preparation the results were very constant. Another important point was the observation that the mutant LPS of the Rc and Re chemotype formed precipitates with polymyxin B which were easily separated by centrifugation or filtration. This property provided a simple experimental approach for investigations into the physicochemical nature of the LPS-polymyxin interaction.

2. Nature of the LPS-polymyxin complex formation

For the following studies, we selected the Rc chemotype LPS from the strain G 30 because it is well characterised and obtained with good purity [15]. It is shown in Fig. 4 that complex formation was

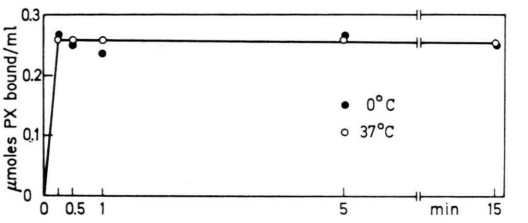

Fig. 4. Kinetics of the LPS-polymyxin complexation at 0 °C and 37 °C. 0.58 mg LPS from strain G 30 in 0.5 ml of distilled water were mixed with 0.5 μmoles of polymyxin B dissolved in 0.1 ml of water. 0.1 ml fractions were filtered through cellulose acetate filters (pore size 0.45 μm) after the noted incubation times. The filter-bound polymyxin was calculated from the residual amount in the filtrate. In the presence of polymyxin B, the input LPS was quantitatively retained on the filters. Without added antibiotic, all the LPS was found in the filtrate as determined by it's phosphate content.

complete within 25 sec at 0 °C or 37 °C. The lack of a temperature dependence suggested a physical rather than a chemical process since neither polymyxin B nor LPS contain highly activated groups being able to build covalent linkages at 0 °C within the short reaction time. Fig. 5 proves that the precipitation was polymyxin dependent leading to complexes which were composed of stoichiometric amounts of polymyxin B and LPS at saturation levels of the antibiotic. Similar isotherms have already been shown for the lipid A precipitate in Fig. 3. At an excess of polymyxin, complex forma-

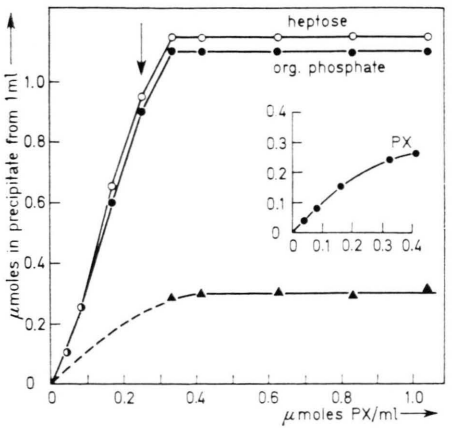

Fig. 5. Precipitation isotherms of the LPS-polymyxin B complexation. 0.55 mg of G 30 LPS in 0.4 ml of distilled water were mixed with 0.1 ml of properly concentrated polymyxin B solutions. After 5 min at 37 °C, the formed precipitate was collected by centrifugation at 2500 rpm. LPS and polymyxin in the precipitate were determined as indicated in Fig. 4. The inset figure shows the polymyxin content of the precipitate at the very low polymyxin concentrations. To avoid the interference of the LPS aminogroups in the polymyxin determination, the not precipitated LPS was removed by precipitation with 0.03 M magnesium chloride [15] and the remaining polymyxin determined in this supernatant.

tion was also linear with the amount of added LPS. However, when the LPS-polymyxin ratio was raised above 3.7 mg dryweight LPS per μmole of polymyxin B, the precipitation was inhibited in analogy to antigen-antibody systems [28]. The LPS-polymyxin precipitates were stable in distilled water and in 0.14 M sodium chloride at pH 7.0. The removal of almost all the LPS or all the antibiotic from the solute by precipitation makes it impossible to calculate a meaningful equilibrium or association constant for the reaction under saturating conditions. If the reaction follows the equation

$$n\,\mathrm{PX} + \mathrm{LPS} \rightleftharpoons \mathrm{PX}_n \cdot \mathrm{LPS}$$

one can calculate an apparent association constant at subsaturating levels of polymyxin B from the results of Fig. 5 which are marked by the arrow, according to

$$K'_{\mathrm{ass.}} = \frac{[\mathrm{PX}_n \cdot \mathrm{LPS}]}{[\mathrm{PX}] \cdot [\mathrm{LPS}]} .$$

Adopting a molecular weight of 10 000 for the G 30 LPS [29], $K'_{\mathrm{ass.}}$ is $4.3 \cdot 10^4$ under these conditions. From the same data of Fig. 5, the number of polymyxin molecules bound per molecule of LPS is determined to be 2.7 at saturating concentrations of polymyxin B. Since every G 30 LPS molecule seems to be composed of 3 basic carbohydrate chains and lipid A moieties [30], the ratio polymyxin-LPS subunit is almost 1 : 1.

The presence of esterified phosphorus in the LPS and the lipid A [27] suggested to us that ionic forces might be responsible for the rapid complex formation. Such a process should be pH dependent according to the pK values of the involved ionisable groups, *i. e.* the γ-aminogroups of the L-α,γ-diaminobutyric acid residues of the polymyxin B [31] and of the KDO-carboxyl- and phosphoryl-groups of LPS. The results depicted in Fig. 6 clearly established the

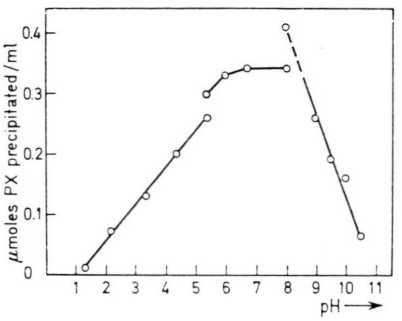

Fig. 6. pH-Dependence of the LPS-polymyxin B complexation. The reaction mixture contained in 0.5 ml final volume 0.25 ml of the desired buffer, 0.42 μmoles polymyxin B sulfate and 0.72 mg of G 30 LPS. After 10 min at room temperature, the precipitate which contained all the LPS (heptose) was collected by centrifugation. The bound polymyxin was calculated from the residual amount in the supernatant. The following buffers were used: 0.1 M Sodium citrate-HCl (pH 1.2—4.9); 0.07 M potassium-sodium phosphate (pH 5.0—8.0); 0.1 M sodium borate-HCl and —NaOH (pH 8.0—10.5). At pH 10.5, only 70% of LPS was precipitated.

expected pH dependence when the amount of polymyxin bound to the precipitated LPS was determined. Since all the LPS was precipitated at all the noted pH values, precipitation must be due to removal of charges from the G 30 LPS molecule by salt formation with polymyxin B at neutral pH, and with protons at low pH values. This loss of electrical charges would transfer this LPS from a hydrophilic form into a hydrophobic state which causes precipitation. A similar effect occurs when the G 30 LPS is treated with divalent cations like magnesium [15]. The wildtype LPS is therefore not precipitated by polymyxin B because the long, hydrophilic *O*-antigenic carbohydrate chain which is missing in the G 30 LPS, keeps this molecule in solution despite its reaction with polymyxin B (Fig. 2, Table I).

If the LPS-polymyxin interaction is ionic in nature, it should also be fully reversed by either changing the pH from 7 to 1 or by addition of magnesium ions. Suspending a G 30LPS-polymyxin complex obtained at pH 7 in buffer of pH 1 actually resulted in complete and rapid disappearence of polymyxin B from the precipitate which still contained all the LPS whereas the antibiotic was found unhydrolysed in the supernatant. Magnesium and sodium ions even at 2 M concentrations, however, were unable to remove all the polymyxin from the complex (Fig. 7). Since the magnesium-polymyxin ratio was maximally 2000 on a molar basis, the polymyxin-LPS interaction can not be explained by ionic forces only. Other responsible forces could either be hydrogen bonding and/or hydrophobic interactions. An indication that hydrophobic forces might be involved is the observation that the complex was completely dissociated by the cationic detergent cetylpyridinium chloride but not by the neutral detergent Triton X100 (Fig. 7). Therefore, both hydrophobic and ionic forces must act together though hydrogen bonding has not yet been excluded. The complete displacement of the antibiotic from the LPS precipitate by cetylpyridinium chloride is final evidence that no covalent linkages are involved. This statement is also confirmed by the experience that over 80% of LPS-bound radioactive polymyxin B are recovered by thinlayer chromatography on cellulose with solvent system I. In addition, the radioactive antibiotic could also be removed from the complex by excess unlabelled polymyxin B (Table II).

In aqueous environments, the LPS molecules are not in a true solution but they are aggregated in membraneous structures [30]. In these vesicle or tube like aggregates, the carbohydrate chains probably extend into the aqueous phase whereas the lipid A portion remains in the hydrophobic interior. Since the experiment with the mutant LPS indicates that the antibiotic binds to the KDO-lipid A region (Table I), the polymyxin molecule must penetrate the carbohydrate regions I and II (Fig. 1) in order to reach it's site of interaction. This question was investigated by proton magnetic resonance spectroscopy. However, while the polymyxin B solution gave a high resolution spectrum at 0.04 M concentration in D_2O, this was not obtained with a colloidal but clear solution of *S. typhimurium* wildtype LPS pointing to an aggregated state of the LPS [32]. Though mixing of LPS and polymyxin B resulted in an extreme broadening of all proton signals of the antibiotic, these data can not be used to discriminate between the carbohydrate or the lipid A portion as a binding site because a tenfold increased polymyxin-LPS ratio (48 mg PX/100 mg LPS/ml D_2O) had to be applied in order to obtain a measurable spectrum at 100 MHz.

Table II. Exchange of LPS-bound mono-*N*-acetyl-^{14}C-polymyxin B by externally added unlabelled polymyxin B. 1.2 mg LPS from *S. typhimurium* G 30 were incubated at 37 °C for 10 min with 0.3 μg mono-*N*-acetyl-^{14}C-polymyxin B in 0.5 ml of distilled water. The LPS was precipitated with 0.03 M magnesium chloride, separated by centrifugation and treated 1 to 3 times with 1.2 mg of unlabeled polymyxin B. The pre-, cipitated LPS-polymyxin complexes were suspended in H_2O and the radioactivity counted.

Treatment	Precipitate from 0.5 ml [cpm]
1. 0.3 M $MgCl_2$	27 436 *
2. 1·1.2 mg polymyxin B	4 486
3. 2·1.2 mg polymyxin B	2 765
4. 3·1.2 mg polymyxin B	1 462

* Constitutes 90% of the input radioactivity.

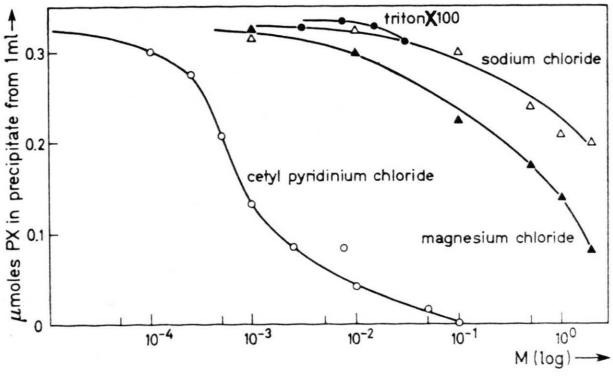

Fig. 7. Release of polymyxin B from the LPS-polymyxin complex by various agents. 0.58 mg of G 30 LPS in 0.1 ml of distilled water were reacted with 0.42 μmoles polymyxin B in 0.25 ml of water for 5 min at room temperature. The resulting LPS-polymyxin precipitates were resuspended and stirred with 0.25 ml of the noted compounds at the desired concentration. After 15 min, the remaining precipitates were collected by centrifugation and the bound polymyxin B calculated from the amount which was present in the supernatant. The formed precipitates contained at all depicted points 100% of the input LPS as determined by the content of organic phosphate. The pH was 7.0 in every case.

More information on the environment of the polymyxin molecule in the LPS matrix could be gained by application of fluorescence techniques [33]. Since blue shifts of the fluorescence emission maxima of dimethylaminonaphthalene sulfonyl groups have been correlated with decreasing polarities of the solvent environment [34], we investigated biologically active, mono-dansyl derivatives of polymyxin B [1, 26]. Fig. 8 shows that the interaction of dansyl-polymyxin with wildtype LPS resulted in a blueshift of 35 nm coupled with an increased relative fluorescence intensity of a factor of 12.7. Similar results were also obtained for complexes of dansyl-polymyxin with LPS from *Proteus mirabilis*, or with RNA and DNA and for solutions of dansyl-polymyxin in *n*-butyl alcohol. No changes, however, were seen in a dansyl-polymyxin mixture with soluble starch which indicates that the antibiotic is unable to combine with neutral carbohydrate chains under these conditions. Since penetration of dansyl-polymyxin into the apolar fatty acid region of lipid A should give a much stronger blueshift and since the observed values are in a very good agreement with the data reported by Waggoner and Stryer [34] for dansyl-groups at a polar-apolar interface, we may conclude that the polymyxin molecules in the complex with LPS are located at the interface between the lipid A and the carbohydrate region of LPS which is also the position of the negatively charged carboxyl- and phosphoryl-groups of LPS (see Fig. 1).

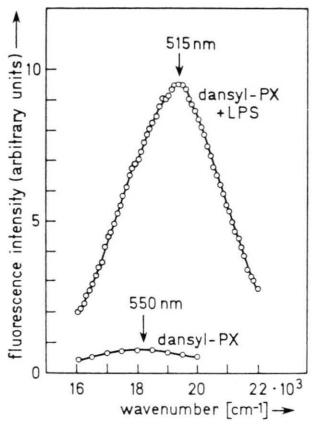

Fig. 8. Fluorescence emission spectra in H_2O of Mono-*N*-dimethylaminonaphthalene sulfonyl polymyxin B (dansyl-PX) at $1.5 \cdot 10^{-6}$ M and of a mixture of dansyl-PX at the same concentration with 1 mg *S. typhimurium* wildtype LPS/ml. The excitation wavelength was 350 nm. The dansyl-PX spectrum was actually recorded at a tenfold sensitivity, but is drawn to scale in order to allow a quantitative comparison of the two spectra.

The so far described properties of the LPS-polymyxin complex proved it to be quite a stable compound under physiologically reasonable conditions. This stability was checked in a biological experiment. Fig. 9 clearly demonstrates that the addition of isolated G 30 LPS to growing cultures of *S. typhimurium* was able to neutralise the antibiotic potency of polymyxin B with good stoichiometry for at least 2 hours at 37 °C. This result is the best evidence that the polymyxin-LPS complex is stable under physiological conditions. This in turn is a prerequisite in order to assign to this reaction a role in the killing process induced by polymyxin B.

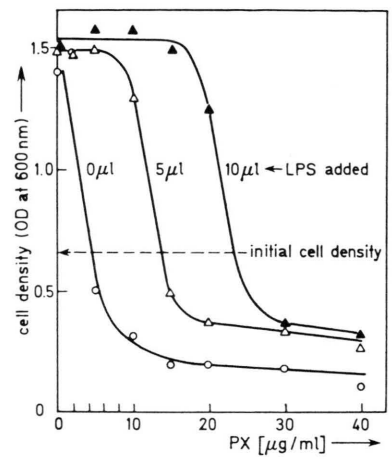

Fig. 9. Neutralisation of the antibiotic potency of polymyxin B by LPS. 1 ml portions of growing cultures of *Salmonella typhimurium* G 30 ($7.5 \cdot 10^8$ cells/ml) were treated with 0.09 ml of properly concentrated polymyxin B solutions containing 5 or 10 μl (= 5 or 10 μg) of G 30 LPS/ml. After 2 additional hours of incubation at 37 °C, the cell density was measured at 600 nm in a Zeiss PMQ II photometer.

Whereas the analysis of the LPS-polymyxin interaction is easily accessible using the isolated components *in vitro*, it is difficult to differentiate between the LPS-polymyxin and phospholipid-polymyxin reactions in whole bacteria. One possible approach is the investigation of the polymyxin susceptibility of bacteria with known mutations in the LPS molecule. The minimal inhibitory concentration of polymyxin B for *S. typhimurium* strain SL 1102 was found to be only 1 μg/ml at a cell density of 10^9/ml while the MIC for the smooth wildtype was 8 μg/ml under the same conditions. This differential

behaviour was also reflected in the binding isotherms for radioactive polymyxin B and in the release isotherms of the periplasmic 3′-nucleotidase which is a measure for the permeability of the outer membrane (Fig. 10). These results, however, point

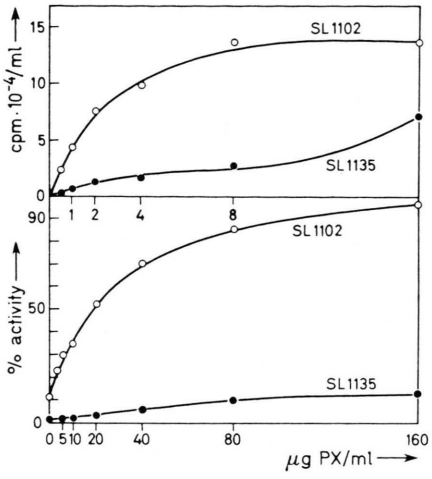

Fig. 10. Binding isotherms for radioactive polymyxin B (top) and release isotherms for the periplasmic 3′-nucleotidase (bottom) as measured with smooth wildtype *Salmonella typhimurium* SL 1135 and the rough strain SL 1102 (see Fig. 1). Binding of mono-*N*-acetyl-^{14}C-polymyxin B was determined by filtration after 1 min incubation at 37 °C with 10^9 cells/ml of neutral 0.14 M sodium chloride. The 3′-nucleotidase activity was determined in the supernatant of 10^{10} cells/ml of 0.14 M sodium chloride after 1 min treatment with polymyxin B at 37 °C. The figure (bottom) shows the percentage of total 3′-nucleotidase which was released into the medium [4].

to a passive role of the LPS, namely a barrier function of the complete carbohydrate chain of LPS for the antibiotic. Whether the LPS-polymyxin complex formation plays an active role for the polymyxin-induced permeability changes in the outer and inner membrane of *Salmonella typhimurium* remains to be established. This question is currently investigated with the aid of polymyxin resistent mutants.

Discussion

The *O*-antigenic LPS of *Salmonella typhimurium* is synthesised in the inner (cytoplasmic) membrane [37]. The *O*-antigen specific carbohydrate chain extends from the cell surface into the aqueous environment of the cell [38] whereas the lipid A portion probably serves to anchor the molecule in the lipid bilayer [39] of the outer membrane [40] which contains also phosphatidylethanolamine and phosphatidylglycerol [37]. The LPS is therefore one of the first cell components which the antibiotic polymyxin B meets on it's way to the ultimate site of action, the cytoplasmic membrane [3]. Several laboratories have provided evidence that penetration of polymyxin B through the outer membrane is an event preceding the destruction of the inner membrane [41–45]. Passage of the outer membrane by polymyxin B is coupled with a permeability change of this structure which allows the penetration of large molecules like lysozyme [2, 3] or periplasmic enzymes (*e.g.* 3′-nucleotidase or ribonuclease I) [46, 47]. The observation of cells having an altered permeability of the outer membrane but an intact cytoplasmic membrane [3] proves that the action on the outer membrane is a prerequisite but not the cause for cell death. Hence, the possible mechanism of LPS as a polymyxin B receptor could be seen in an impairment of the contribution of LPS to the conformational stability of the outer membrane. Direct evidence that polymyxin-induced alterations of LPS do occur *in vivo* is the severe inhibition of LPS-specific phage absorption in polymyxin-treated bacteria [7, 8]. Another point is the polymyxin-dependent appearence of morphological alterations of the outer membrane which gives rise to blebs and projections extending from the cell surface into the medium [48–50]. These alterations, however, are not present when the cells previous to the polymyxin treatment had been challenged with LPS-specific phages [7]. This suggests two things: 1. The polymyxin-induced morphological alterations of the cell surface occur at sites which contain LPS. Since the area of the cell surface is a function of the cell size, incorporation of polymyxin into a fixed area must result in an increased surface area which can only extend into the environment but not the cell interior. 2. The formation of distinct projections in polymyxin-treated bacteria could be due to clustering or mosaic distribution of LPS on the cell surface. This possibility has recently been discussed [51].

Regarding the binding site of LPS for polymyxin B, an ultimate understanding of the exact mechanism will be dependent on the analysis of the exact primary and secondary structure of the lipid A and the adjoining core polysaccharide. In addition, the mechanism how the lipid A is incorporated into the cell surface of Gram-negative bacteria has to be established.

As to the binding sites in the polymyxin B molecules, the importance of the 5 free aminogroups is evident from the pH-dependence of the reaction (Fig. 6). Furthermore, fully *N*-acetylated radioactive polymyxin B derivatives [25] did not complex with isolated LPS (J. Bader, unpublished observations). The aminoterminal fatty acid and the D-phenylalanine-L-leucine regions of the molecule could be involved in hydrophobic interactions [52] whereas the carbonyl- and amide-groups of the peptide bonds could participate in hydrogen bonding. A requirement to understand the role of these residues would be the knowledge of the conformation of polymyxin B in aqueous solvents which is still uncertain [53].

A differentiation between the LPS-polymyxin and the phospholipid-polymyxin interactions in the membranes of whole bacteria could be aided by investigations into the polymyxin-binding properties of isolated inner and outer membranes. We have isolated these membranes from *Salmonella typhimurium* G 30 according to the recently published procedure of M. J. Osborn's laboratory [37] and are currently studying their response towards polymyxin B.

The isolation of polymyxin-phospholipid complexes could help to obtain more information on the nature and function of the polymyxin receptors of sensitive bacteria [54].

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