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Mechanism and Improvement of Complex Formation Between LPS and Polymyxin B That is Immobilized on an Ion Exchanger

Convenient Immobilization Procedures for Spacer and Polymyxin B

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ABSTRACT

Polymyxin B was immobilized on the ion exchanger, Amberlite IRC-50. The modified support was used as an adsorbent for lipopolysaccharide of *E. coli* from a phosphate buffer at pH = 7.0 and $T = 20°C$. The insertion of a spacer, 6-amino hexanoic acid, improved the adsorption capacity of the modified carrier significantly. Upon partial acetylation (25%) of primary amino groups of immobilized Polymyxin B, the adsorption capacity of the support was halved. From our results, it is concluded that the complex formation between lipopolysaccharide and immobilized Polymyxin B is based on both lipophilic, as well as electrostatic, interactions. Convenient procedures for the immobilization of the ligands and the characterization of the ion exchanger are described.

Index Entries: Lipopolysaccharide; Polymyxin B; immobilization; spacer effects; 6-amino hexanoic acid ethyl ester.

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INTRODUCTION

Lipopolysaccharide (LPS) is a characteristic amphiphilic component of the outer membrane of Gram-negative bacteria. The molecule is built up of a strain-dependent heteropolysaccharide part covalently bound to a rather strain-independent lipid part, called lipid A (*1–5*). LPS molecules are exclusively located in the outer layer of the outer cell wall of Gram-negative bacteria, where they protect the cell against leakage of cytoplasmic material and harmful external components, such as detergents, bile salts, and antibiotics.

On one hand, if Gram-negative bacteria reside in an organism, their LPS molecules cause some beneficial biological effects. For example, the heteropolysaccharide part of LPS plays an important role in the activation of the organism's immune system, which starts to produce bacterial antibodies as soon as this part of LPS is recognized (*4*). On the other hand, if LPS is released from disintegrating bacteria, it is also responsible for many endotoxic properties of Gram-negative bacteria, e.g., fever, diarrhea, changes in the white blood cell count (*1,4*), hypotension, and complement activation (*3*). Almost all of these endotoxic activities reside in the lipid A part of LPS (*2,3*).

Normally, LPS is detoxified by the liver and may not be found in the organism's blood. But, in cases of a reduced liver function, the blood concentration of LPS may rise to such a level that endotoxic activities become noticeable ("endotoxemia"). Some other causes for endotoxemia are a severe burn injury, surgery, and chemotherapy (*6*). If too much LPS is present in the blood and it cannot be detoxified fast enough by the organism, it is important to have a suitable way to detoxify LPS artificially so that the amount of LPS is reduced to an acceptable level (*12–14*).

An artificial way to detoxify LPS is the administration of an antibiotic. A widespread antibiotic against most strains of Gram-negative bacteria is Polymyxin B (PMB), which is derived from *Bacillus polymyxus* (*16*). This antibiotic belongs to a closely related group of cationic amphiphilic antibiotics, the polymyxins. These antibiotics are characterized by a heptapeptide ring, to which a C:8 or C:9 fatty acid is attached through an amide bond (*7–9*).

PMB may spontaneously incorporate into the outer cell wall, where it forms a very stable complex with LPS through hydrophobic and electrostatic interactions (*7,10*). After extensive scientific studies, it is generally accepted that the antibiotic property of PMB resides in the incorporation of PMB into the bacterial membrane. This spontaneous process increases the membrane permeability and thereby inhibits the bacterial respiration. Morrison and Jacobs (*11*) found that the positively-charged primary amino groups, as well as the lipid part of PMB, are essential to establish a strong interaction between PMB and LPS.

The use of PMB as an in vivo antibiotic is limited to oral and local administration, because PMB is strongly toxic to the central nervous system

and kidneys of the organism (*6,8*). This problem of toxicity can be solved if the antibiotic is kept outside the organism by its covalent immobilization on a solid support and utilizing hemoperfusion. In this way, PMB has lost its toxicity and blood, or any other LPS-containing solution and can be brought into contact with this LPS-removing material.

For our investigations, PMB was covalently bound to the ion exchanging resin, Amberlite IRC-50, via condensation reactions between primary amino groups of PMB and carboxyl groups of the solid support. This modified solid support was used to adsorb LPS of *E. coli* from a buffer solution at pH=7.0. The influence on the LPS adsorption capacity of an inserted spacer arm between PMB and the support was also investigated. 6-Amino hexanoic acid (EACA) functioned as a spacer. A fraction of PMB's primary amino groups was acetylated to show the importance of their protonated state in the complex formation with LPS. We report about the convenient modification procedure, the performance of this endotoxin adsorbent, and the mechanism of binding between PMB and LPS.

MATERIALS AND METHODS

Chemicals

Both Polymyxin B Sulphate (no. P 1004) and Lipopolysaccharide from *E. coli* 0111:B4, phenol-extracted (no. L 2630) were purchased from Sigma, St. Louis, MO. All other chemicals were reagent grade and used as received.

IMMOBILIZATION PROCEDURES

Immobilization of Spacer Arms

Before the spacer, EACA, was covalently coupled to the ion exchanger, we synthesized its ethyl ester. The esterification was based on the method of Rothe et al. (*23*). We adjusted this method by using a two-molar excess of thionylchloride regarding the spacer quantity. After evaporation of most of the ethanol, the HCl salt of the spacer's ethyl ester precipitated almost quantitatively. As a result, we omitted the HCl-elimination step and the vacuum distillation. Table 1 shows some relevant physical properties of the synthesized product, the HCl salt of EACA ethyl ester.

In order to regain the spacer as a free amine, we dissolved 1 g each of the spacer's HCl salt in 5 mL dry dioxane. Then, we added an equimolar amount of tri-ethylamine, and the precipitated HCl salt of triethyl amine was filtered off. The clear dioxane solution, containing the free spacer, was used in the immobilization procedure.

The carboxyl groups of the ion exchanger were activated through conversion into reactive esters with *N*-hydroxy-succinimide (NHS) and di-cyclohexyl-carbodiimide (*20–22*). Both spacer immobilization and sup-

Table 1
Relevant Physical Properties of 6-Amino Hexanoic Acid Ethyl Ester

Structure:	$CH_3 - CH_2 - O - \overset{O}{\overset{\|}{C}} - (CH_2)_4 - NH_2 \cdot HCl$			
Molecular weight:	195 g/mol			
Melting point:	109–110°C			
Elemental analysis:		N	C	H
	Theor.	7.2%	49.2%	9.2%
	Exp.	7.1%	50.1%	9.5%
^{13}C-NMR data:	Solvent: $CDCl_3$			
	Carbon Atom			δ, ppm
	$-CH_3$			14.4
	$-CH_2-O-$			60.4
	$>C=O$			173.5
	$-CO-{}^*CH_2-$			33.9
	$-CO-CH_2-{}^*CH_2-$			25.9
	$-{}^*CH_2-CH_2-NH_2 \cdot HCl$			27.3
	$-CH_2-NH_2$			39.7

port activation were carried out in a one-pot synthesis. About 1 g ion exchanger was washed with 7 mL dry dioxane and suspended in 10 mL of this solvent. Then, 1 g *N*-hydroxy-succinimide, 2 g di-cyclohexyl-carbodiimide, and about 10 mmol of the spacer's free amine in 10 mL dry dioxane were added. After 16 h of stirring, the solid support was washed twice with dichloromethane/methanol (25:1) and five times with 10 mL dioxane. Next, the ester was hydrolyzed with 10 mL of 0.1*M* NaOH at 40°C during 2 h.

The modified, solid support was prepared for elemental analysis by washing it thoroughly with 10 mL portions of a 0.1*M* HCl solution during several hours. The time interval between two washing batches was about half an hour, because equilibrium is not immediately established (33). After the washing procedure, drying of the support was carried out under vacuum (1 mm Hg) at 30°C for at least 16 h.

Via mass balances, we derived an equation with which the ligand content (either spacer or PMB) on the ion exchanger could be calculated from the weight fractions of nitrogen, carbon, or hydrogen in the carrier (Eq. (1)). These weight fractions were estimated with elemental analysis before and after an immobilization.

$$C = X_{i,2} - X_{i,1} / n_i \cdot A_i - X_{i,2} \cdot (M - 18) \quad (1)$$

where C is the ligand content in mol/g support before reaction, X_i is the weight fraction of element i, M is the ligand's molecular weight in g/mol, A_i is the atomic weight of element i in g/mol and n_i is the number of atoms

i in the ligand. The indices 1 and 2, respectively, indicate the weight fraction determined before and after immobilization reaction.

For the derivation of this expression, we assumed that only one ligand (spacer or PMB) at a time was coupled to the carrier by way of condensation reactions. If the ligand content on the carrier is to be calculated from hydrogen weight fractions, then n_i is the number of hydrogen atoms in the ligand minus two for each of the ligand's amino group that is involved in a condensation reaction.

Immobilization of Polymyxin B

PMB was covalently bound to the carrier by way of a condensation reaction between one or more of the PMB's primary amino groups and the carboxylic groups of the solid support. This reaction was effected by the water-soluble carbodiimide (*17*), 1-ethyl-3-(3-di-methyl aminopropyl)-carbodiimide (EDCI). For the coupling procedure, we dissolved 96.3 mg PMB in 5 mL of a diluted HCl solution (pH=5.6). This PMB solution was added to 569 mg wet ion exchanger and then 1.5 g EDCI was added. The reaction was allowed to proceed for 16 h under stirring.

The effluent from the condensation reaction was collected in a 50 mL flask. Then, the ion exchanger was carefully washed with several 5 mL portions of 0.05*M* HCl containing 1*M* NaCl. These solutions were combined in the same 50 mL flask, and the volume was made up to 50 mL with distilled water. The PMB content on the carrier was determined with two methods.

On one hand, the PMB content on the carrier was estimated by differential measurement. The PMB concentration in the collected solution was determined with HPLC and substracted from the PMB amount that was added before the immobilization reaction. The HPLC analysis of PMB was performed with the same kind of column used by Elverdam et al. (*18*). We adapted the column's length, 10 cm in our case, and the mobile phase composition, which was the same as described by Whall (*19*). The unknown PMB concentration in samples was derived from a calibration curve.

On the other hand, the PMB content on the carrier was calculated from elemental analysis data. Portions of the modified support subjected to elemental analysis were prepared following the same procedure for the carrier with immobilized spacer arms. Again, the PMB content on the solid support was calculated with Eq. (1).

After the immobilization procedure, a part of PMB's primary amino groups was acetylated to show the importance of their protonated state in the binding of LPS. The acetylation procedure was carried out as described by Hiraki et al. (*29*).

The degree of acetylation of the primary amino groups of immobilized PMB was calculated from the difference in primary amino group quantity on the carrier before and after the acetylation procedure. The amount of primary amino groups on the ion exchanger was estimated with the 2-hydroxy-1-naphtaldehyde method (*30–32*).

LPS Adsorption Experiments

The adsorption of LPS was performed in batch experiments with portions of ion exchanger that were differently modified, that is, either with or without spacer arms or with or without PMB. The adsorption time was 16 h. The solid support portions were carefully washed before use with several 10 mL portions of 0.1*M* HCl and once with 10 mL distilled water.

The amount of adsorbed LPS was determined by differential measurement of the dissolved LPS quantity. After acid hydrolysis and successive reaction with phenol, LPS could be quantitatively detected with a spectrophotometer (*28*). The unknown LPS concentration was derived from a calibration curve. The standards contained between 50 and 400 μg LPS.

There are many analytical methods for the quantitation of LPS in solutions (*1,25–27*). However, most of these methods are not suitable for standard use, because they are too complex or sensitive for other components than just LPS. The analysis method with phenol/sulphuric acid is suitable for standard use and the method is sensitive enough for our experiments. The detection limit is about 10 μg LPS from *E. coli* 0111:B4.

RESULTS AND DISCUSSION

The main aim of our research can be separated into two parts. First, the ion exchanging resin Amberlite IRC-50 was to be modified with PMB in such a way that it could be used as an adsorbent for bacterial LPS. This Amberlite IRC-50 was macroporous in structure and contained 10 mmol carboxyl groups/g dry resin. Second, we wanted to investigate the binding mechanism between LPS and immobilized PMB and show the relevance of the primary amino groups of immobilized PMB in establishing a strong interaction with LPS.

IMMOBILIZATION OF SPACER ARMS

Before the spacer, EACA, was covalently coupled to the ion exchanger, its corresponding ethyl ester was synthesized. This esterification has two important functions. The most evident function is to prevent polymerization of the spacer in a one-pot immobilization procedure. But, the most striking function may be the solubilization of the spacer. Normally, EACA would be only soluble to a great extent in water, but after esterification the spacer is soluble in many organic solvents.

We adjusted the method of Rothe et al. (*23*) for the synthesis of EACA's ethyl ester. In our procedure, a rapid, almost quantitative precipitation (>95%) of the HCl-salt of the spacer's ethyl ester occurs. This product can easily be purified by washing it with ethyl ether and then drying it

under vacuum. A ^{13}C-NMR spectrum of the spacer revealed that its ethyl ester had been synthesized (Table 1) and that the product was of a high purity state.

The main advantage of the adapted procedure is that the total synthesis time is shortened, because two time consuming purification steps can be eliminated (the HCl removal with ammonia and a vacuum distillation).

The free base of the spacer's ethyl ester, necessary for the coupling reaction, can be liberated by the addition of an equimolar amount of triethyl amine to a dioxane solution that contains the ester's HCl salt. The triethyl amine HCl salt will be formed and this will precipitate. After filtration, the ester's free amine will remain in the dioxane.

The activation of carboxyl groups with di-cyclohexyl carbo-diimide (DCC) and *N*-hydroxy succinimide (NHS) is a widely used method in peptide synthesis (*20–22*). This procedure was also applied in our experiments to activate the carboxyl groups of Amberlite IRC-50.

One disadvantage of this activation procedure is that DCC not only reacts with carboxyl groups, but also with NHS (*24*). In this way, a byproduct, succinimidoxycarbonyl-β-alanine-hydroxy-succinimide ester, is formed as a precipitate. Although this byproduct is hardly soluble in almost every organic solvent, it could be removed with a mixture of dichloromethane and methanol (25:1).

Although it was expected that the byproduct would plug up the pores of the ion exchanger, it turned out from our calculations, which are to be discussed later, that the conversion is 70%. This is high enough to assure, finally, that most of the antibiotic is bound to the carboxyl groups of the spacer arm and not to the ion exchanger itself.

QUANTITATIVE ANALYSIS FROM ELEMENTAL ANALYSIS DATA

Carriers that are to be subjected to elemental analysis must meet some requirements. First, the number and structure of immobilized ligands must be known. Second, all carboxyl groups must have the same counter ion. In our experiments, the solid support is carefully washed with a very pure HCl solution so that protons function as counter ions after the support was dried.

The accuracy of the calculations from elemental analysis data can be estimated theoretically. A convenient parameter for this accuracy is the relative error in the spacer concentration, *RE*, caused by an uncertainty in the involved weight fraction, δX. *RE* can be derived by differentiation of Eq. (1) in X_2. This yields Eq. (2).

$$RE = 1 / C \cdot dC / dX_2 \cdot \delta X_2$$

$$= n_i \cdot A_i - X_{i,1} \cdot (M - 18) / [X_{i,2} - X_{i,1}] \cdot [n_i \cdot A_i - X_{i,2} \cdot (M - 18)] \cdot \delta X_2 \quad (2)$$

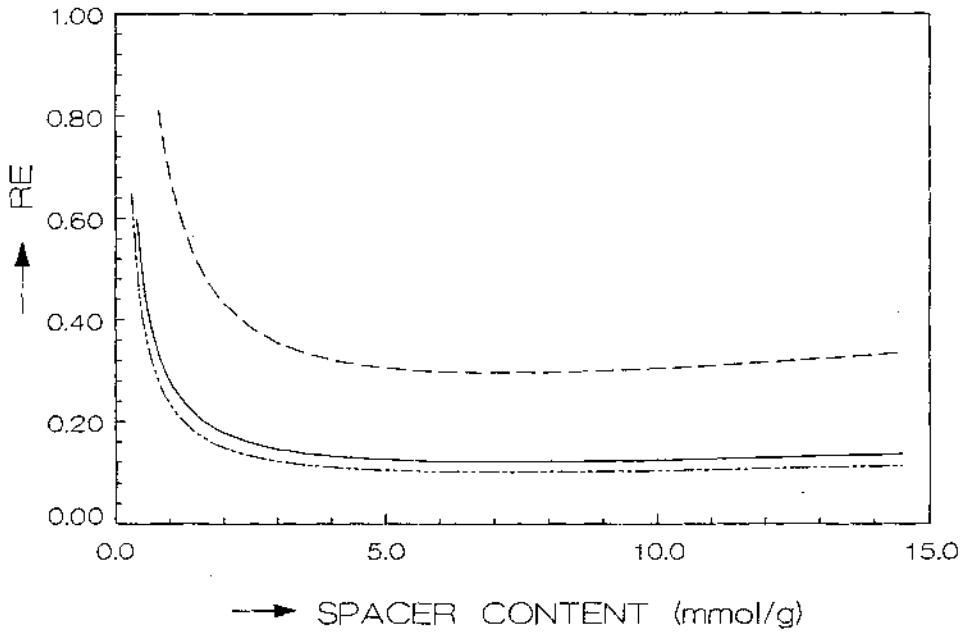

Fig. 1. Theoretically estimated relative error (RE) in the carrier's spacer content calculated from elemental analysis data. RE is shown for calculations from the weight fractions of nitrogen (———), carbon (---), and hydrogen (———).

A maximum value of $\delta X_2 = 0.003$ was found by experience. By combination of Eqs. (1) and (2), RE can be calculated as a function of C, which was carried out for the immobilized spacer (Fig. 1). It may be clear from Fig. 1 that calculations from elemental analysis data are not accurate if the ligand concentration on the carrier does not exceed a certain minimum value. Another fact that appears from Fig. 1 is that an accurate spacer content should be calculated from carbon and nitrogen and not from hydrogen contents. Finally, it can be calculated that each curve exhibits a minimum in RE that is located at $C = (M - 18)^{-1}$. So, the minimum in RE only depends on the molecular weight of the immobilized ligand.

We calculated with Eqs. (1) and (2) that 7.0 mmol spacer was coupled per 1 g dry ion exchanger ($RE = 10\%$) and that 0.18 mmol PMB was coupled to 1 g of this dry support ($RE = 13\%$). From HPLC analysis, we determined that this PMB content was 0.20 mmol/g dry, spacer-containing ion exchanger. The latter content is the most reliable one, because there are two uncertainties in the calculations from elemental analysis data: PMB's exact molecular weight is not known (the PMB we used consisted of PMB_1, PMB_2, and PMB_3 (*7,8*)), and a little *N*-acyl urea from EDCI is probably formed on the carrier. But, although the PMB content is calculated from HPLC analysis data rather than elemental analysis data, the latter gives a very good approximation.

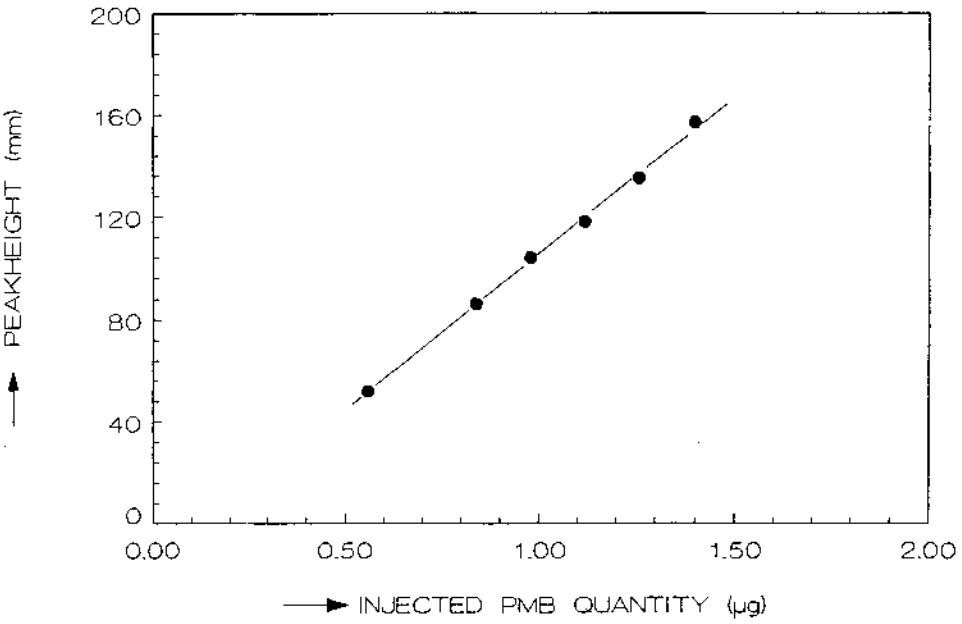

Fig. 2. A linear calibration line for the quantitative determination of PMB was produced with the adapted HPLC system.

If another component than the spacer or PMB is immobilized simultaneously on the support, all necessary equations can be derived in an analogous way. As these equations become very complicated, they are not mentioned here.

QUANTITATIVE ANALYSIS OF PMB WITH HPLC

The choice of a Nucleosil RP 18 column and a phosphate buffer as mobile phase resulted in an effective, rapid, and cheap chromatographic system for the quantitative analysis of PMB. The number of theoretical plates per meter ($N = 23{,}500$) was in the same order of magnitude as the value obtained by Whall (*19*). The resolution was high enough for complete chromatographic separation of PMB and the peak heights formed a linear function of the injected amount of PMB (Fig. 2).

In the procedure of coupling PMB with EDCI, the excess of EDCI regarding PMB should not exceed 100, because this may result in an incomplete chromatographic separation of PMB from EDCI's urea derivative. This urea derivative is formed during the immobilization procedure and elutes before PMB. If the excess of EDCI used for the reaction is too high, the elution parameters must be adapted to maintain complete chromato-

Table 2
Adsorption of LPS from *E. coli* from a Phosphate Buffer at pH = 7.0 and T = 20°C during 16 h With Four Differently Modified Portions Amberlite IRC-50[a]

Immobilized ligands		LPS adsorption
EACA, mmol/g	Polymyxin B, mmol/g[b]	Adsorbed LPS, mg/g
—	—	0.04
—	0.12	0.19
7	—	0.21
7	0.20	2.4
7	0.20	1.2[c]

[a]Ligand contents are defined per gram dry support, whereas the amount of adsorbed LPS is defined per gram wet support. Water content is about 50%.
[b]Determined with HPLC.
[c]After 25% acetylation of PMB's primary amino groups.

graphic separation of PMB. Generally, this will result in an increased analysis time that is not suitable for standard PMB analysis.

LPS ADSORPTION EXPERIMENTS

PMB was immobilized on Amberlite IRC-50, eventually after immobilization of a spacer. Four differently modified portions Amberlite IRC-50 were used in batch experiments to adsorb LPS of *E. coli* during 16 h from a buffer solution at pH = 7.0 and $T = 20°C$ (Table 2).

The LPS adsorption under the same conditions as a function of time was carried out in batch experiments with several portions of ion exchanger that contained 7 mmol/g spacer arms and 0.20 mmol/g PMB (Fig. 3). The results reveal that spacer arms are necessary to obtain a pronounced LPS adsorption. Nevertheless, it is clear that the LPS adsorption is a rather slow process, probably owing to a restricted diffusion of LPS into the adsorbent.

MECHANISM OF COMPLEX FORMATION

The binding sites for the formation of a strong complex between PMB and bacterial LPS in solution have already been determined by analytical techniques such as NMR (*34–36*). It is generally accepted that the formation of a strong complex between both macromolecules is based on lipophilic, as well as electrostatic, interactions. Morrison and Jacobs (*11*) already showed that the primary amino groups are essential in establishing a strong interaction between PMB and LPS.

One might expect that the long sugar backbone of LPS would prevent its negatively-charged groups to approach the protonated primary amino

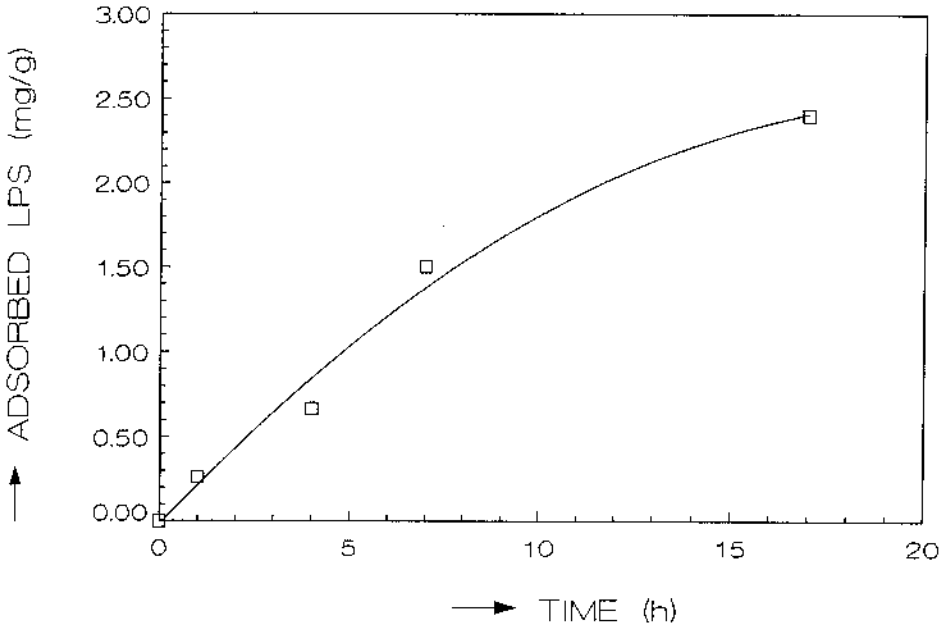

Fig. 3. Time course of the adsorption of LPS from *E. coli* with the modified ion exchanger. The LPS was adsorbed during 16 h from a buffer at pH = 7.0 and $T = 20°C$. The modified solid support contained 7 mmol/g spacer and 0.20 mmol/g PMB.

groups of immobilized PMB close enough to establish significant electrostatic interactions. In that case, the binding between LPS and immobilized PMB would be owing to lipophilic interactions between the acyl chains in both macromolecules. We found that steric hindrance is clearly present as the LPS adsorption capacity is significantly improved upon insertion of the spacer. In all other experiments, when no spacer is used, the adsorption capacity is much less. But, on the basis of the results mentioned in Table 2, it is not clear whether electrostatic interactions have to be taken into consideration in the complex formation. It is still possible that the binding only arises from hydrophobic interactions between the fatty acid chains of both LPS and immobilized PMB.

To investigate the role of electrostatic interactions in the complex formation, a part of the primary amino groups of immobilized PMB was acetylated. It turned out that the LPS adsorption capacity halved upon 25% acetylation. Thus, it can be concluded that the mechanism of binding between LPS and immobilized PMB is based on the same kind of interactions as if both macromolecules were freely dissolved. This implies that a very stable and strong complex is formed between LPS and immobilized PMB, confirmed by the fact that the LPS adsorbent could not be regenerated with the commonly used sodium deoxycholate (*15*).

The formation of a strong and stable complex between LPS and immobilized PMB is in favor of the adsorbent's selectivity toward LPS in the presence of other components, such as phospholipids and proteins. The performance of the LPS adsorbent in the presence of other dissolved components has yet to be investigated. If the selectivity is still significant in that case, the adsorbent finds its application in the LPS removal from serum and solutions that are used in biological experiments.

SUMMARY

Convenient immobilization procedures are reported for both spacer and PMB on Amberlite IRC-50, which is a chemically- and mechanically-stable ion exchanging resin with a macroporous structure. Furthermore, we describe the performances of this modified support as adsorbent for bacterial LPS from aqueous solutions. Finally, we report that electrostatic interactions are important for complex formation between LPS and immobilized PMB.

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REFERENCES

1. Mayer, H., Tharanathan, R. N., and Weckesser, J. (1985), *Methods Microbiol.* **18,** 157.
2. Galanos, C., Lüderitz, O., Rietschel, E. T., and Westphal, O. (1977), *Int. Rev. Biochem* **14,** 239.
3. Rietschel, E., Brade, H., Wollenweber, H., Zähringer, U., and Lüderitz, O. (1986), *Riv. Immunol. Immunofarmacol.* **6,** 32.
4. Rietschel, E., Brade, H., Brade, L., Kaca, W., Kawahara, K., Lindner, B., Lüderitz, T., Tomita, T., Schade, U., Seydel, U., and Zähringer, U. (1985), *Prog. Clin. Biol. Res.* **189,** 31.
5. Lugtenberg, B., and van Alphen, L. (1983), *Biochim. Biophys. Acta* **737,** 51.
6. Kodama, M., Tani, T., Hanazawa, K., Oka, T., Teramoto, K., and Nishiumi, S. (1985), *Eur. Pat. Appl. EP 129173.*
7. Storm, D. R., Rosenthal, K. S., and Swanson, P. E. (1977), *Ann. Rev. Biochemistry* **46,** 723.
8. Srinivasa, B. R. and Ramachandran, L. K. (1979), *J. Sci. Ind. Res.* **38,** 695.
9. Laporte, D. C., Rosenthal, K. S., and Storm, D. R. (1977), *Biochemistry* **16,** 8.
10. Jain, M. K. and Zakim, D. (1987), *Biochim. Biophys. Acta* **906,** 33.
11. Morrison, D. C. and Jacobs, D. M. (1976), *Immunochemistry* **13,** 813.

12. Hanasawa, K., Tani, T., Oka, T., Yoshioka, T., Endo, Y., Horisawa, M., Nakane, Y., Kodama, M., Teramoto, K., and Nishiumi, S. (1984), *Therapeutic Apheresis: A Critical Look*, Nosé, Y., Malchesky, P. S., and Smith, J. W., eds., ISAO Press, Cleveland, OH.
13. Maitra, S. K., Yoshikawa, T. T., Guze, L. B., and Schotz, M. C. (1981), *J. Clin. Microbiol.* **13,** 49.
14. Gaffin, S. L. and Abraham, C. (1980), *Hemoperfusion: Kidney Liver Support Detoxification* [Proc. Int. Symp. 1979], Sideman, S. and Chang, T. M., eds., Hemisphere, Washington DC.
15. Issekutz, A. C. (1983), *J. Immunol. Methods* **61,** 275.
16. Kluger, M. J., Singer, R., and Eiger, S. M. (1985), *J. Immunol. Methods* **83,** 201.
17. Tengblad, A. (1981), *J. Biochem.* **199,** 297.
18. Elverdam, I., Larsen, P., and Lund, E. (1981), *J. Chromatogr.* **218,** 653.
19. Whall, T. J. (1981), *Chrom.* **13,** 567.
20. Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1963), *J. Am. Chem. Soc.* **85,** 3039.
21. Selinger, Z. and Lapidot, Y. (1966), *J. Lipid Res.* **7,** 175.
22. Buzas, A., Egnell, C., and Fréon, P. (1962), *C.R. Séances Acad. Sci* **255,** 945.
23. Rothe, M. and Gehrke, K. (1965), *Makromol. Chem.* **83,** 1.
24. Gross, H. and Bilk, L. (1968), *Tetrahedron* **24,** 6935.
25. Keler, T. and Nowotny, A. (1986), *Anal. Biochem.* **156,** 189.
26. Valois, F. W. (1979), *Prog. Clin. Biol. Res.* **29,** 415.
27. Goldman, R. C. and Leive, L. (1980), *Eur. J. Biochem.* **107,** 145.
28. Gerhardt, P., Murray, R. G., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., and Phillips, G. B. (1981), *Manual Methods for General Bacteriology*, ASM, Washington DC, pp. 333,334.
29. Hiraki, Y., Oda, T., and Tai, A. (1983), *Bull. Chem. Soc. Jpn.* **56,** 2330.
30. Esko, K., Karlsson, S., and Jerker, P. (1968), *Acta Chem. Scand.* **22,** 3342.
31. Lustenberger, P., Formstecher, P., and Dautrevaux, M. (1980), *J. Chromatogr.* **193,** 451.
32. Manecke, G. and Polakowski, D. (1981), *J. Chromatogr.* **215,** 13.
33. Hanley, M. B., Churms, S. C., and Leisegang, E. C. (1967), *Chem. Commun.* **2,** 78.
34. Sixl, F. and Watts, A. (1985), *Biochemistry* **24,** 7906.
35. Sixl, F. and Galla, H. (1982), *Biochim. Biophys. Acta* **693,** 466.
36. Schindler, M. and Osborn, M. J. (1979), *Biochemistry* **18,** 4425.