THE INTERACTION OF POLYMYXIN E WITH BACTERIAL AND OTHER LIPIDS

by

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INTRODUCTION

In previous studies on the bactericidal action of polymyxin E[1,2,3] it has been shown that the treatment of suspensions of susceptible bacteria with the antibiotic results in a leakage of soluble cellular material from the organisms. To account for this effect, it has been suggested that polymyxin E combines with and thereby disorganises a cellular membrane within the organisms[4]. Such a mechanism presumes the presence of a membrane within the cell wall, which is capable of retaining low molecular weight cell solutes. The work of WEIBULL[5] has provided direct evidence for the presence of such a membrane underlying the cell wall of *Bacillus megaterium*. In addition, the examination of mechanically disrupted bacterial cells in the electron microscope has indicated the existence of a cellular membrane in other Gram positive[6] and Gram negative bacteria[4].

There is very little information concerning the chemical composition of bacterial membranes or of the nature of the sites which may be involved in the reaction with polymyxin E. Such information as is available suggests the presence of both lipid and protein in the membranes[7,8]. Whilst from a comparison of the competition between polymyxin and certain metallic cations for sites within the bacterial cell, NEWTON[9] has suggested that the polymyxin-combining groups in *Pseudomonas aeruginosa* may be polyphosphates.

In order to obtain further information on the mechanism by which polymyxin E exerts its bactericidal action, an investigation has been made of the interaction of polymyxin E with orientated monolayers of phospholipids and lipids of bacterial origin. The effects of the lipids in antagonizing the bactericidal action of polymyxin E has also been studied.

MATERIALS AND METHODS

Polymyxin E. A crystalline sample of polymyxin E sulphate, kindly given by Dr. J. F. TREVAN, was used.

Organisms. A polymyxin E sensitive strain of *Pseudomonas denitrificans* and a resistant strain of *Staphylococcus aureus*, previously investigated[1,3,4], were used. The growth conditions and harvesting were as described elsewhere[3,4].

Phospholipids. Highly purified samples of lecithin and cardiolipin were supplied by Dr. M. PANGBORN. Cephalin was obtained from a chloroform extract of ox red cell stroma by precipitation with ethanol[10]. Paper chromatography of acid hydrolysates of this lipid fraction demonstrated the

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presence of serine in addition to ethanolamine. Thus this preparation contained both phosphatidyl serine and phosphatidyl ethanolamine but has been referred to as cephalin.

Preparation of bacterial fractions for lipid extraction

Suspensions of *Ps. denitrificans* in 0.01 *M* phosphate buffer pH 6.3 were disrupted in a Mickle disintegrator by the method described previously[3,4]. Care was taken to limit the shaking process so that the inner secondary layer, (cytoplasmic membrane[4]) was retained largely within the cell walls of the organism. The disrupted bacteria were washed free from contaminating cell cytoplasm and intact cells by differential centrifugation with the phosphate buffer, and were finally resuspended in distilled water and freeze dried.

Buffered suspensions of *Staph. aureus* were similarly disrupted. On centrifugation of the suspension at 3000 *g* for 20 min two layers were deposited, the lower being white and opaque and the upper dark yellow and opalescent. The pigmented layer was redispersed in the supernatant fluid, which was then decanted. The lower layer, which consisted of cell walls and a small quantity of intact cells, was submitted to differential centrifugation with the phosphate buffer to remove the intact cells. The cell walls were then resuspended in distilled water and freeze dried. The original supernatant, obtained after centrifugation of the suspension of disrupted *Staph. aureus*, was then centrifuged at 18,000 *g* for 1 h. This caused the deposition of the pigmented material, which was washed by centrifugation with the phosphate buffer, resuspended in distilled water and freeze dried. This dark yellow fraction has been isolated previously from *Staphylococci* by MITCHELL AND MOYLE[8] and COOPER[11] and has been shown by these authors to consist mainly of very small spherical particles. It has been suggested by MITCHELL AND MOYLE[8] that this small particle fraction, which contains a large proportion of lipid, may form a continuous layer or membrane beneath the cell wall of the intact *Staphylococci*.

Lipid extraction

The lipid extraction procedure was carried out on approximately 100 mg samples of the three bacterial fractions.

The lipids were obtained by first refluxing each of the bacterial fractions with 2 ml of 95% methanol for 1 h to disrupt any lipo-protein complexes (REICHERT[12]). The methanol was distilled off and the fractions dried *in vacuo* over P_2O_5 for 2 h. They were then each refluxed with 4 ml of Na dried diethyl ether for 30 min. After this time the ethereal extracts were collected and the process of refluxing repeated with fresh samples of ether. It was found that three extractions were sufficient to remove the ether soluble lipids from the fractions. The ethereal extracts from each fraction were transferred quantitatively to weighing bottles. After evaporation of the ether, the lipids were dried for 16 h *in vacuo* over P_2O_5, weighed, and dissolved in ether.

It was found that only a very small amount of lipid could be ether-extracted from the *Staphylococcal* cell walls, and it was possible only to investigate the properties of monolayers of this lipid and their interaction with polymyxin E.

Total nitrogen and total phosphorus analyses were made in duplicate on 1 mg samples of the lipids using the micro-methods described by UMBREIT, BURRIS AND STAUFFER[13]. These analyses were not carried out on the *Staphylococcal* cell wall lipid.

Paper chromatography

2 mg samples of the bacterial lipids were hydrolysed in sealed tubes containing 1 ml of 6 *N* HCl for 48 h at 100°. The chilled and filtered hydrolysates were evaporated and dried *in vacuo* over P_2O_5 and KOH pellets, redissolved in a small volume of distilled water and again dried. The residues were then dissolved in 0.025 ml of distilled water and samples submitted to uni-dimensional paper chromatography. Chromatograms were run for 18 h using Whatman No. 1 filter-paper with the solvent system *n*-butanol/acetic acid (10:3 v/v) saturated with water. Test mixtures of serine, ethanolamine and choline were also run with the lipid hydrolysates. The primary amino compounds were detected by the ninhydrin method. Choline was detected on separate chromatograms by the phosphomolybdic acid method of CHARGAFF, LEVINE AND GREEN[14].

Monolayer technique

Monomolecular films of the lipids were studied at the air/liquid interface using a Langmuir-Adam surface balance. Spreading solutions of the phospholipids were prepared using a mixed alcohol/benzene (1:9 v/v) solvent; the bacterial lipids were generally spread from 60–80° petroleum ether. Measurements were made at 20° ± 1. The reactions of spread monolayers of the lipids were investigated by injecting aqueous solutions of polymyxin E, with thorough mixing, into the trough solution underlying the monolayers. The reactions were followed by noting the increase in surface pressure (decrease of interfacial tension) with time at a constant monolayer area, *i.e.* "constant area penetration"[15]. Where necessary, corrections were applied to the observed surface pressure readings to compensate for the change in surface tension on the reference side of the surface balance

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when polymyxin E was added to the trough solution. Since polymyxin E is only slightly surface active at the air/liquid interface[3] this correction, added to the observed surface pressure, amounted to only 0.8 dyne/cm at the highest concentration of polymyxin E used in the penetration experiments.

Bulk reactions of the lipids with polymyxin E

The lipids were dissolved at 1 mg/ml in a mixed ether/alcohol solvent. 0.05 ml samples were added to a series of 1 ml sterile solutions of polymyxin E (0–80 μg/ml) in 0.01 *M* phosphate buffer, pH 6.3, contained in 1 × 7.5 cm tubes. After 15 min the tubes were inoculated with 1 ml suspensions of the polymyxin sensitive strain of *Ps. denitrificans* at 0.02 mg bacterial dry wt/ml, and the tubes incubated at 25° for 20 min. Suitable controls without the addition of the lipids were also set up and incubated. Dilutions of the suspensions were then made in nutrient broth, and the broth suspensions plated out on agar medium. The plates were read after 24 h incubation at 37°.

RESULTS

Table I shows the results obtained for the lipid extraction of the bacterial fractions and some properties of the lipids. Choline was not detected among the nitrogenous constituents of the *Pseudomonas* lipid and *Staphylococcal* small particle fraction lipid, whereas both serine and ethanolamine were identified in hydrolysates of these lipids. In addition to these two constituents another unidentified ninhydrin-reacting substance was present in hydrolysates of the *Staph. aureus* small particle fraction lipid. In the solvent system used for the chromatography this substance had an R_F value approximately half that of ethanolamine. LEVINE AND CHARGAFF[16] have also reported the presence of minor nitrogenous constituents, in addition to choline, ethanolamine and serine, in hydrolysates of beef brain phosphatides.

TABLE I

LIPID EXTRACTION OF THE BACTERIAL FRACTIONS

Bacterial fraction	*Lipid* extracted*	*Total N***	*Total P***	*Choline*	*Ethanolamine*	*Serine*
Disrupted cells of *Ps. denitrificans*	12.8	1.1	4.3	—	+	+
Cell walls of *Staph. aureus*	0.8	not determined				
Small particle fraction of *Staph. aureus*	22.3	0.8	1.8	—	+	+

* Lipid extraction expressed as the percentage dry wt of the appropriate bacterial fraction.
** N and P analyses given as the percentage contained in the lipid material.

The lipid material isolated from the disrupted cells of *Ps. denitrificans* was found to contain a high percentage of phosphorus, this being of the same order as that found in many phosphatides. However, the nitrogen analyses indicated that this lipid cannot consist entirely of nitrogen-containing phosphatides, and it is probable that a significant proportion (c. 50%) of this lipid mixture consists of phosphatidic acids. It is relevant here to mention that FOLCH[17] has identified phosphatidyl inositol as a constituent of the phosphatides present in brain cephalin.

The nitrogen and phosphorus analyses of the lipid mixture isolated from the small particle fraction of *Staph. aureus* show that these elements were present in equimolecular amounts. The quantities of these elements present would suggest that phosphatides could account for only approximately 50% of the lipids in the mixture. Other ether-soluble materials such as the sterols and fatty acids may contribute to this lipid mixture.

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Monolayer characteristics of the lipids

The properties of monolayers of lecithin, cephalin and cardiolipin have been reported elsewhere[10].

Monolayers of the bacterial lipids were investigated on trough sub-solutions of 0.145 *M* NaCl adjusted with HCl or NaOH to give solutions at pH 2, 6 and 10. Fig. 1 gives the force/area (F/A) curves of the *Ps. denitrificans* lipid. The monolayers were progressively expanded as the trough solution pH was increased from 2 to 10, although at surface pressures greater than 16 dynes/cm the F/A curves converged and above this surface pressure showed little if any dependence upon pH. This effect of pH can be ascribed to the ionization of acidic groups (phosphate, carboyxl) present in the monolayer, which results in an increased electrostatic repulsion between these groups when the trough solution pH is increased. A similar pH dependence has been observed with monolayers of cardiolipin and cephalin[10].

The F/A curves of the lipid isolated from the small particle fraction of *Staph. aureus* are given in Fig. 2. Monolayers of this lipid were unaffected by a change of the sub-solution pH from 2 to 6, whilst at pH 10 expansion of the monolayer occurred. Fig. 3 shows the F/A curves of the lipid obtained from the cell walls of *Staph. aureus*. Very little dependency upon trough sub-solution pH was observed with this lipid, although slightly greater areas were found for the monolayers at pH 2 when compared with those at pH 6 and 10.

It was considered that in view of the very low lipid content of the *Staphylococcal* cell wall preparation this lipid might have been derived from a contamination of the cell walls with traces of the lipid-rich small particle fraction. However, the properties of monolayers of the two *Staphylococcal* lipids demonstrate that these lipids differ considerably in their overall composition. The presence of very small quantities of lipid material in the cell walls of several other Gram positive bacteria has been reported by SALTON[18].

Constant area penetration of lipid monolayers by polymyxin E.

For the penetration experiments, the lipids were spread upon 0.01 *M* phosphate buffer at pH 6.8 and the monolayers compressed to a surface pressure of 5.6 dyne/cm. The effect of adding polymyxin E to the trough sub-solution underlying monolayers of the *Ps. denitrificans* lipid compressed initially to 5.6 dyne/cm. is recorded in Fig. 4. After the addition of polymyxin E the surface pressure rose rapidly. This occurred as a result of the electrostatic binding of the lipid polar groups with those of the polymyxin E, and the association between the non-polar portions of the lipid and the penetrating molecule. The surface pressure continued to rise for several minutes, and then reached a steady value. Similar results were obtained for the other lipids, with the exception of monolayers of lecithin which were found to be unreactive to the injected polymyxin E. The polymyxin E/lipid monolayer reactions were studied with a range of polymyxin E concentrations and the resultant surface pressure rises (interfacial tension decreases) noted by extrapolating the linear portions of the reaction isotherms to zero time. By this means penetration isotherms were obtained by plotting the extrapolated pressure rises against the concentration of polymyxin E present. Fig. 5 shows the constant area penetration isotherms for all the lipids studied. The small surface pressure changes observed with lecithin monolayers are no greater than those expected for the free adsorp-

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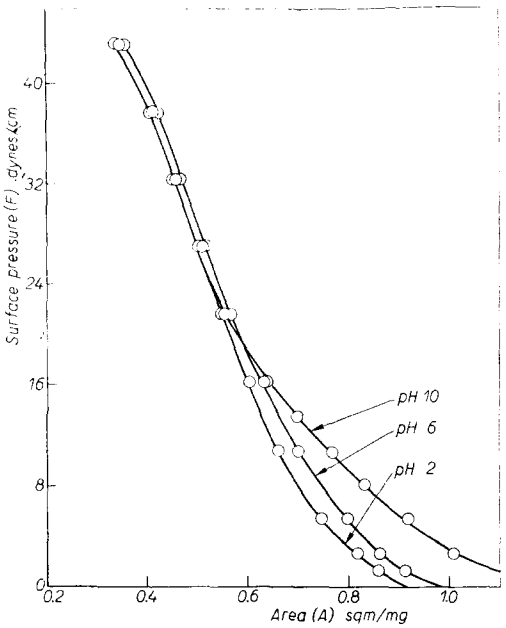

Fig. 1. Force/area curves of lipid derived from disrupted cells of *Ps. denitrificans*, spread upon 0.145 *M* NaCl, pH adjusted with NaOH or HCl. T = 20°.

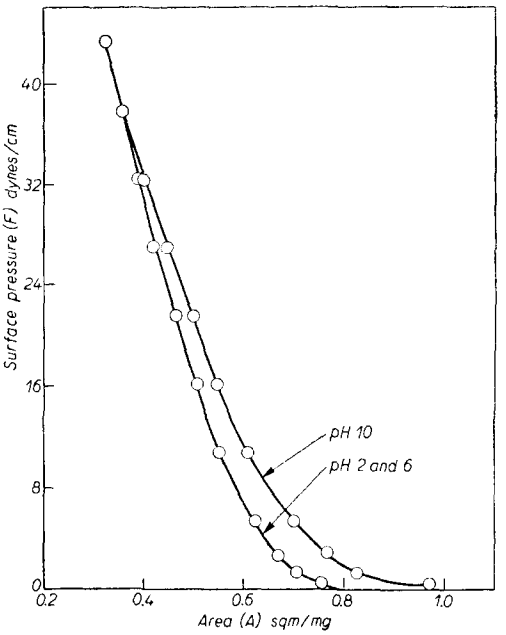

Fig. 2. Force/area curves of lipid derived from the small particle fraction of *Staph. aureus*, spread upon 0.145 *M* NaCl, pH adjusted with NaOH or HCl. T = 20°.

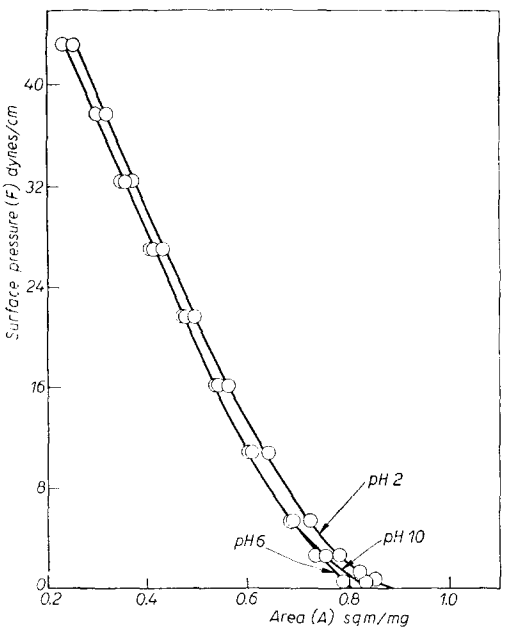

Fig. 3. Force/area curve of lipid derived from the cell walls of *Staph. aureus*, spread upon 0.145 *M* NaCl, pH adjusted with NaOH or HCl. T = 20°.

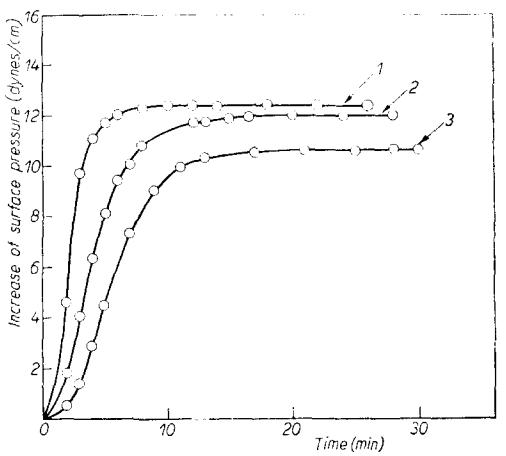

Fig. 4. The penetration of polymyxin E into monolayers of the lipid isolated from disrupted cells of *Ps. denitrificans*. Lipid monolayers spread on 0.01 *M* phosphate buffer pH 6.8 and compressed initially to 5.6 dynes/cm. Polymyxin E added to the trough solution to give concentrations of 5 μg/ml (Curve 1), 2.5 μg/ml (Curve 2), and 1.25 μg/ml (Curve 3). T = 20°.

tion of polymyxin E at the interface. The presence of the lecithin monolayer apparently has no effect on this adsorption.

Greatest reactivity towards polymyxin E was obtained with monolayers of cephalin and the *Ps. denitrificans* lipid. Complex formation also occurred with the monolayers of cardiolipin and the lipids derived from the *Staphylococcal* fractions, although in these cases the resulting changes in interfacial tension were not so great. Direct comparisons of the effect of polymyxin in E upon monolayers of the lipids can be made since the monolayers all possess similar compressibilities over the surface pressure range studied. The changes in surface pressure can be interpreted as a measure of the ability of the polymyxin E to enter the surface phase and complex with the film forming molecules.

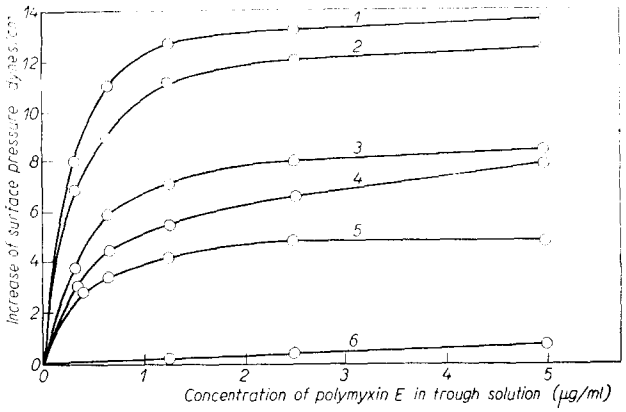

Fig. 5. The penetration of lipid monolayers by polymyxin E. Lipids spread upon 0.01 *M* phosphate buffer pH 6.8 and compressed to 5.6 dynes/cm. Curve 1 = Cephalin, Curve 2 = Lipid from disrupted cells of *Ps. denitrificans*, Curve 3 = Lipid from small particle fraction of *Staph. aureus*, Curve 4 = Cardiolipin, Curve 5 = Lipid from the cell walls of *Staph. aureus*, Curve 6 = Lecithin. T = 20°.

Bulk reactions of the lipids with polymyxin E

The effects of the lipids upon the bactericidal action of polymyxin E are shown in Table II. With the exception of lecithin, all the lipids studied caused the bactericidal end point to be raised. The phosphatidic acid, cardiolipin, was found to be the most effective with the given experimental conditions. The presence of 25 μg/ml of this lipid was found to raise the bactericidal concentration of polymyxin E from between 1.25 and 2.5 μg/ml, without lipid, to between 10 and 15 μg/ml. Thus approximately 10 μg of polymyxin E is rendered biologically inactive by 25 μg of cardiolipin. This ratio corresponds closely with that expected if the three ionizing phosphate groups in the cardiolipin molecule (M.W. 2,200)[19] react with the four available amino groups of the polymyxin E molecule (M.W. 1,250)[20]. The lipid isolated from the disrupted cells of *Ps. denitrificans* and cephalin also caused a significant inactivation of the polymyxin E, whilst the lipid obtained from the *Staphylococcal* small particle fraction produced a slight increase in bactericidal end point. In this connection, BLISS, CHANDLER AND SCHOENBACH[21] found that soap and phospholipid extracts of soya bean exerted a slight antagonistic action towards polymyxin. Prior to the addition of the bacterial suspensions, it was observed that insoluble complexes were formed between the lipids and polymyxin E in the tubes containing the higher concentrations of polymyxin E. This occurred in all cases with the exception of systems containing lecithin. The formation of water insoluble complexes

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between polymyxin and unspecified phospholipids has also been reported by LATTERADE AND MACHEBOEUF[22].

TABLE II

EFFECT OF LIPIDS ON POLYMYXIN E ACTIVITY

Bacteria: *Ps. denitrificans* 0.01 mg dry wt/ml., 0.01 *M* Phosphate buffer pH 6.3. Lipids at 25 μg/ml.

Polymyxin E conc. μg/ml	*0*	*0.31*	*0.63*	*1.25*	*2.5*	*5.0*	*10*	*20*	*40*
Control (no lipid)	+	+	+	+	—	—	—	—	—
Lecithin	+	+	+	+	—	—	—	—	—
Cardiolipin	+	+	+	+	+	+	+*	—*	—*
Ps. denitrificans lipid	+	+	+	+	+	+	—*	—*	—*
Staph. aureus small particle fraction lipid	+	+	+	+	+	—	—*	—*	—*
Cephalin	+	+	+	+	+	+	—*	—*	—*

* Precipitate formed in tubes before addition of bacteria.
\+ Growth on subculture; — No growth on subculture.

DISCUSSION

The data presented here show that the lipid monolayers vary considerably in their reactivity towards polymyxin E. Of particular interest is the case of lecithin. The absence of any surface complex formation with the lecithin monolayers indicates that the positively charged choline group effectively shields the ionized phosphate group. This prevents an electrostatic bonding between the monolayer phosphate groups and the amino groups of the polymyxin E molecule, which would be necessary for penetration of the monolayer to occur. It has been observed that monolayers of cephalin are more strongly penetrated by polymyxin E than those of the phosphatidic acid, cardiolipin. With the pH conditions used, monolayers of both these lipids possess a net negative charge, which is due to the phosphate groups, and which facilitates interaction with the cationic polymyxin E. However, it is evident that, with cephalin monolayers, the specific interaction of polymyxin E with other hydrophilic groups, in addition to that occurring with the lipid phosphate group, leads to an enhancement of the penetration.

Strong surface complex formation has been shown to take place between polymyxin E and monolayers of the lipid derived from the disrupted cells of *Ps. denitrificans*, a polymyxin sensitive organism. It is possible, therefore, that the observed bactericidal action of polymyxin E[1,3] may be due to the reaction of the antibiotic with the lipid components of the bacterial membrane. It is considered that this reaction produces an increase in surface pressure (decrease in interfacial tension) at the membrane which is sufficient to cause a modification of the membrane structure. In support of this suggestion it has been shown that the reaction between polymyxin E and monolayers of the lipid isolated from the disrupted cells of *Ps. denitrificans* occur, and are completed, at antibiotic concentrations which are bactericidal for susceptible organisms[1].

Moderate complex formation also occurs with polymyxin E and monolayers of the lipid obtained from the small particle fraction (cellular membrane[8]) of *Staph. aureus*, an organism resistant to polymyxin E. However, the demonstration of a reaction between polymyxin E and isolated structural components of the bacterial cell does not necessarily imply that such a reaction will occur in the intact cell. The antibiotic must be able to

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penetrate readily to the susceptible site. For the case of polymyxin E, it has been observed previously[1,3] that whereas sensitive bacteria rapidly absorb large quantities of the antibiotic, resistant organisms, under the same conditions, only poorly absorb polymyxin E. In addition, a study of haemolysis by ionic detergents[23], a process analogous to the action of ionic surface active agents upon bacteria[24], has shown that complete lysis only occurs when a critical surface pressure is attained at the red cell surface. The critical surface pressure is reached by complex formation between the stroma cholesterol and detergent lysin. If a similar mechanism applies for bacteriolysis by surface active compounds, the absence of any rapid cytolytic action when resistant bacteria are exposed to polymyxin E can be attributed to both the poor absorption of the antibiotic and to the inability of polymyxin E to produce a sufficient increase in surface pressure at the membrane.

The ability of the lipids to antagonize the bactericidal action of polymyxin E did not follow exactly the reaction sequence found for the penetration of the lipid monolayers by polymyxin E. This is not unexpected in view of the differences in particle size and accessibility of the reactive groups of the lipids when present in aqueous dispersions. Thus the polymyxin E reactivity sequence found for the penetration of the lipid monolayers, which are orientated at the interface, must be regarded as giving a more precise indication of the molecular associations. It has been suggested by BAKER, HARRISON AND MILLER[25] that phospholipids may protect the bacterial cell from the toxic effects of detergents by altering the structure of the cellular membrane so as to prevent penetration by the detergents. It does not appear necessary to suggest such a mechanism in the present case. The action of the lipids can be adequately explained by the formation of biologically inactive complexes with polymyxin E in the suspension medium. Similarly, SCHULMAN AND RIDEAL[26] have shown that haemolysis by sodium cetyl sulphate is inhibited by the simultaneous addition of cholesterol, the inhibition being due to the formation of a strong complex between cholesterol and the detergent.

ACKNOWLEDGEMENTS

The author wishes to thank the Medical Research Council for a personal grant, and Dr. J. H. SCHULMAN, O.B.E., for helpful discussions.

SUMMARY

The reactions of polymyxin E with several lipids have been investigated using the monolayer technique. Strong complex formation has been shown to occur between polymyxin E and monolayers of a cephalin preparation and with monolayers of the lipid material isolated from disrupted cells of *Ps. denitrificans*, a polymyxin-susceptible organism. Moderate complex formation also occurred between polymyxin E and monolayers of cardiolipin and the lipid material obtained from two *Staphylococcal* fractions, whilst lecithin monolayers were unreactive towards polymyxin E. The relationship of these results with the suggestion that polymyxin E causes a disorganisation of the cellular membrane of susceptible organisms is discussed.

The antagonistic effects of several of the lipids upon the bactericidal action of polymyxin E has been ascribed to the formation of biologically inactive complexes in the bacterial suspension medium.

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RÉSUMÉ

Les réactions de la polymyxine E avec divers lipides ont été étudiées par la technique des couches monomoléculaires. La polymyxine E forme aisément des complexes avec des couches monomoléculaires de céphaline, ainsi qu'avec des couches monomoléculaires d'un lipide isolé de cellules désagrégées de *Ps. denitrificans*, organisme sensible à la polymyxine. Elle forme moins facilement des complexes avec des couches monomoléculaires de cardiolipine et d'un matériel lipidique extrait de deux fractions de *staphyloccoques*; des couches monomoléculaires de lécithine ne réagissent pas avec la polymyxine E. La relation entre ces résultats et l'hypothèse selon laquelle la polymyxine E provoquerait la désorganisation de la membrane cellulaire des organismes sensibles est discutée.

L'action antagoniste de quelques lipides sur l'activité bactéricide de la polymyxine E est attribuée à la formation de complexes biologiquement inactifs dans le milieu de suspension des bactéries.

ZUSAMMENFASSUNG

Es wurden die Reaktionen von Polymixin E mit verschiedenen Lipoiden unter Verwendung der Oberfächenfilmtechnik untersucht. Starke Komplexbildung wurde beobachtet zwischen Polymixin E und Filmen einer Cephalinpräparation und Filmen aus Lipoiden, die aus zerstörten Zellen von *Ps. denitrificans*, einem Polymyxin empfindlichen Organismus, hergestellt wurden. Eine mittlere Komplexbildung trat auf zwischen Polymyxin E und Filmen aus Cardiolipin und Lipoiden, die aus *Staphylococcen*-Fraktionen erhalten wurden. Filme aus Lezithin dagegen reagierten nicht mit Polymyxin E. Es werden diese Ergebnisse mit der Vorstellung, dass Polymyxin E eine Zerstörung der Zellmembran der empfindlichen Organismen hervorruft, diskutiert.

Die antagonistischen Wirkungen einiger Lipoide auf die baktericide Wirkung von Polymyxin E wird der Bildung von biologisch unwirksamen Komplexen in dem Suspensionsmedium zugeschrieben.

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Received August 21st, 1954