ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1983, p. 107–113
0066-4804/83/070107-07$02.00/0

Vol. 24, No. 1

Polycations Sensitize Enteric Bacteria to Antibiotics

MARTTI VAARA[1]* AND TIMO VAARA[2]

National Public Health Institute, SF-00280 Helsinki 28,[1] and Department of Microbiology, University of Helsinki, SF-00710 Helsinki 71,[2] Finland

Received 20 December 1982/Accepted 6 April 1983

Polymyxin B nonapeptide, a polymyxin B derivative which lacks the fatty acyl part and the bactericidal activity of polymyxin, was shown to sensitize smooth encapsulated *Escherichia coli* (O18:K1) and smooth *Salmonella typhimurium* to hydrophobic antibiotics (novobiocin, fusidic acid, erythromycin, clindamycin, nafcillin, and cloxacillin). The polymyxin B nonapeptide-treated bacteria were as sensitive to these antibiotics as are deep rough mutants. A lysine polymer with 20 lysine residues ($lysine_{20}$) had a largely similar effect. Larger lysine polymers and the protamine salmine were bactericidal but, at sublethal concentrations, sensitized the strains to the antibiotics mentioned above, whereas $lysine_4$, streptomycin, cytochrome *c*, lysozyme, and the polyamines cadaverine, spermidine, and spermine had neither bactericidal nor sensitizing activity.

Gram-negative bacteria have an exceptionally efficient barrier against many external influences: the outer membrane (OM), a unique structure located outside the peptidoglycan. This membrane makes gram-negative bacteria resistant to host defense factors, such as lysozyme, which are toxic to gram-positive bacteria. In gram-negative bacteria that live in the gut, the OM is an effective barrier, also giving protection from bile and digestive enzymes (17, 21). At the same time, their OM excludes many antibiotics (e.g., erythromycin, lincomycin, clindamycin, novobiocin, fusidic acid, nafcillin, and cloxacillin) (10, 21) and reduces the penetration of several others (especially penicillin, ampicillin, carbenicillin, and most cephalosporins) (21, 24, 29, 39). The well-known difficulty of treating gram-negative infections is partially due to the permeability barrier properties of the OM, suggesting the possibility of making the bacteria sensitive to many more antibiotics by disorganizing their OM.

But is this disorganizing possible? Polymyxin, a polycationic amphipathic antibiotic, is bactericidal to gram-negative bacteria by virtue of a dual mechanism of action: it disorganizes and penetrates the OM to reach its final bactericidal target, the cytoplasmic membrane (28, 31). Another OM-disorganizing agent is EDTA (reviewed by Leive in reference 16). Unfortunately, both polymyxin and EDTA are also toxic to eucaryotic cells and therefore do not seem useful as therapeutic agents.

Chihara et al. (5, 6) have previously shown that the removal of the fatty acid tail from polymyxin significantly reduces its toxicity to eucaryotes; however, the antibacterial activity of the drug is lost at the same time. Could this modified but still cationic polymyxin B nonapeptide derivative (PMBN) still disorganize the OM to facilitate the entry of antibiotics?

In this paper, we show that a number of polycationic agents, including PMBN, sensitize smooth *Escherichia coli* and *Salmonella typhimurium* strains to several antibiotics by a factor of 100 or more. We suggest that these agents do so by reducing the permeability barrier function of the OM. In an accompanying paper (34), we describe in more detail the action of these polycations on the OM permeability barrier and on the OM morphology which supports this suggestion.

MATERIALS AND METHODS

Bacterial strains. In most experiments, smooth *S. typhimurium* and *E. coli* strains were used. The *S. typhimurium* strain was SL696 (*metA22 trpB2 H1-b H2-e,n,x fla-66 rpsL120*) (38) of the LT2 cell line, and the *E. coli* strain was IH3080 (EM40) (23), an O18:K1 strain isolated from the cerebrospinal fluid of a neonate with meningitis. Strain SL696 was sensitive to the smooth-specific phages of *S. typhimurium* (P22C2 and 9NA) (38) but resistant to the R-specific phages (6SR, Ffm, Br2, Br60, P221, and C21) (38). Strain IH3080 was sensitive to the lipopolysaccharide-specific phage C21 and to the K1 capsule-specific phage K1a (12) and agglutinated latex particles coated with K1 antibodies (Orion Diagnostica, Helsinki, Finland). These O and K properties of the strains were verified throughout the study. In some experiments, the deep rough Re mutant SL1102 (38) of the *S. typhimurium* LT2 cell line was also used.

Cultivation of bacteria. Bacteria were grown in Luria broth (18) at 37°C on a rotary shaker (220 rpm) until they reached the early logarithmic phase of growth (40 Klett units; Klett-Summerson colorimeter, red filter),

washed with 0.9% NaCl, and resuspended in 0.9% NaCl (final optical density, 120 Klett units, corresponding to ~10^9 cells per ml). This suspension was used as the inoculum for all experiments.

Sensitivity determinations. The minimal inhibitory concentrations (MICs) of the antibiotics in the presence of polycationic agents were measured as follows. Davis minimal medium (25) with added glucose (1 g/liter), Casamino Acids (1 g/liter; Difco Laboratories, Detroit, Mich.); L-tryptophan (20 mg/liter), and increasing amounts of the antibiotic to be tested was inoculated with 10^4 cells per ml from a fresh suspension of the bacteria. Samples (200 μl) of this inoculated medium were pipetted into wells of a microtiter plate (Titertek, catalog no. 76-21305; Flow Laboratories, Inc., Rockville, Md.). Each well already contained increasing amounts of the polycationic agent to be tested in 30 μl of 0.9% NaCl. The plates were sealed with adhesive tape and incubated at 37°C for 18 h. The lowest concentration of antibiotic that completely inhibited visible growth was recorded and interpreted as the MIC.

Davis minimal medium was used to avoid precipitation of some polycations (salmine, polylysines), which took place in complex media. The MIC of each polycationic agent was measured in a similar manner (antibiotics were omitted in the growth medium). If the growth was less turbid in the presence of the polycation than in the control tubes, the polycation was regarded to retard growth. Bactericidal action and bacteriostatic action were differentiated by viable count determination after incubation.

Polycationic agents. Polymyxin B sulfate, cytochrome *c*, egg white lysozyme, spermine-hydrochloride, spermidine-hydrochloride, cadaverine-hydrochloride, and poly-L-lysine hydrobromides (with approximately 20, 50, 115, or 300 lysine residues) were obtained from Sigma Chemical Co., St. Louis, Mo.; streptomycin sulfate was obtained from Hoechst AG, Frankfurt (Main), West Germany; L-Lysine$_4$-HCl was obtained from Miles-Yeda Ltd., Rehovoth, Israel; and salmine sulfate (protamine) was obtained from BDH Ltd., Poole, England. Thermolysin-treated salmine was prepared by incubating 150 mg of salmine with 7 mg of thermolysin (type x; Sigma) in 3.5 ml of 0.03 M Tris-hydrochloride (pH 8.0) at 37°C for 16 h. Thin-layer chromatography of the hydrolysate (cellulose-coated aluminium foil; *n*-butanol–pyridine–acetic acid–water [30:20:6:24, vol/vol]; Sakaguzi staining for arginyl residues) did not reveal the salmine spot at origin but showed a new heterogenous spot with an R_f of ~0.10. The preparation of PMBN is described below.

PMBN. (i) Preparation. PMBN was prepared by an enzymatic hydrolysis of polymyxin B as originally developed by Chihara et al. (6) for preparing colistin nonapeptide from colistin (polymyxin E).

Fifty-two milligrams of polymyxin B sulfate (corresponding to ~37 mg of polymyxin B) was dissolved in 4 ml of 0.07 M potassium phosphate buffer (pH 7.2), after which 1 ml of ficin suspension (EC 3.4.22.3; P 4125, containing 25 mg of protein; Sigma) was added. The mixture was incubated at 37°C for 3 h with light shaking and then stirred in boiling water for 5 min, and the formed precipitate (denatured ficin) was removed.

(ii) Purification. The solution was acidified with 50 μl of 1 N HCl, washed twice with 2.5 ml of *n*-butanol, and then alkalified with 150 μl of 1 N NaOH and again washed twice with 2.5 ml of *n*-butanol. A 2.6-ml amount of the solution was then mixed with a suspension of 44 ml of CM-Sephadex C 50 (0.25 g/100 ml of deionized water; Pharmacia Fine Chemicals AB, Uppsala, Sweden), incubated at 37°C for 5 min with shaking, centrifuged (1,000 × *g*, 10 min), and washed with 20 ml of deionized water. PMBN was eluted from the Sephadex beads by two lots of 20 ml of 10% pyridine–10% acetic acid in water, lyophilized, dissolved in deionized water, and stored at −20°C.

(iii) Yield and purity. The PMBN concentration was calculated from the amount of free amino groups found in an amino group determination with a molecular weight of 950. Polymyxin B sulfate was used as a control. The yield was 63%. The aqueous solution of PMBN preparation (1 mg/ml) was neutral (pH 7).

For purity analysis, the preparation was run in thin-layer chromatography with cellulose-coated aluminium foil (E. Merck AG, Darmstadt, West Germany) and the solvent system *n*-butanol–pyridine–acetic acid–water (30:20:6:24, vol/vol). The spots were visualized by ninhydrin. The R_fs were 0.56, 0.48, and 0.77 for polymyxin B, PMBN, and fatty acyl diaminobutyric acid (formed in the enzymatic treatment), respectively. Thin-layer chromatography of the PMBN preparation (maximal amount, 20 μg) revealed a spot with an R_f of 0.48, whereas 0.05 μg of polymyxin B still gave a visible spot with an R_f of 0.56, indicating that the PMBN preparation contained less than 0.25% polymyxin. When 0.1 μg of polymyxin B sulfate was mixed with 10 μg of the PMBN preparation, it still migrated separately (R_f = 0.56) from PMBN.

Antibiotics. The following antibiotics were used: novobiocin (sodium salt), Sigma; fusidic acid (sodium salt), Lovens Kemiske Fabrik, Copenhagen, Denmark; erythromycin ethylsuccinate, Orion, Helsinki, Finland; clindamycin hydrochloride, The Upjohn Co., Kalamazoo, Mich.; nafcillin (sodium salt), Wyeth Laboratories, Philadelphia, Pa.; cloxacillin (sodium salt), Astra, Södertälje, Sweden; and benzylpenicillin (sodium salt), Novo Industri, Copenhagen, Denmark. The stock solution of erythromycin was prepared by dissolving 5.5 mg of erythromycin ethylsuccinate with 2 ml of 96% ethanol, after which deionized water was added to a final volume of 5.5 ml. Other antibiotics were readily dissolved in deionized water.

RESULTS

We have previously shown that salmine (the protamine isolated from salmon sperm) and polylysine, as well as polymyxin, sensitize the rough Rb_2-type *S. typhimurium* strain to deoxycholate (33). In the present study, a larger selection of different polycationic agents was included: polyamines with 2 (cadaverine), 3 (spermidine), or 4 (spermine) amino groups; lysine polymers with 4, ~20, ~50, ~115, and ~300 free ε-amino groups; the basic proteins lysozyme (molecular weight [mw], 14,000; 13% basic amino acids) and cytochrome *c* (mw, 12,000; 20% basic amino acids) as well as the protamine salmine (mw, 4,000; containing 21

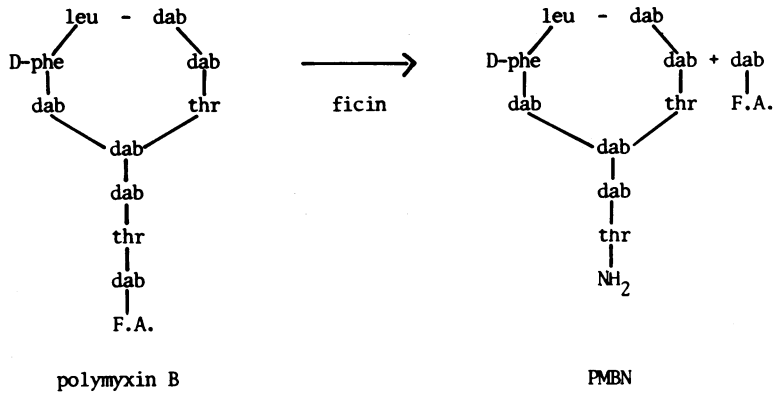

FIG. 1. Structural formulas of polymyxin B and its derivative, PMBN, prepared by enzymatic (ficin) treatment. Abbreviations: dab, diaminobutyric acid; phe, phenylalanine; thr, threonine; leu, leucine; F.A., fatty acyl group. Polymyxin E differs from polymyxin B in having D-leucine instead of D-phenylalanine.

arginine residues) and a hydrolysate of it (fragments with only 4 to 6 arginines) (1). Polymyxin B was used as a reference compound. In addition, we prepared PMBN (Fig. 1). Purity analyses showed that the PMBN preparation contained no detectable amounts of polymyxin B (polymyxin B content, <0.25%).

As test bacteria, we used a smooth strain of *S. typhimurium* (LT2 strain SL696) and an encapsulated, smooth strain of *E. coli* (O18:K1 strain IH3080) isolated from the cerebrospinal fluid of a neonate with meningitis.

Intrinsic antibacterial activities of polycations. The intrinsic antibacterial activities of the polycations are shown in Table 1. Polymyxin B had by far the most potent antibacterial activity. Protamine and the larger polylysines (lysine polymers with ≥50 lysine residues [$lysine_{\geq 50}$]) were also antibacterial. $Lysine_{20}$ was clearly less active than these against *E. coli* and totally inactive against *S. typhimurium*. PMBN did not have any effect on *S. typhimurium* but retarded the growth of *E. coli*. $Lysine_4$, polyamines, lysozyme, cytochrome *c*, and the protamine fragments did not inhibit the growth of either strain. All the antibacterial polycations were bacteriostatic at the MIC but exhibited a clear-cut bactericidal activity at a concentration 3 to 10 times the MIC. No synergy or antagonism could be observed; the MIC of protamine (against *S. typhimurium*) was unchanged when it was tested in the presence of concentrations of up to 100 μg of the noninhibitory polycations $lysine_{20}$ or PMBN per ml.

Effect of polycations on sensitivity of smooth *S. typhimurium* to novobiocin and fusidic acid. Novobiocin and fusidic acid are hydrophobic antibiotics to which enteric bacteria are resistant. The deep rough mutants of enteric bacteria which have a very defective lipopolysaccharide structure and concomitantly an abnormal OM are, however, strikingly sensitive to these drugs (21, 33), as are also a number of less well-characterized mutants with defective OM (2, 7). These antibiotics were therefore used to probe the effect of the cationic agents on outer membrane permeability.

PMBN, which did not have any intrinsic antibacterial action, sensitized smooth *S. typhimurium* to novobiocin by a factor of ≥100 (Table 2). $Lysine_{20}$ gave similar results (≥100-fold increase in sensitivity). Subinhibitory concentrations (0.3 to 1.0 μg/ml) of protamine and $lysine_{50}$ were also effective in sensitization (≥100-fold increase in sensitivity to novobiocin), whereas $lysine_4$, all the tested polyamines, lysozyme, cytochrome *c*, and the protamine fragments did not sensitize

TABLE 1. MICs of various polycationic agents

Polycation	MIC (μg/ml) for[a]:	
	S. typhimurium SL696	*E. coli* IH3080
Polymyxin B	1	1
PMBN	≥100	≥100[b]
Protamine	3	3
Protamine fragments	≥100	≥100
$Lysine_4$	≥100	≥100
$Lysine_{20}$	≥100	30
$Lysine_{50}$	3	3
$Lysine_{115}$	3	3
$Lysine_{300}$	3	3
Cadaverine	≥100	≥100
Spermidine	≥100	≥100
Spermine	≥100	≥100
Lysozyme	≥100	ND[c]
Cytochrome *c*	≥100	≥100

[a] The MIC was defined as the lowest concentration of the agent that prevented visible growth of the test bacterium incubated for 18 h at 37°C.

[b] Retarded growth at ≥1 μg/ml.

[c] ND, Not done.

TABLE 2. MICs of novobiocin for *S. typhimurium* in the presence of various polycationic agents

Polycation	Novobiocin MIC (μg/ml) in the presence of polycation concn (μg/ml) of[a]:					
	0.0 or 0.1	0.3	1	3	10	30
Polymyxin B	≥100	≥100	0	0	0	0
PMBN	≥100	10	3	3	1	1
Protamine	≥100	30	3	0	0	0
Protamine fragments	≥100	≥100	≥100	≥100	≥100	≥100
$Lysine_4$	≥100	≥100	≥100	≥100	≥100	≥100
$Lysine_{20}$	≥100	3	1	1	1	1
$Lysine_{50}$	≥100	30	1	0	0	0
Cadaverine	≥100	≥100	≥100	≥100	≥100	≥100
Spermidine	≥100	≥100	≥100	≥100	≥100	≥100
Spermine	≥100	≥100	≥100	≥100	≥100	≥100
Streptomycin	≥100	≥100	≥100	≥100	≥100	≥100
Lysozyme	≥100	≥100	≥100	≥100	≥100	≥100
Cytochrome *c*	≥100	≥100	≥100	≥100	≥100	≥100

[a] The MIC was defined as the lowest concentration of novobiocin that prevented visible growth of *S. typhimurium* incubated for 18 h at 37°C. 0. The polycationic agent alone inhibited growth.

the bacterium. Because *S. typhimurium* SL696 has a ribosomal resistance to the basic aminoglycoside streptomycin, the possible effect of streptomycin on OM permeability was also tested and found to be absent. In the assay system used, sublethal concentrations (≤0.3 μg/ml) of polymyxin did not have any action on OM permeability.

The results obtained when fusidic acid was used as a probe were very similar (Table 3). Again, PMBN and $lysine_{20}$ increased the OM permeability. A slight sensitization was also caused by polymyxin B. $Lysine_4$, the polyamines, lysozyme, and streptomycin were inactive.

Sensitization of smooth *S. typhimurium* to other antibiotics by PMBN and $lysine_{20}$. Because PMBN and $lysine_{20}$ had minimal intrinsic antibacterial activities but were potent sensitizers to novobiocin and fusidic acid, the sensitization of smooth *S. typhimurium* to other antibiotics was tested in the presence of these agents (Table 4). PMBN sensitized the bacterium to all the hydrophobic antibiotics tested (erythromycin, clindamycin, cloxacillin, nafcillin). As a rule, 3 to 10 μg of PMBN per ml was needed to make the smooth strain as sensitive to these antibiotics as is the deep rough Re strain of *S. typhimurium* (Table 4). A slight sensitivity increase to penicillin, which is believed to diffuse across the OM through hydrophilic pores in the absence of cationic agents (21), was also observed.

The results were basically similar when $lysine_{20}$ was used instead of PMBN. A striking difference, however, was the poor sensitization to erythromycin and clindamycin, which are monobasic antibiotics.

Sensitization of smooth *E. coli* to antibiotics by PMBN and $lysine_{20}$. In the absence of polycations, smooth *E. coli* was slightly more sensitive than *S. typhimurium* to the antibiotics tested. PMBN sensitized the strain to all these antibiotics and also retarded bacterial growth at concentrations of ≥1 μg/ml (Table 5). At a concentration of as low as 0.3 μg/ml, PMBN increased the sensitivity of the strain to fusidic acid and erythromycin by factors of 100 and 30, respectively.

$Lysine_{20}$ was as effective in sensitization as when *S. typhimurium* was used as a test organism. When compared with PMBN, its capacity to sensitize bacteria to erythromycin and clindaycin was low, as it was in *S. typhimurium*.

DISCUSSION

A number of cationic agents have been reported as inhibitory or lethal to enteric bacteria. Besides polymyxins (28), these include protamine (14, 33) polylysine (3, 14, 33), histones (14), and the cationic polypeptides isolated from polymorphonuclear leukocytes (19, 37). A puri-

TABLE 3. MICs of fusidic acid for *S. typhimurium* in the presence of various polycationic agents

Polycation	Fusidic acid MIC (μg/ml) in the presence of polycation concn (μg/ml) of[a]:					
	0.0 or 0.1	0.3	1	3	10	30
Polymyxin B	300	100	0	0	0	0
PMBN	300	100	30	30	10	>3
Protamine	300	300	100	0	0	0
$Lysine_4$	300	300	300	300	300	300
$Lysine_{20}$	300	100	100	30	30	30
$Lysine_{50}$	300	300	30	0	0	0

[a] The MIC was defined as the lowest concentration of fusidic acid that prevented visible growth of *S. typhimurium* incubated for 18 h at 37°C. In addition, the following polycationic agents did not sensitize the test bacterium to fusidic acid: cadaverine, spermidine, spermine, streptomycin, and lysozyme. 0, The polycationic agent alone inhibited growth.

TABLE 4. MICs of various antibiotics against deep rough *S. typhimurium* (Re), smooth *S. typhimurium*, and Smooth *S. typhimurium* in the presence of PMBN or lysine$_{20}$

Antibiotic	MIC (μg/ml) for[a]:								
	S. typhimurium Re	Smooth *S. typhimurium* without polycations	Smooth *S. typhimurium* in the presence of indicated concn (μg/ml) of:						
			PMBN				Lysine$_{20}$			
			0.3	1	3	10	0.3	1	3	10
Novobiocin	3	≥100	10	3	3	1	3	1	1	1
Fusidic acid	10	300	100	30	30	10	300	100	30	30
Erythromycin	3	≥100	30	10	10	3	≥100	≥100	≥100	≥100
Clindamycin	3	100	30	10	10	10	100	100	100	30
Cloxacillin	30	≥1,000	300	100	100	30	≥1,000	100	100	30
Nafcillin	30	≥1,000	≥1,000	100	100	30	≥1,000	100	100	30
Benzylpenicillin	3	10	10	10	3	3	10	3	3	3

[a] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth of the test bacterium incubated for 18 h at 37°C.

fied β-lysin-like peptide of normal rabbit serum (mw, ~1,800; four basic amino acids) is bactericidal to both gram-positive and -negative bacteria (4). Recently, two basic bactericidal peptides (mw, ~7,000; 13 to 14 basic amino acids) have been isolated from hemolymph of the pupae of giant silk moths vaccinated with enteric bacteria (15).

Previous reports on OM permeability increase by polycationic agents other than the antibiotic polymyxin, however, are scarce. We have previously shown that protamine and polylysine, as well as polymyxin, sensitize a rough (Rb_2) strain of *S. typhimurium* to deoxycholate (33). Furthermore, the cationic leukocyte protein BP (mw, ~50,000; 15% basic amino acids) has been shown to increase the sensitivity of *E. coli* and *S. typhimurium* to actinomycin (37). Interestingly, rough mutants were significantly more sensitive to this effect than smooth strains.

In the present paper, we have shown that low concentrations (0.3 μg/ml) of various polycationic agents sensitize smooth enteric bacteria to antibiotics such as novobiocin, fusidic acid, erythromycin, clindamycin, nafcillin, cloxacillin, and penicillin. In fact, the polycations made the smooth strains as sensitive to antibiotics as is the deep rough Re strain of *S. typhimurium*, which has a very abnormal OM (21). These polycationic agents included protamine (mw, ~4,000; 21 arginine residues) and lysine$_{50}$-HBr (mw, ~10,000; 50 free NH_2 groups), which are growth inhibitory or bactericidal at higher concentrations (3 to 10-μg/ml), and PMBN (mw, ~950; 5 free NH_2 groups) and lysine$_{20}$-Hbr (mw, ~4,000; 20 free NH_2 groups), which have lower or no antibacterial activities of their own. It is likely that the permeability-increasing action of polycations is due to their strong binding to the acidic core and lipid A part of lipopolysaccharide (26, 35), which disorganizes the OM, as discussed in the accompanying paper (34).

In the assay system used, sublethal concentrations (≤0.3 μg/ml) of polymyxin caused only slight sensitization to antibiotics. We suggest that the permeability-increasing action of poly-

TABLE 5. MICs of various antibiotics against *E. coli* in the presence of PMBN or lysine$_{20}$

Antibiotic	MIC (μg/ml) for[a]:							
	E. coli	*E. coli* in the presence of indicated concn (μg/ml) of:						
		PMBN				Lysine$_{20}$		
		0.3	1[b]	3[b]	10[b]	0.3	1	3
Novobiocin	30	10	10	3	3	10	3	1
Fusidic acid	100	3	3	3	1	100	30	10
Erythromycin	30	1	1	1	1	30	30	10
Clindamycin	30	3	3	3	3	30	10	10
Cloxacillin	≥300	100	100	30	30	ND[c]	30	10
Penicillin	10	10	10	3	3	ND	3	3

[a] The MIC was defined as the lowest concentration of antibiotic that prevented visible growth of the test bacterium incubated for 18 h at 37°C.

[b] PMBN alone retarded growth.

[c] ND, Not done.

myxin is masked by its direct bactericidal action. However, it is also possible that low concentrations of polymyxin were inactivated by absorption onto the walls of the wells in the plastic microtitration plates (32, 36).

Cationic agents possessing two (cadaverine) three (spermidine, streptomycin), or four (spermine, lysine$_4$) net basic charges were totally inactive as both OM permeability increasing agents and antibacterial agents, although the polyamines, streptomycin, and lysine$_4$ interact with isolated lipopolysaccharide (9; M. Vaara and T. Vaara, unpublished data) and streptomycin has been shown to increase the permeability of *Pseudomonas aeruginosa* OM to lysozyme (13). Apparently, an interaction with lipopolysaccharide is not sufficient to increase OM permeability. In fact, cations possessing only a few basic charges could stabilize the OM and decrease its permeability, as do the divalent cations Mg^{2+} and Ca^{2+} (20, 22, 27). Spermine, spermidine, and streptomycin have been described to stabilize lysozyme-induced *E. coli* spheroplasts against lysis in water, although the molecular mechanism of this stabilization is not known (30). The basic proteins cytochrome *c* (mw, 12,000; 20% basic amino acids) and egg white lysozyme (mw, 14,000; 13% basic amino acids) have also been reported to interact with lipopolysaccharide (8, 11). They did not, however, increase the permeability of the OM (Table 2 and 3). This might be explained by a lower content of basic amino acids in these proteins than in protamine, polylysines, and PMBN. Furthermore, protamine fragments with only four to six arginine residues did not have any permeability-increasing activity, although PMBN, with only five amino groups, was active.

As a whole, the results suggest that even as few as five basic charges are sufficient to increase OM permeability if the charges are in a favorable conformation, as they are in PMBN, a direct derivative of the natural OM-active antibiotic polymyxin. If the basic charges are in a less proper conformation, more of them are needed, and the sensitization to some antibiotics (erythromycin, clindamycin; Tables 4 and 5) still remains poor.

Polymyxin might be an ideal agent to disorganize the OM structure. It is a potent bactericidal drug and may facilitate its own permeation through the OM. Thus, its permeability-increasing effects are largely masked by its direct lethal action. Chihara et al. (5, 6) tried to develop a polymyxin derivative lacking toxic action on eucaryotic membranes. The significantly less toxic derivative PMBN, however, had lost its antibacterial activity. We have shown that PMBN retains OM permeability-increasing properties.

ACKNOWLEDGMENTS

We thank P. H. Mäkelä for critically reading the manuscript.

This work was supported by a grant from the Emil Aaltonen Foundation to M.V.

LITERATURE CITED

1. **Ando, T., and K. Suzuki.** 1967. The amino acid sequence of the third component of clupeine. Biochim. Biophys. Acta **140**:375–377.
2. **Boman, H. G., K. Nordström, and S. Normark.** 1974. Penicillin resistance in *Escherichia coli* K-12: synergism between penicillinase and a barrier in the outer part of the envelope. Ann. N.Y. Acad. Sci. **235**:569–586.
3. **Buchanan-Davidson, D. J., C. V. Seastone, and M. A. Stahmann.** 1960. Action of synthetic polylysine on the growth and phagocytosis of bacteria in vitro. J. Bacteriol. **80**:590–594.
4. **Carroll, S. F., and R. J. Martinez.** 1981. Antibacterial peptide from normal rabbit serum. 1. Isolation from whole serum, activity, and microbial spectrum. Biochemistry **20**:5973–5981.
5. **Chihara, S., A. Ito, M. Yahata, T. Tobita, and Y. Koyama.** 1974. Chemical synthesis, isolation and characterization of α-N-fattyacyl colistin nonapeptide with special reference to the correlation between antimicrobial activity and carbon number of fattyacyl moiety. Agr. Biol. Chem. **38**:521–529.
6. **Chihara, S., T. Tobita, M. Yahata, A. Ito, and Y. Koyama.** 1973. Enzymatic degradation of colistin. Isolation and identification of α-N-acyl-α,Y-diaminobutyric acid and colistin nonapeptide. Agr. Biol. Chem. **37**:2455–2463.
7. **Coleman, W. G., Jr., and L. Leive.** 1979. Two mutations which affect the barrier function of the *Escherichia coli* K-12 outer membrane. J. Bacteriol. **139**:899–910.
8. **Day, D. F., M. L. Marceav-Day, and J. M. Ingram.** 1978. Protein-lipopolysaccharide interactions. 1. The reaction of lysozyme with *Pseudomonas aeruginosa* LPS. Can. J. Microbiol. **24**:196–199.
9. **Galanos, Ch.** 1975. Physical state and biological activity of lipopolysaccharides. Z. Immunitaetsforsch. Exp. Klin. Immunol. **149**:214–229.
10. **Garrod, L. P., H. P. Lambert, and F. O'Grady.** 1973. Antibiotic and chemotherapy. Churchill Livingstone, Edinburgh, United Kingdom.
11. **Geyer, R., C. Galanos, O. Westphal, and R. J. Golecki.** 1979. A lipopolysaccharide-binding cell-surface protein from *Salmonella minnesota*. Isolation, partial characterization and occurrence in different *Enterobacteriaceae*. Eur. J. Biochem. **98**:27–38.
12. **Gross, R. J., T. Cheasty, and B. Rowe.** 1977. Isolation of bacteriophage specific for the K1 polysaccharide antigen of *Escherichia coli*. J. Clin. Microbiol. **6**:548–550.
13. **Hancock, R. E. W., V. J. Raffle, and T. I. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19**:777–785.
14. **Harold, F. M.** 1970. Antimicrobial agents and membrane permeability. Adv. Microbiol. Physiol. **4**:45–104.
15. **Hultmark, D., H. Steiner, T. Rasmuson, and H. G. Boman.** 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur. J. Biochem. **106**:7–16.
16. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–127.
17. **Mäkelä, P. H. (Rapporteur), D. J. Bradley, H. Brandis, M. M. Frank, H. Hahn, W. Henkel, K. Jann, S. A. Marse, J. B. Robbins, L. Rosenstreich, H. Smith, K. Timmis, A. Tomasz, M. J. Turner, and D. C. Wiley.** 1980. Evasion of host defense group report, p. 174–197. *In* H. Smith, J. J. Skehel, and M. J. Truner (ed.), The molecular basis of microbial pathogenicity. Dahlem Konferenzen 1980. Weinheim, Verlag Chemie GmbH.

18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Modrzakowski, M. C., M. H. Cooney, L. E. Martin, and J. K. Spitznagel.** 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes. Infect. Immun. **23**:587–591.
20. **Nikaido, H., P. Bavoil, and Y. Hirota.** 1977. Outer membranes of gram-negative bacteria. XV. Transmembrane diffusion rates in lipoprotein-deficient mutants of *Escherichia coli*. J. Bacteriol. **132**:1045–1047.
21. **Nikaido, H., and T. Nakae.** 1979. The outer membrane of gram-negative bacteria. Adv. Microbiol. Physiol. **20**:163–250.
22. **Nikaido, H., S. Ah. Song, L. Shaltiel, and M. Nurminen.** 1977. Outer membrane of *Salmonella*. XIV. Reduced transmembrane diffusion rates in porin deficient mutants. Biochem. Biophys. Res. Commun. **76**:324–330.
23. **Paakkanen, J., E. C. Gotschlich, and P. H. Mäkelä.** 1979. Protein K: a new major outer membrane protein found in encapsulated *Escherichia coli*. J. Bacteriol. **139**:835–841.
24. **Richmond, M. H., and S. Wotton.** 1976. Comparative study of seven cephalosporins: susceptibility to beta-lactamases and ability to penetrate the surface layers of *Escherichia coli*. Antimicrob. Agents Chemother. **10**:219–222.
25. **Sanderson, K. E., H. Ross, L. Ziegler, and P. H. Mäkelä.** 1972. F^+, Hfr, and F′ strains of *Salmonella typhimurium* and *Salmonella abony*. Bacteriol. Rev. **36**:608–637.
26. **Schindler, M., and M. J. Osborn.** 1979. Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry **18**:4425–4430.
27. **Stan-Lotter, H., M. Gupta, and K. E. Sanderson.** 1979. The influence of cations on the permeability of the outer membrane of *Salmonella typhimurium* and other gram-negative bacteria. Can. J. Microbiol. **25**:475–485.
28. **Storm, D. R., K. S. Rosenthal, and P. E. Swanson.** 1977. Polymyxin and related peptide antibiotics. Annu. Rev. Biochem. **46**:723–763.
29. **Strominger, J. L., K. Izaki, M. Matsuhashi, and D. J. Tipper.** 1967. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Fed. Proc. **26**:4–28.
30. **Tabor, C. W.** 1962. Stabilization of protoplasts and spheroplasts by spermine and other polyamines. J. Bacteriol. **83**:1101–1111.
31. **Teuber, M.** 1974. Action of polymyxin B on bacterial membranes. III. Differential inhibition of cellular function in *Salmonella typhimurium*. Arch. Microbiol. **100**:131–144.
32. **Teuber, M., and J. Bader.** 1976. Action of polymyxin on bacterial membranes. Binding capacities for polymyxin B of inner and outer membranes isolated from *Salmonella typhimurium* G30. Arch. Microbiol. **109**:51–58.
33. **Vaara, M.** 1981. Increased outer membrane resistance to ethylene-diaminetetraacetate and cations in novel lipid A mutants. J. Bacteriol. **148**:426–434.
34. **Vaara, M., and T. Vaara.** 1983. Polycations as outer membrane-disorganizing agents. Antimicrob. Agents Chemother. **24**:114–122.
35. **Vaara, M., T. Vaara, J. Jensen. I. Helander, M. Nurminen, E. Th. Rietschel, and P. H. Mäkelä.** 1981. Characterization of the lipopolysaccharide from the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*. FEBS Lett. **129**:145–149.
36. **Vaara, M., T. Vaara, and M. Sarvas.** 1979. Decreased binding of polymyxin by polymyxin-resistant mutants of *Salmonella typhimurium*. J. Bacteriol. **139**:664–667.
37. **Weiss, J., P. Elsbach, I. Olson, and H. Odeberg.** 1978. Purification and characterization of a potent bactericidal and membrane-active protein from the granules of human polymorphonuclear leukocytes. J. Biol. Chem. **253**:2664–2672.
38. **Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker.** 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70**:527–554.
39. **Zimmerman, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. **12**:368–372.