

## Binding of Polycationic Antibiotics and Polyamines to Lipopolysaccharides of *Pseudomonas aeruginosa*

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**Polycations, such as aminoglycoside and peptide antibiotics, and naturally occurring polyamines were found to bind to the lipopolysaccharide of *Pseudomonas aeruginosa* and alter its packing arrangement. Binding of cations was measured by the displacement of a cationic spin probe from lipopolysaccharide into the aqueous environment upon addition of competitive cations. The level of probe displacement was dependent on the concentration and charge of the competing cation, with the more highly charged cations being more effective at displacing probe. The relative affinity of several antibiotics for lipopolysaccharide correlated with their ability to increase outer membrane permeability, while the relative affinity of several polyamines correlated with their ability to stabilize the outer membrane. Probe mobility within the lipopolysaccharide head group was shown to be decreased by cationic antibiotics and unaltered or increased by polyamines. We propose that antibiotic permeability and disruption of outer membrane integrity by polycationic antibiotics results from binding of the antibiotic to anionic groups on lipopolysaccharide with a consequent change in the conformation of lipopolysaccharide aggregate structure.**

The outer membrane of gram-negative bacteria forms an effective barrier to many antibiotics. The synergism of some antibiotics may lie in the ability of certain drugs to increase the permeability of the membrane, facilitating the entry of themselves and other compounds. Cationic antibiotics, such as gentamicin (in the case of *Pseudomonas aeruginosa*) and polymyxin B (for many bacteria) have been reported to increase the permeability of the outer membrane to lysozyme and hydrophobic compounds (11, 12, 31). The initial action of these antibiotics may be to disrupt outer membrane structure, allowing themselves and other compounds to enter the cell and inhibit specific metabolic processes.

Leive (18) and Nikaido (24) have proposed that the high resistance of gram-negative bacteria to hydrophobic antibiotics and the low permeability of the outer membrane of hydrophobic compounds are a result of the presence of anionic lipopolysaccharides (LPS). The outer monolayer of the outer membrane, made up of LPS and protein (14), forms a rigid, highly charged surface which is stabilized by divalent cations (1, 18). Although the highly anionic nature of LPS from enteric bacteria may deter the association of hydrophobic compounds, cationic compounds may preferentially bind.

Studies with model phospholipid systems have shown that both aminoglycosides and polymyxin-like peptides bind to acidic lipids and rearrange their packing structure (9, 19). In addition, both classes of antibiotics induce blebbing of bacterial outer membranes (13, 15, 26, 28). Polymyxin B may also decrease the size of *Escherichia coli* LPS aggregates as visualized by electron microscopy (21).

Våara and coworkers have reported that LPS from polymyxin-resistant strains of *Salmonella typhimurium* binds less polymyxin B than does the LPS from susceptible strains (32) and that the peptide portion of polymyxin B

sensitizes *E. coli* to serum (33). To ascertain the initial action of polycationic antibiotics, we measured the relative affinities of various polycationic antibiotics and polyamines for purified preparations of *P. aeruginosa* LPS by using a cationic spin probe. This spin probe partitions into anionic sites on LPS aggregates (4, 5), and by competitive cation titrations, the relative affinities of a variety of cations for LPS were obtained. In addition, the mobility of the probe was measured, and results indicated that polycationic antibiotics rigidify LPS aggregates. Thus, this probing technique may be a useful approach for characterizing compounds which alter the LPS barrier function.

### MATERIALS AND METHODS

**LPS samples.** LPS was isolated from *P. aeruginosa* strains H103 (12) and Z61 (17, 36) by the ethanol and Mg<sup>2+</sup> precipitation procedures of Darveau and Hancock (7) and twice extracted in an equal volume of chloroform-methanol (1:1) to remove residual phospholipids. Some of the LPS samples were further purified by extensive dialysis in 0.2 M NaCl-1 mM EDTA-0.02% NaN<sub>3</sub>-10 mM Tris (pH 8.0) to remove residual sodium dodecyl sulfate, followed by dialysis against 10 mM MgCl<sub>2</sub> and finally distilled water. All experiments were done with LPS suspended in 50 mM potassium hydroxide-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0) at 37°C. LPS was quantitated by weighing lyophilized samples and using an average molecular mass of 9,000. This molecular mass was derived by assaying for 2-keto-3-deoxyoctulosonic acid (KDO) (8) and assuming two reactive KDO residues per LPS (16). Elemental analysis of LPS was done by inductively coupled plasma emission spectroscopy of wet ashed samples as described previously (6).

**Electron spin resonance.** Electron spin resonance spectroscopy was carried out with a Varian X-band spectrometer, model E-112. Titrations of LPS (10 mg/ml) were carried out by using the probe 4-dodecyl dimethyl ammonium-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT<sub>12</sub>; probe-to-

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TABLE 1. Elemental composition of purified *P. aeruginosa* LPS isolates<sup>a</sup>

Strain	Composition ratio			
	Ca/LPS (mol/mol)	Mg/LPS (mol/mol)	P/LPS (mmol/g)	P/LPS (mol/mol)
H103	0	9.9	1.4	12.7
Z61	0.4	9.0	1.3	12.1

<sup>a</sup> There was less than 0.05 mol of Na<sup>+</sup>, K<sup>+</sup>, Fe<sup>3+</sup>, Al<sup>3+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, Mo<sup>2+</sup>, Cd<sup>2+</sup>, Cr<sup>2+</sup>, Pb<sup>2+</sup>, Co<sup>2+</sup>, Ba<sup>+</sup>, and Hg<sup>2+</sup> per mol of LPS associated with the samples.

<sup>b</sup> An average molecular mass of 9,000 is assumed for LPS.

LPS molar ratio, 1:18) through successive additions of cations to the sample.

Spectra were analyzed for  $2T_{\parallel}$ , the hyperfine splitting parameter, which is a measure of probe mobility, and for  $\psi_i$ , the partitioning of probe between aqueous (*F*) and LPS-bound (*B*) environments. Differences in  $2T_{\parallel}$  of greater than 0.5 gauss are considered significant.  $\psi_i = -\log ([B]/[F])/C_i$ , where  $C_i = [B]/[F]$  with no added ions (4), and [*B*] and [*F*] are concentrations of bound and free probe, respectively.

**Chemicals.** Polymyxin B nonapeptide (PMBN) was a gift of M. Vaara; EM 49 was a gift of the Squibb Institute, Princeton, N.J.; SCH21561 (2'-*N*-ethyl-netilmicin) was a gift of Schering Corp., Bloomfield, N.J.; all other cations used for titrations except MgCl<sub>2</sub> were purchased from Sigma Chemical Co., St. Louis, Mo., as sulfate salts; 5-doxyl stearate was purchased from Molecular Probes, Inc., Portland, Ore.; and CAT<sub>12</sub> was synthesized as described previously (4).

## RESULTS

The intrinsic metal ion content of the *P. aeruginosa* LPS isolates was analyzed by using an inductively coupled plasma emission spectrometer. Results (Table 1) indicate that all of the samples studied were in the magnesium salt

form, as expected, based on the method of LPS purification, and had 12 to 13 phosphate residues per molecule of LPS.

The relative affinities of added cations for LPS and their ability to alter the fluidity of LPS aggregates were measured by using the spin probe CAT<sub>12</sub>. Analysis of Scatchard and Hill plots indicated that there were approximately 2.2 CAT<sub>12</sub> binding sites per molecule of LPS from *P. aeruginosa* H103, with each binding site having a dissociation constant ( $K_d$ ) of approximately 0.65  $\mu$ M (Fig. 1). Probe binding to these sites appeared to be noncooperative, as indicated by a Hill coefficient of 0.9 (Fig. 1B). Furthermore, the affinity of CAT<sub>12</sub> for LPS appeared to be dependent on the cations present in solution, with measured  $K_d$  values for CAT<sub>12</sub> of between 0.2 and 11  $\mu$ M, depending on the experimental conditions. Increasing concentrations of Mg<sup>2+</sup> or K<sup>+</sup> in the sample increased the measured  $K_d$ , suggesting competition between CAT<sub>12</sub> and other cations for the same site(s) (data not shown).

Titration of LPS samples containing CAT<sub>12</sub> with various cations displaced different amounts of the probe as indicated by the increase in the magnitude of the partitioning parameter  $\psi_i$  (Fig. 2A). In contrast, titration of LPS samples containing the negatively charged spin probe 5-doxyl stearate with spermine or polymyxin B did not displace detectable levels of this probe (data not shown). The specific displacement of CAT<sub>12</sub> by cations suggested that the partitioning of CAT<sub>12</sub> from the LPS-bound state to the free state could be used to measure the relative affinity of cations for LPS. This partitioning may result from competition between probe, added cations, and intrinsic cations (Table 1) for binding sites. The sigmoidal CAT<sub>12</sub> displacement curves noted here were similar to the binding curves of polymyxin B and gentamicin described by other investigators (20, 29), which have been interpreted as positive cooperative binding of the antibiotics to LPS.

Generally, addition of the highly charged cations (net charge of +4 or +5; e.g., polymyxin B and gentamicin) displaced greater amounts of CAT<sub>12</sub> from LPS aggregates (as

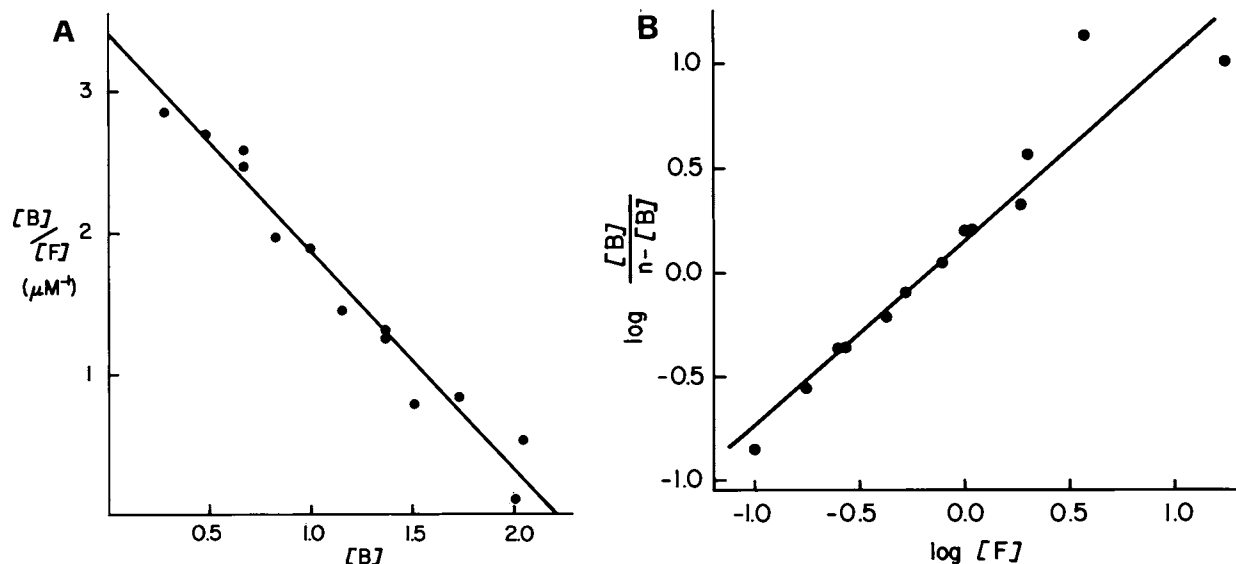


FIG. 1. CAT<sub>12</sub> binding to *P. aeruginosa* H103 LPS presented as Scatchard (A) and Hill (B) plots. LPS samples (5  $\mu$ M), suspended in 39 mM potassium-HEPES, were mixed with different concentrations of CAT<sub>12</sub>. The concentrations of bound [*B*] and free [*F*] probe were determined from the resultant electron spin resonance spectra. Bound probe is displayed as moles of bound CAT<sub>12</sub> relative to moles of LPS, and *n* is the number of probe-binding sites per LPS molecule (2.2) determined from the Scatchard plot.

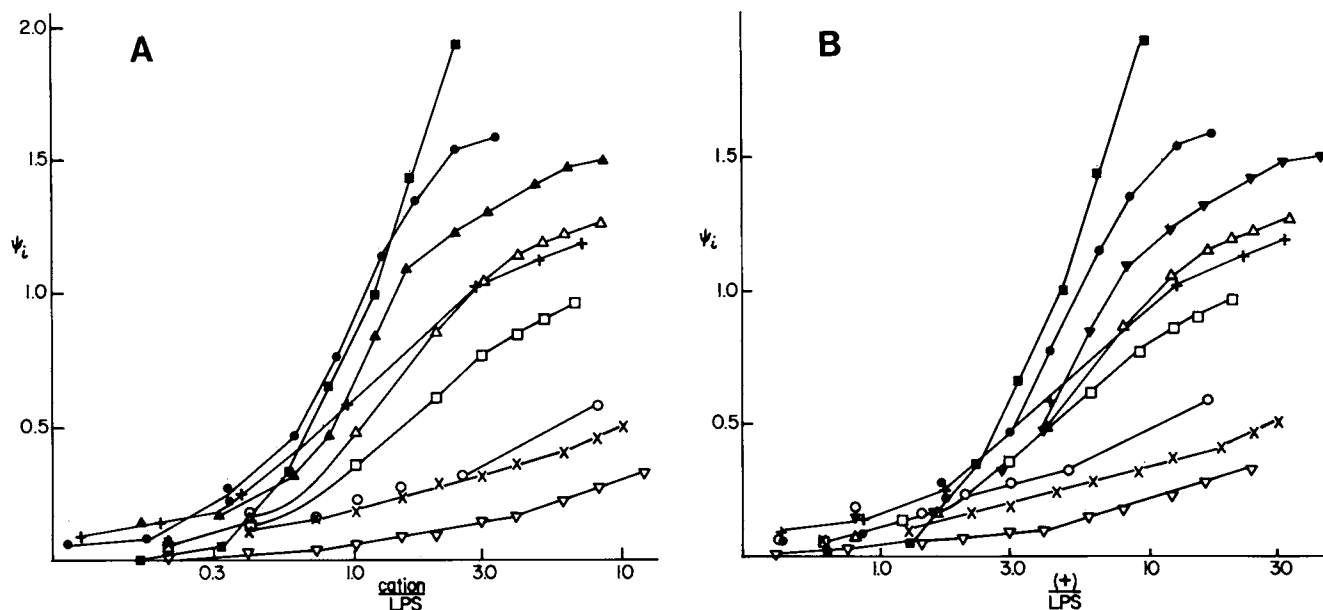


FIG. 2. Partitioning of the spin probe  $CAT_{12}$  upon addition of cations to H103 LPS. The partition function  $\psi_i$  was measured as a function of the cations with the following charges: EM 49 (■), +4; polymyxin B (●), +5; PMBN (▼ or ▲), +5; spermine (△), +4; gentamicin (+), +4.5; spermidine (□), +3; streptomycin (×), +3; Mg (○), +2; putrescine (▽), +2. The amount of cations (abscissa) is plotted as the number of moles of cations added per mole of LPS (A) and as the number of positive charges added per LPS (B).

judged by the large  $\psi_i$ ) than did cations with less charge (e.g.,  $Mg^{2+}$  and putrescine), although  $CAT_{12}$  was not displaced in amounts equal to the added charge. Analysis of the titrations on the basis of added charge per LPS showed that  $CAT_{12}$  displacement was dependent on the competing cation and not just the concentration of competitive charge (Fig. 2B). When cations of similar charge were compared, the peptide antibiotics were more effective at displacing  $CAT_{12}$  than were the polyamines, which, in turn, were slightly more

effective than were the aminoglycosides. Compounds similar to gentamicin, differing slightly in charge and structure, had similar affinities toward LPS on an added charge per LPS basis (Fig. 3).

The change in LPS head group fluidity upon addition of cations appeared to depend on the class of cation added (antibiotic or polyamine) rather than on the relative affinity (Fig. 4). All added cations rigidified LPS aggregates slightly at concentrations of up to approximately 0.5 mol of added cation per mol of LPS. At higher levels, polyamines fluidized slightly while antibiotics induced further rigidification. Of all the cations added, PMBN rigidified LPS aggregates the most, consistent with the reported rigidification of dimyristyl phosphatidic acid by the nonapeptide derivative of polymyxin E (29). We have observed analogous antibiotic-induced rigidification and polyamine-induced fluidization of LPS from *E. coli* and *S. typhimurium* (unpublished data).

The ability of cations to displace  $CAT_{12}$  from LPS depended on the strain from which the LPS was isolated. To demonstrate this, we used the mutant strain, Z61, which has a >100-fold increase in susceptibility to  $\beta$ -lactam antibiotics and a 2-fold increase in susceptibility to aminoglycosides (36). The LPS of this strain has an altered sugar content compared with that of the parent strain (17). Polymyxin B displaced similar amounts of probe from LPS isolated from either strain H103 or strain Z61. In contrast, the displacement of probe by gentamicin, neomycin, and spermine was less when LPS from strain Z61 was used, as compared with results obtained with LPS from strain H103 (Fig. 5).

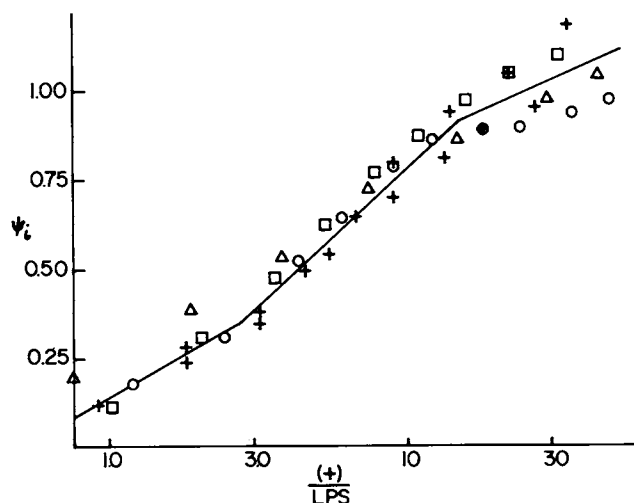


FIG. 3. Partitioning of  $CAT_{12}$  onto LPS from strain H103 with the addition of aminoglycoside antibiotics. The partition parameter  $\psi_i$  was measured as a function of the concentration of added cations with the following charges: neomycin (○), +6; SCH21561 (□), +5; gentamicin (+), +4.5; amikacin (△), +6. The level of added cations (abscissa) is presented as the number of positive charges added per molecule of LPS.

## DISCUSSION

The results from the Scatchard analysis of  $CAT_{12}$  binding to LPS and from the competitive displacement of  $CAT_{12}$  from LPS by cations indicate that there are specific binding sites on LPS for which  $CAT_{12}$  and other cations compete. Analysis of Scatchard plots indicate that there are approximately two  $CAT_{12}$ -binding sites on *P. aeruginosa* H103 LPS,

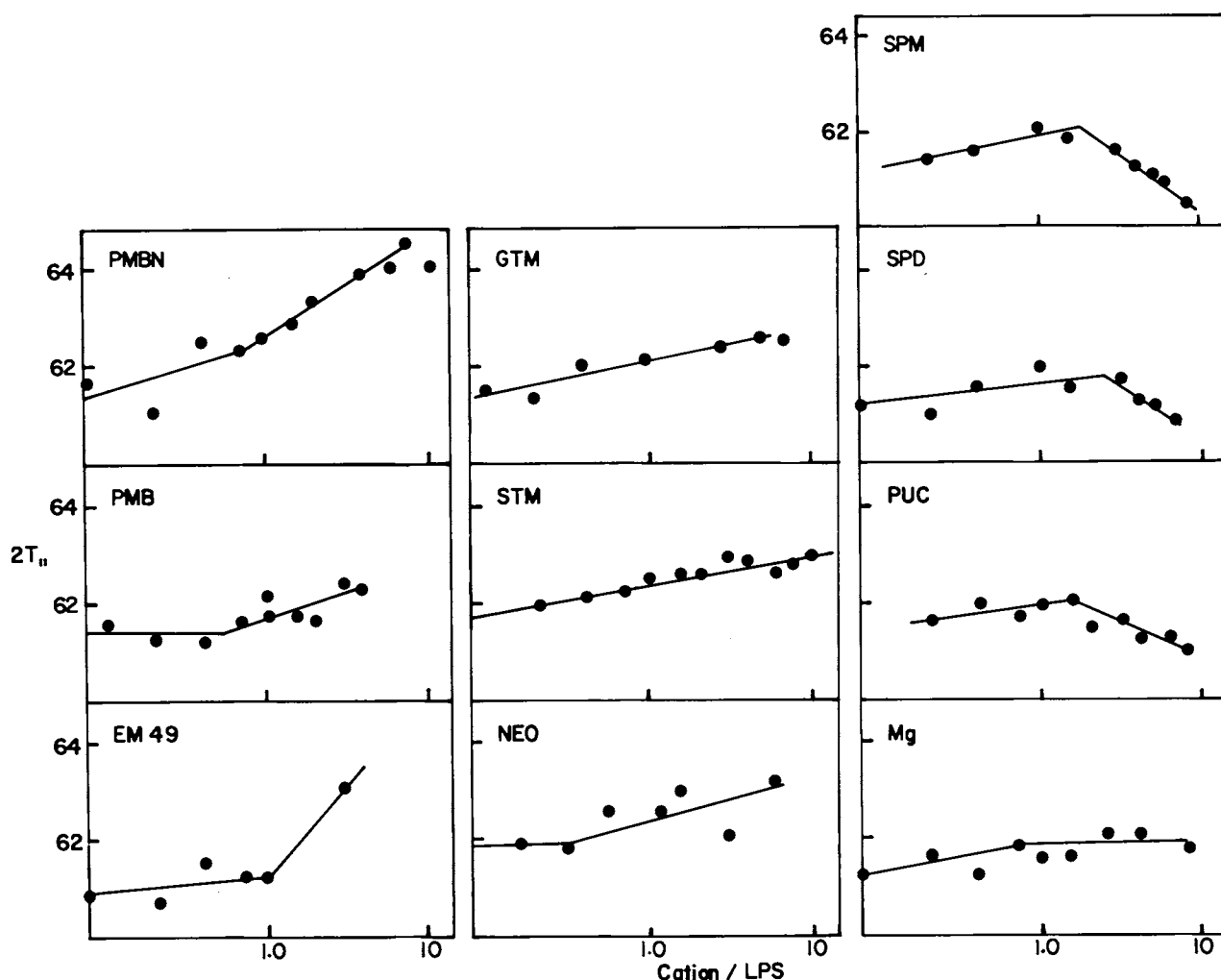


FIG. 4. Head group mobility of LPS from *P. aeruginosa* H103 was measured by the hyperfine splitting parameter ( $2T_{||}$ ) in gauss of bound  $\text{CAT}_{12}$ . This parameter was measured as a function of increasing concentrations of: PMBN, polymyxin B (PMB), EM 49, gentamicin (GTM), streptomycin, (STM), neomycin (NEO), spermine (SPM), spermidine (SPD), putrecine, (PUC), and Mg.

with an average  $K_d$  of approximately  $0.65 \mu\text{M}$ . Schindler and Osborn (27) detected a single divalent cation-binding site on LPS from *S. typhimurium* which had a  $K_d$  for  $\text{Ca}^{2+}$  of  $3 \mu\text{M}$ . However, this  $K_d$  for  $\text{Ca}^{2+}$  was measured in the presence of a high concentration of Tris, which may have raised its observed  $K_d$ ; in addition, LPS from *S. typhimurium* has about half as many phosphate groups as does LPS from *P. aeruginosa* (3, 23, 34). Thus, the affinity of  $\text{CAT}_{12}$  for LPS may be similar to that of divalent metals for LPS. Since addition of  $\text{Mg}^{2+}$  to *P. aeruginosa* LPS increased the apparent  $K_d$  of  $\text{CAT}_{12}$ , we propose that  $\text{CAT}_{12}$  competes with  $\text{Mg}^{2+}$  for divalent cation sites.

The LPS samples used were initially charge neutralized by  $\text{Mg}^{2+}$  (Table 1). Assuming that each  $\text{Mg}^{2+}$  ion neutralized two negative charges in the LPS, two monovalent  $\text{CAT}_{12}$  molecules would be needed to charge neutralize a single  $\text{Mg}^{2+}$  site. Thus, the two high-affinity sites for  $\text{CAT}_{12}$  may reflect the single high-affinity divalent cation site described previously (27). Since  $\text{CAT}_{12}$  possesses a long hydrophobic acyl chain, its reporter group probably sits near the hydrocarbon-water interface and interacts predominantly with the phosphate substituents of lipid A. The  $\text{Ca}^{2+}$ -binding site in *S.*

*typhimurium* (27) was detected in both Re and Rc LPS, also suggesting that its binding site was on lipid A phosphates or KDO residues. In such a position, the spin probe would be sensitive to cations bound to these sites, to the LPS packing arrangement, and to the overall aggregate surface potential (25). The LPS preparations used in this study are representative of the LPS present on the intact bacteria; they are heterogeneous in the phosphate substitutions in the core-lipid A, and in the length of O-antigen attached to the core. Thus, cation binding to these aggregates represents an average of the interaction with molecules with different levels of phosphate and different lengths of O-antigen.

The ability to displace the probe from LPS aggregates varied among the cations tested. The affinity of cations to LPS results, in part, from ionic attraction, with differences in binding reflecting both the net positive charge on the cation and the arrangement of the positive charges on the molecules. The LPS of *P. aeruginosa* has approximately 12 phosphates (3, 34) and two to three KDO residues (3, 16, 34), allowing numerous arrangements of cation-binding sites, some of which may have high affinity for certain cations. Competition between cations could be for the same site or

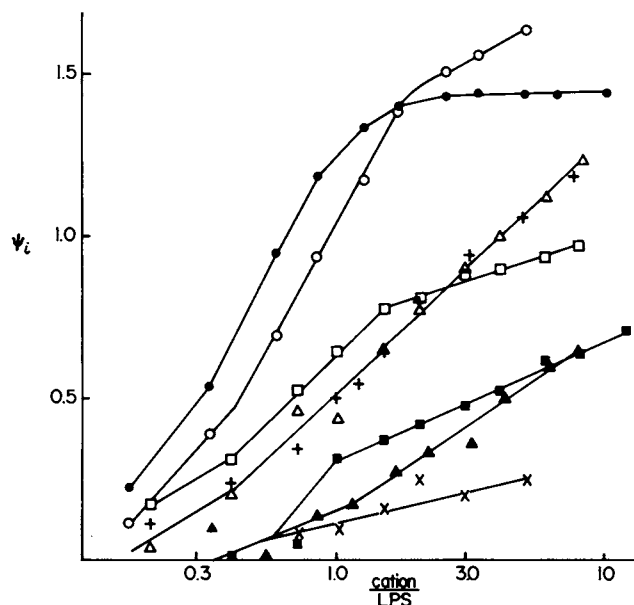


FIG. 5. Partitioning of CAT<sub>12</sub> onto LPS of strains H103 and Z61. The partition parameter  $\psi_i$  was measured as a function of increasing concentrations of: polymyxin B (○, ●) neomycin (□, ■), spermine (△, ▲), and gentamicin (+, ×) onto the H103 (open symbols and +) and Z61 (closed symbols and ×) LPS. The amount of cation added per LPS is plotted as moles per mole.

for overlapping sites or could result from charge repulsion resulting from a change of surface potential. Cations with a higher net charge would, in general, be better competitors because of the large number of negative charges they could sequester. In addition, as any given cation binds to LPS, the LPS aggregate may alter its conformation to maximize ionic interactions, and this change may alter the binding sites of other cations. This induced change in conformation is suggested by the different physical properties of different LPS salt forms (6, 10). Thus, partitioning of CAT<sub>12</sub> upon cation addition may be sensitive to the affinity of the added cation for LPS as well as to the preferential site to which the cation bound.

CAT<sub>12</sub> was readily displaced by polymyxin B and EM 49, each of which possesses an acyl chain that may enhance its affinity for LPS aggregates through hydrophobic interactions with the lipid region. It is not surprising that the polycationic peptide antibiotics have a high affinity for LPS, since polymyxin B and EM 49 have been reported to bind to and perturb the packing arrangement of membranes and acidic phospholipids (26, 29, 31), and since polymyxin B has been reported to have a 20-fold-lower  $K_d$  for *S. typhimurium* LPS than does Ca<sup>2+</sup> (27).

Removal of the acyl chain from polymyxin B to form PMBN reduced the affinity of the compound for LPS to a level closer to that of similarly charged cations. The different affinities, noted for compounds with and without acyl chains, may also indicate that the two classes interact with different regions of the LPS molecule. Cations with acyl chains may compete for the same sites as CAT<sub>12</sub> on the lipid A, while cations lacking acyl chains may also bind to phosphate or KDO residues in the core-polysaccharide region. That the binding sites for cations with and without acyl chains differ is also suggested by the different affinities observed for the two classes of cations added to LPS from strains H103 and Z61. The nonacylated polycations neomycin, gentamicin, and

spermine displaced much less probe from the LPS of strain Z61 than they did that from the LPS of strain H103, while polymyxin B displaced similar levels of CAT<sub>12</sub> from the two samples. Strain Z61 is supersusceptible to many antibiotics, and our results suggest that its isolated LPS has a lower affinity for nonacylated polycationic antibiotics. Perhaps the alterations in LPS, which decrease resistance to hydrophobic antibiotics, affect ionic interactions within the LPS and thus differentially alter the barrier function of the intact outer membrane to polycationic and hydrophobic or zwitterionic compounds.

The relative affinities of the aminoglycoside antibiotics gentamicin and streptomycin for LPS, as indicated by CAT<sub>12</sub> partitioning, reflect both their relative abilities to increase outer membrane permeability and their relative MICs for strain H103 (20). The low affinity of streptomycin for LPS of strain H103 may result from two of the charges of streptomycin being delocalized on guanido groups which may be poor counterions. The aminoglycoside antibiotics which lacked guanido groups all displaced similar amounts of CAT<sub>12</sub> from LPS on a charge-per-LPS basis (Fig. 3), indicating that the more highly charged compounds had greater affinities for LPS on a molar basis. This is consistent with the finding that neomycin increases outer membrane permeability to a greater degree than does gentamicin (12) and that the aminoglycosides with less charge have lower MICs than do the more highly charged drugs (20). The high affinity of neomycin for LPS of strain H103 may allow it to bind to and increase the permeability of outer membranes at low concentrations, but due to the high affinity, neomycin may remain bound to LPS and be less available to enter the cell and inhibit protein synthesis.

The increase in rigidity of LPS aggregates upon addition of cationic antibiotics indicates an antibiotic-induced alteration of the LPS packing structure. Polyamines naturally present in the outer membrane bind tightly to LPS yet do not immobilize LPS head groups or increase the permeability of the outer membrane. Possibly, the small size of the polyamines allows them to bind to LPS aggregates, neutralizing some of the charges without disrupting the LPS packing arrangement, while large antibiotics rearrange LPS packing, forming cracks in the overall membrane structure. The association of divalent cations and polyamines with LPS may allow the aggregate to assume its native conformation and stabilize the structure. Such stabilization by these cations is also suggested by the ability of polyamines to protect spheroplasts against lysis (22, 30) and by the ability of the divalent cation chelator EDTA to disrupt and increase the permeability of outer membranes (18). The longer, more highly charged polyamines were more effective at stabilizing spheroplasts (22) and were found to bind to LPS with a higher affinity.

Although different antibiotics may have different mechanisms of action once inside the cell, all antibiotics must pass through the outer membrane to reach their site of action in gram-negative cells. We have found that polycationic antibiotics bind to and rigidify isolated, purified LPS and that their affinity for LPS appears to correlate with their reported ability to increase outer membrane permeability (11, 12). That cationic antibiotics can increase outer membrane permeability to other compounds implies that they perturb overall membrane integrity rather than passively diffuse through the membrane.

In accordance with results of others (2, 31, 35), our data support the idea that positive charges on polycationic antibiotics are attracted to the negatively charged groups on

LPS, and upon binding, alter the LPS aggregate structure by disrupting LPS-LPS interactions. In the intact bacterium, this alteration of LPS-LPS interactions, and perhaps LPS-protein interactions, may lead to permeabilization of the outer membrane.

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