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Interactions of linear dicationic molecules with lipid A: structural requisites for optimal binding affinity

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SUMMARY. **The structural determinants of the binding affinity of linear dicationic molecules toward lipid A have been examined with respect to the distance between the terminal cationic functions, the basicity, and the type of cationic moieties using a series of spermidine derivatives and pentamidine analogs by fluorescence spectroscopic methods. The presence of two terminal cationic groups corresponds to enhanced affinity. A distinct sigmoidal relationship between the intercationic distance and affinity was observed with a sharp increase at 11 Å, levelling off at about 13 Å. The basicity (pK) and nature of the cationic functions are poor correlates of binding potency, since molecules bearing primary amino, imidazolino, or guanido termini are equipotent. The interaction of pentamidine, a bisamidine drug, with lipid A, characterized in considerable detail employing the putative intermolecular excimerization of the drug, suggests a stoichiometry of 1:1 in the resultant complex. The binding is driven almost exclusively by electrostatic forces, and is dependent on the ionization states of both lipid A and the drug. Under conditions when lipid A is highly disaggregated, pentamidine binds specifically to bis-phosphoryl- but not to monophosphoryl-lipid A indicating that both phosphate groups of lipid A are necessary for electrostatic interactions by the terminal amidininium groups of the drug. Based on these data, a structural model is proposed for the pentamidine-lipid A complex, which may be of value in designing endotoxin antagonists from first principles.**

Endotoxin, or lipopolysaccharide, a structural component of Gram-negative bacteria is a major factor in the pathogenesis of endotoxic shock, a common and relatively serious clinical problem for which therapeutic modalities of documented value are not available at the present time. The incidence of endotoxic shock is rising worldwide[1] and mortality due to this syndrome has essentially remained unchanged during the past decade at around 50–70%.[2] Our understanding of the structural aspects of lipopolysaccharide, and of its toxic moiety, lipid A,[3] the mechanisms by which the toxin interacts with and activates target cells, and the subsequent cellular events that ultimately manifest in the shock state is steadily increasing and has perhaps reached a level that might mandate systematic and rational approaches toward combating endotoxicity. One of the many conceivable therapeutic options would be to remove, or sequester, circulating endotoxin, and a large body of work has accumulated on the use of antibodies directed against structurally conserved regions of lipopolysaccharide or lipid A (Ref. 4 and references therein). We have been interested in exploring the possibility of sequestering endotoxin using small molecules. Earlier work from our laboratories has examined the interaction of cationic, amphiphilic peptides[5,6] and nonpeptidic molecules[7] with endotoxin with a view to delineating those structural features that correspond to maximal af-

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finity and endotoxin-antagonistic properties in such molecules. We have recently reported that pentamidine, an aromatic diamidine antiprotozoal drug, binds lipid A with an apparent dissociation constant of 0.12 μM and attenuates endotoxicity in several bioassays.[7] The relatively high affinity of the drug was attributed to the presence of two strongly basic cationic termini with the intervening distance comparable to the interphosphate distance of lipid A, suggesting that the interaction of the drug was a consequence of electrostatic interactions involving both the phosphate groups on lipid A and the amidinium groups of pentamidine. Preliminary evidence was also presented to show that the affinity of α,ω-diamino-alkanes and polyamines toward lipid A correlated with the distance between the terminal primary amino groups.[7] This paper evaluates in greater detail the requirement of two cationic functions, the nature of the cationic groups, and of the distance between them as determinants of the affinity of linear dibasic molecules toward lipid A. Derivatives of spermidine with either terminal primary amino group acetylated were used to test the premise that both cationic groups participate in recognizing the anionic lipid A phosphates, and N^4-benzylspermidine was used to examine the effect of bulky hydrophobic nonterminal substituents. An N^1,N^8-diguanido analog of spermidine was synthesized in order to evaluate if the guanido group could provide superior ion-pairing interactions. The affinities of analogs of pentamidine of varying linear dimensions and differing basicity of the cationic groups were also compared. The binding of pentamidine, a model dibasic therapeutically useful compound, to lipid A was characterized using fluorescence spectroscopic methods. These results may be of value in the rational design of endotoxin sequestering agents.

MATERIALS AND METHODS

Reagents

Mono- and bis-phosphoryl lipid A from *Salmonella minnesota* Re595 were purchased from Ribi Immunochemicals and were solubilized in water containing 0.5% triethylamine by vigorous vortexing and heating the suspension above the phase transition temperature of lipid A (60°C) for 10 min. N^1-acetyl-, N^8-acetyl-, and N^4-benzyl-spermidine were obtained from Sigma Chemical Co. Inc., St Louis, MO, USA. N^1,N^8-diguanidospermidine was synthesized by reacting a 10-molar excess of *o*-methylisourea (Aldrich) with aqueous spermidine at pH 10.5 for 6 h. The crystalline end product was purified as the free base by repeated crystallization in distilled water, converted to the hydrochloride salt, and lyophilized. ^{13}C NMR verified the guanidation of both terminal amino functions, and not the N^4-imino group (data not shown).

The structures of the pentamidine analogs are shown in Figure 1. Berenil (Sigma), propamidine (Wellcome Laboratories, Beckenham, Kent, UK), and pentamidine (Sigma) constitute one set of analogs with varying interamidinium distances (12.82–19.56 Å) whereas methoxypentamidine and imidazolinopentamidine (provided by Dr S. Mahato, Indian Institute of Chemical Biology, Calcutta, India) are analogs whose basic strength (pK) are lower than that of pentamidine. The electron-donating methoxy group decreases the pK of the amidines, and the imidazolino group is considerably less basic than the amidinium group.

Dansylcadaverine (5-dimethylaminonaphthalene-1-(N-(5-aminopentyl)) sulfonamide) was synthesized as reported earlier.[8] Concentrations of dansylcadaverine in water were determined by spectrophotometry at 330 nm using a molar extinction coefficient of 3300 for the dansyl moiety.[9] 12-Doxylstearate, dimyristoyl-phosphatidylcholine and dipalmitoyl-phosphatidylcholine were purchased from Sigma. Terbium trichloride hexahydrate was from Aldrich. CAT_{12} (4-dodecyl-dimethylammonium-1-oxyl-2,2,6,6-tetramethylpiperidine bromide) was a gift from Dr E.J. McGroarty, East Lansing, MI, USA.

Fluorescence experiments

Fluorescent probe displacement

Experiments were performed in 50 mM Tris-HCl, pH

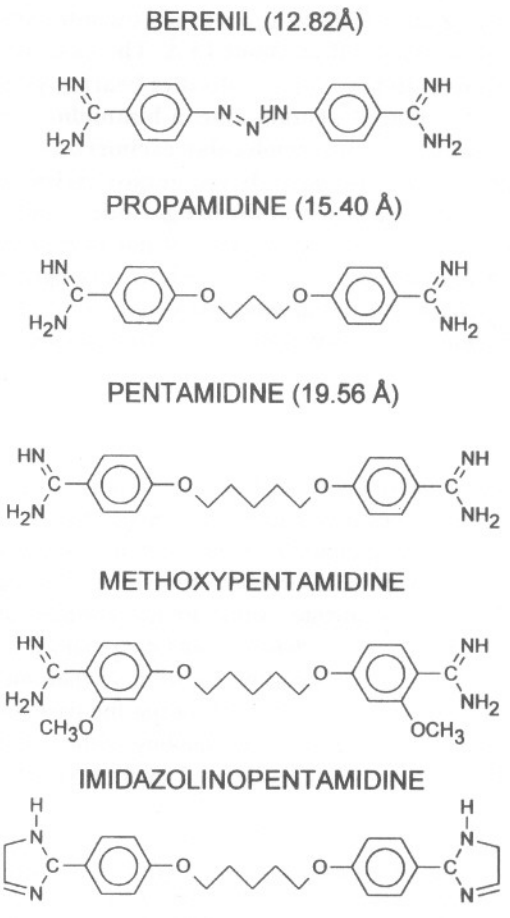

Fig. 1 — Structure of pentamidine analogs. Interamidinium distances (in Angstrom units) were obtained by molecular modeling assuming all-*trans* C–C bonds.

7.4 using a Shimadzu RF-5000 spectrofluorimeter at 25°C (below the phase-transition temperature of lipid A). Bandpasses for excitation and emission were 5 nm in all experiments. The use of dansylcadaverine as a fluorescent displacement probe to characterize the interaction of lipid A-binding molecules has been described earlier.[9] Briefly, the binding of the probe to lipid A results in a blue-shift and intensity enhancement in the emission spectrum of dansylcadaverine. Compounds which bind lipid A displace the probe, resulting in quenching of fluorescence. Probe displacement is analyzed as a function of displacer concentration. Comparisons of dansylcadaverine displacement curves to evaluate relative potencies of the compounds were performed by simultaneous curve-fitting with a four-parameter logistic equation to obtain accurate ED_{50} values (displacer concentration corresponding to 50% probe displacement) using the ALLFIT program[10] (provided by Dr P.J. Munson, National Institutes of Health, Bethesda, MD, USA). For the dansylcadaverine displacement experiments, the excitation wavelength was 340 nm. The wavelengths of maximal emission of free and lipid A-bound probe in buffer were 535 nm and 515 nm, respectively.

Characterization of pentamidine binding using intrinsic drug fluorescence

Aqueous solutions of pentamidine are weakly fluorescent[11] (excitation, 270 nm; emission, 335 nm) and it was noted in preliminary experiments that the addition of lipid A to pentamidine resulted in concentration-dependent biphasic intensity enhancements and spectral shifts accompanied by the appearance of a red-shifted emission band at 383 nm; these features, suggested the formation of excited-state dimers (excimers), and, therefore, presented additional possibilities of investigating drug-lipid A interactions. The fluorescence properties of the drug were, therefore, first characterized, and the results used in interpreting the data on the binding of the drug to lipid A.

The excitation and emission spectra of pentamidine were examined under conditions of varying pH and ionic strength, and relative quantum yields (ratio of emission intensity to absorbance) were measured in solvents of different dielectric constants and viscosities. The spectral changes occurring as a consequence of the interaction of the drug with lipid A were employed to examine the stoichiometry of interaction, and to delineate differences between mono- and bis-phosphoryl lipid A with respect to their modes of interaction with pentamidine. Titration experiments varying either drug or lipid A concentrations were performed. The effect of temperature on the putative excimerization process was examined by conventional Arrhenius plots (logarithm of the ratio of excimer/monomer intensities plotted against reciprocal absolute temperature). The location of the bound drug on lipid A was also examined by 'depth profiling' using site-specific quenchers that quench fluorophores selectively with respect to the their location in the lipid assembly. These quenchers included iodide and acrylamide which permeate into the hydrocarbon regions, 12-doxyl stearate whose spin label penetrates into the apolar domain, the trivalent cation terbium, which binds to the negatively charged headgroups, and CAT_{12} a cationic spin probe which also binds to the headgroup via electrostatic interactions.[12] All the above experiments were carried out in 50 mM Tris-HCl buffer, pH 7.4, except in experiments designed to evaluate the effect of mono- and divalent cations on pentamidine binding to lipid A; these latter experiments were performed in double-distilled, deionized water; stock solutions of the salts were unbuffered, and the pH of these stock solutions were in the range of 5.5–6.1 (uncorrected for ionic strength). Some experiments were also performed with lipid A solubilized in 1:1 chloroform/methanol mixtures to evaluate drug binding to disaggregated lipid A.

Molecular modeling

The inter phosphate distance in *Escherichia coli* type (hexacyl) lipid A was obtained from the atomic coordinates of an energy minimized molecular model of core glycolipid kindly provided by Dr M. Kastowsky, Institute for Crystallography, Berlin, Germany.[13] Distances between terminal amino groups in alkyldiamines and polyamines, and the terminal amidinium groups in the pentamidine analogs were obtained by modeling with INSIGHT (version 2.51; Biosym Technologies Inc., CA, USA) on a Silicon Graphics Iris workstation as described earlier.[7]

RESULTS

Effect of varying intercationic distance and basicity of cationic groups

The dansylcadaverine displacement activities of spermidine and of its derivatives are shown in Figure 2. The concentrations corresponding to 50% displacement of bound probe (ED_{50}) of both N^1- and N^8-monoacetylspermidine are about 7 times higher than that of spermidine, showing that the presence of two terminal cationic functions correspond to enhanced affinity. The ED_{50} for N^4-benzylspermidine is about 2.4 times that of spermidine, pointing to possible steric hindrance imposed by the benzyl moiety. N^1,N^8-diguanidospermidine is no more effective than spermidine, suggesting that the replacement of the primary amino groups with the strongly basic guanido groups confer no additional enhancement in affinity.

The displacement curves of lipid A-bound dansylcadaverine by the pentamidine analogs are shown in Figure 3. The affinities of all 5 analogs toward lipid A are virtually indistinguishable, suggesting that neither varying the interamidinium distance in the range of 13~20 Å (berenil, propamidine and pentamidine), nor decreasing the basicity of the terminal cationic groups

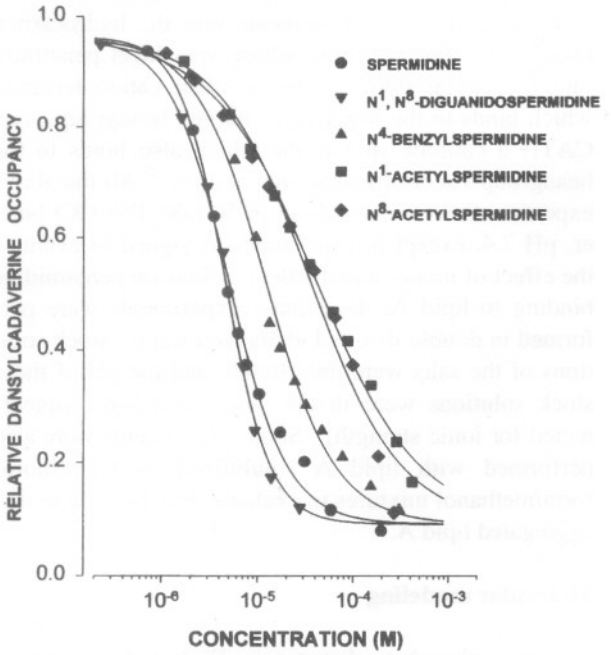

Fig. 2 — Displacement of bis-phosphoryl lipid A-bound dansylcadaverine by spermidine derivatives. [Lipid A]: 7 µM; [Dansylcadaverine]: 35 µM. Successive aliquots of spermidine derivatives were added to the mixture of lipid A and probe in 50 mM Tris-HCl, pH 7.4. The X-axis represents the final molar concentrations of the spermidine derivatives. Values of relative probe occupancy denoted on the Y-axis were calculated as described.[9] Relative probe displacement potencies (ED_{50}[derivative]/ED_{50}[spermidine]) are: N^1-acetyl-spermidine, 8.131; N^8-acetylspermidine, 7.074; N^4-benzyl-spermidine, 2.427; N^1, N^8,-diguanidospermidine, 0.979.

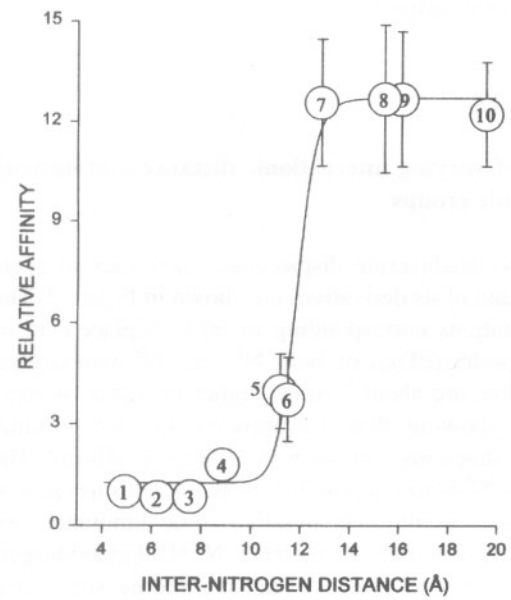

Fig. 4 — Relationship between inter-nitrogen distance and binding affinity to bis-phosphoryl lipid A of α,ω-diaminoalkanes, polyamines and bisamidine compounds. The affinities denoted on the Y-axis are expressed relative to that of 1,3-diaminopropane (C_3) and is calculated as ED_{50}[C_3]/ED_{50}[Compound], where ED_{50} is the analyte concentration corresponding to 50% probe displacement in dansylcadaverine displacement experiments and were obtained from ALLFIT. Plotted values are means ± SE. Values for the diamines and polyamines are taken from.[7]. A sigmoidal curve was fit by nonlinear regression. 1, 1,3-diaminopropane; 2, putrescine; 3, cadaverine; 4, 1,6-diaminohexane; 5, spermidine; 6, 1,8-diaminooctane; 7, berenil; 8, propamidine; 9, spermine; 10, pentamidine.

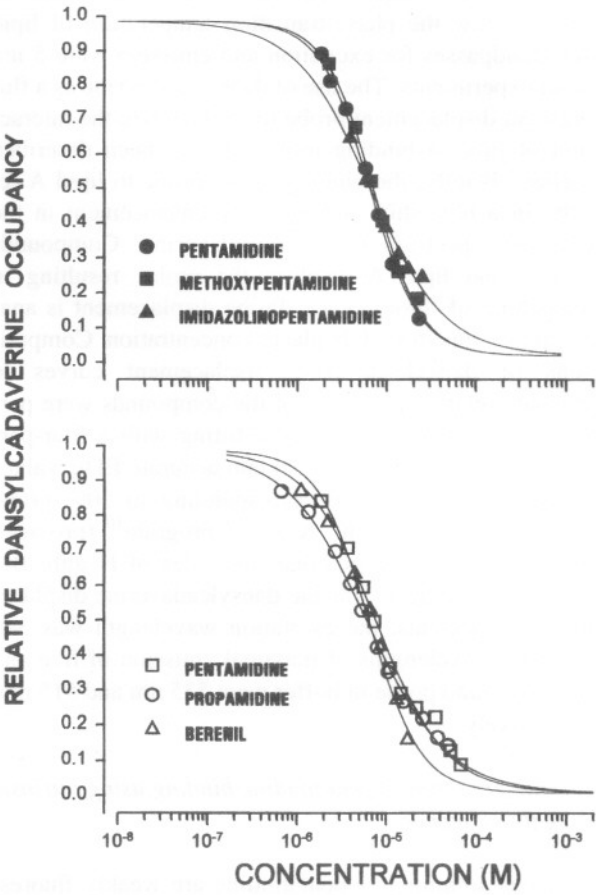

Fig. 3 — Displacement of bis-phosphoryl lipid A-bound dansylcadaverine by pentamidine analogs. Relative probe displacement potencies (ED_{50}[analog]/ED_{50}[pentamidine]) are: methoxypentamidine, 0.986; imidazolinopentamidine, 0.992; propamidine, 0.995; berenil, 1.067. Experimental conditions as in caption to Figure 2.

manifest in altered affinity. We had earlier shown that the affinity of alkyldiamines and polyamines toward lipid A exhibited an exponential relationship with respect to the distance between the cationic termini, spermine (16.1 Å) displaying maximal affinity.[7] This earlier observation was unsatisfactory since there was only a single data point in the 12.5–16 Å range.[7] Combining the present observations obtained with the pentamidine analogs with the earlier results yields a distinct sigmoidal relationship between the relative affinities of dibasic molecules and the distance between their cationic termini (Fig. 4), with a sharp increase from 11 Å, leveling off at about 13 Å. The interphosphate distance in the energy minimized model of lipid A is 13.9Å.[7,13]

The fluorescence spectra of pentamidine in the presence of varying concentrations of lipid A are shown in Figure 5. The emission maximum of aqueous pentamidine (excitation, 270 nm) is 343 nm. Successive additions of lipid A to pentamidine result in the progressive increase in the intensity of a new, red-shifted emission band centered at 383 nm. The intensity of this band is saturable, and further additions of lipid A result in attenuated intensity which is concomitant with intensity en-

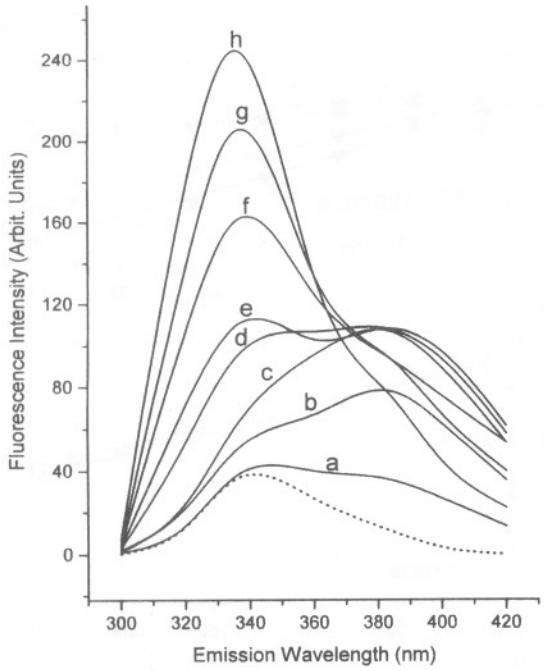

Fig. 5 — Fluorescence emission spectra of pentamidine (15 μM in 50 mM Tris-HCl buffer, pH 7.4) (dotted line) and the effect of addition of lipid A (solid lines). Final concentrations of added bis-phosphoryl lipid A (in μM) are: (a) 3.0; (b) 6.0; (c) 9.0; (d) 12.0; (e) 15.0; (f) 22.5; (g) 60.0; (h) 100. Excitation, 270 nm. The putative excimer band is centered at 383 nm.

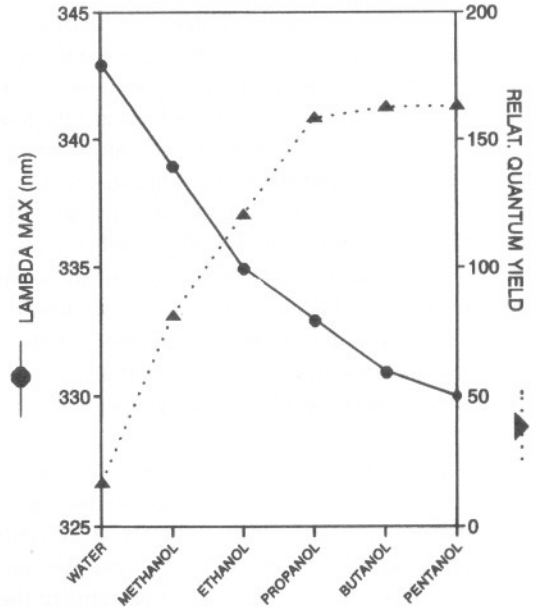

Fig. 6 — Effect of solvent polarity on the wavelength of maximal emission and on the relative quantum yield (emission intensity/ absorbance; arbitrary units) of pentamidine isethionate (25 μM).

hancements at 335 nm. Examination of the spectra indicates that this latter band is the original pentamidine emission that is slightly blue-shifted (*see* Fig. 5); this blue-shifted band is probably a consequence of the drug sensing a microenvironment of a low dielectric constant in the presence of the lipid, since decreasing solvent polarity results in qualitatively similar blue shifts of pentamidine emission (Fig. 6). The red-shifted band at 383 nm did not appear under conditions of varying pH (2.0–12), ionic strength (0–5M NaCl), viscosity (0–40% glycerol added), or in the presence of phosphate-containing compounds (NADP), and appeared to be specific for anionic phospholipid suspensions, since phosphatidylserine, but not phosphatidylcholine, induced this spectral shift (*see* Fig. 9) and other polyanionic compounds, such as sheared DNA, hyaluronic acid and heparin, were only marginally effective (data not shown). In identifying the possible photophysical basis of this band, we considered three possibilities: (i) a review of the literature suggested the possibility of *intra*molecular excited-state dimerization (excimerization) since this property appears to be generally exhibited by pseudo-bichromophoric compounds.[14–17] However, intramolecular excimerization is a unimolecular process and is essentially concentration-independent.[14] The data presented in Figure 5 show a clear concentration dependence of the fluorescence intensity of the red-shifted band, ruling out intramolecular excimerization. (ii) The presence of strongly polarizable N and O atoms on the phenyl ring systems suggested the possibility of electrochromism,[18] otherwise called the Stark effect.[19–21] However, difference absorption spectra (reference, aqueous pentamidine: sample, identical concentrations of pentamidine in the presence of 0–50 μM concentrations of lipid A) did not result in spectral changes characteristic of electrochromism[19,20] (data not shown). (iii) The third possibility that we considered was the phenomenon of *inter*molecular excim-

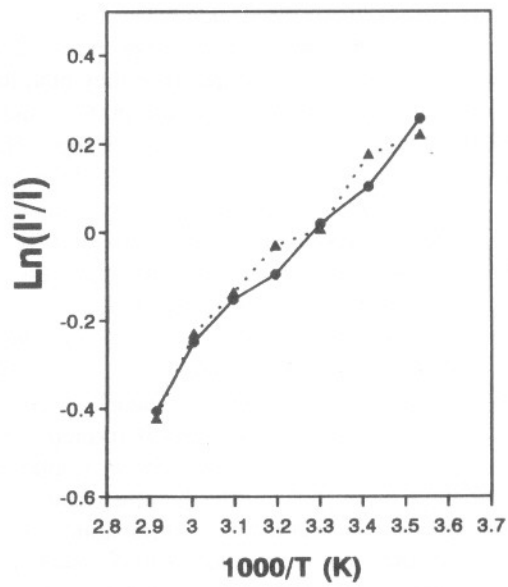

Fig. 7 — Arrhenius plots of the excimerization of bis-phosphoryl lipid A-bound pentamidine at 1:1 molar ratio. The Y-axis is the natural logarithm of the ratio of excimer intensity at 383 nm (I′) to that of monomer intensity at 335 nm (I). The X-axis is proportional to the inverse of the absolute temperature. The hysteresis between the heating (dotted line) and cooling (solid line) profiles is negligible.

erization, a well known property of such fluorescent probes as pyrene.[22] We reasoned that the binding of pentamidine at the interface between the anionic lipid assemblies and the solvent results in a saturable accumulation, and consequently to very high local *effective* concentrations of the drug; such high concentrations are probably not obtainable in free solution because of Coulombic repulsive effects induced by the strongly cationic amidinium groups, or when bound to non-lamellar polyanions, such as DNA, because of different binding geometry.[23,24] The high effective concentration of lipid A-bound pentamidine would facilitate excited-state-to-ground-state nonradiative energy transfer processes and may thus result in intermolecular excimerization.[22]

Arrhenius plots of the 383/335 nm emission intensity ratios reveal a strong temperature dependence (Fig. 7). The inverse relationship of the intensity at 383 nm with temperature would be consistent with excimerization since increase in temperature would destabilize the bound state of the drug due to enhanced thermal motion. The concentration dependence of the intensity at 383 nm and, more importantly, the decrease in intensity above threshold lipid A concentrations (Fig. 5) would be consistent with intermolecular excimerization; the latter phenomenon is probably a consequence of redistribution of pentamidine leading to a decrease in its effective concentration under conditions of excess lipid A.

In an attempt to further delineate the origins of these two emission bands, experiments designed to probe the location of the drug with respect to the lipid were performed. Lipid A was added to pentamidine at a concentration which resulted in a spectrum with the monomer and excimer components of near equal magnitudes, and the effects of various quenchers were then studied. Figure 8 shows the results of three such experiments. Terbium, a trivalent lanthanide cation would be expected to bind to the acidic phosphate groups since they bind divalent cations.[25] The addition of terbium preferentially quenches the excimer fluorescence (Fig. 8 top). The quenching by a cationic spin probe CAT_{12}, which has been reported to bind to the headgroup region of lipid A and LPS,[12] is also very similar to that of terbium (Fig. 8 top). This suggests that the excimerization process is spatially associated with the headgroup region. Iodide was found to quench both excimer and monomer fluorescence non-selectively (Fig. 8 middle). This is to be expected for although the I^- ion is a quenching agent normally used to probe solvent exposed tryptophanyl residues in proteins, it also permeates the hydrophobic domains of lipid assemblies.[26] On the other hand, 12-doxyl stearate selectively attenuates the monomer (Fig. 8 bottom). The spin label on this fatty acid is distally located and, therefore, probes the interior of the hydrocarbon regions. This is consistent with the solvent dielectric-dependent emission properties (Fig. 6) and verifies that the blue-shifted monomer band at 335 nm arises from the chromophore of the drug in the vicinity of the hydrocarbon chains.

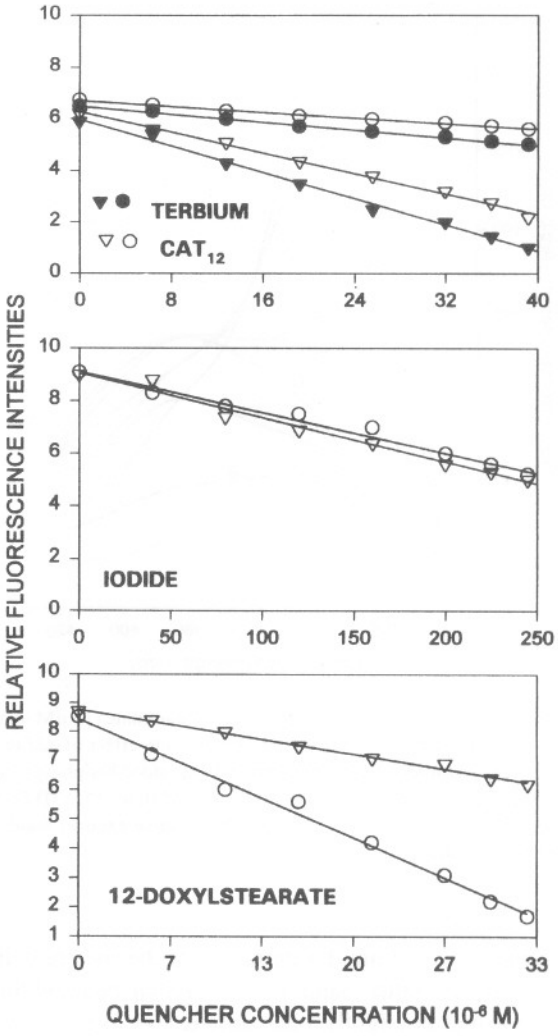

Fig. 8 — Differential quenching of monomer intensity at 335 nm (circles) and excimer intensity at 383 nm (triangles) of pentamidine (5 μM) bound to bis-phosphoryl lipid A (8.5 μM, in buffer).

The assignment of the fluorescence bands now allowed the interaction of the drug with lipid A to be examined in greater detail. Figure 9 shows the changes in excimer and monomer fluorescence intensities of pentamidine plotted as a function of lipid A/drug molar ratios. These experiments were carried out in 50 mM Tris-HCl, pH 7.4. 4′-Mono- and 1,4′-bis-phosphoryl lipid A were used to specifically test the hypothesis that the two cationic groups of the drug recognize the two anionic phosphates on lipid A. As controls, phosphatidylserine and dimyristoyl-phosphatidylcholine were used. Surprisingly, excimer fluorescence enhancements are observed with both mono- and bis-phosphoryl lipid A; both these derivatives bind pentamidine with an apparent stoichiometry of 1:1 as is evident from the inflection points of the excimer (383 nm) intensity curves (Fig. 9 top). The excimer intensity at equimolar drug:lipid A mixtures is higher for bis-phosphoryl- than for monophosphoryl-lipid A. At higher lipid A/drug molar ratios, the excimer intensities fall off, suggesting a dilution effect with respect to the effective drug concentration at the lipid A-solvent interface. This effect is

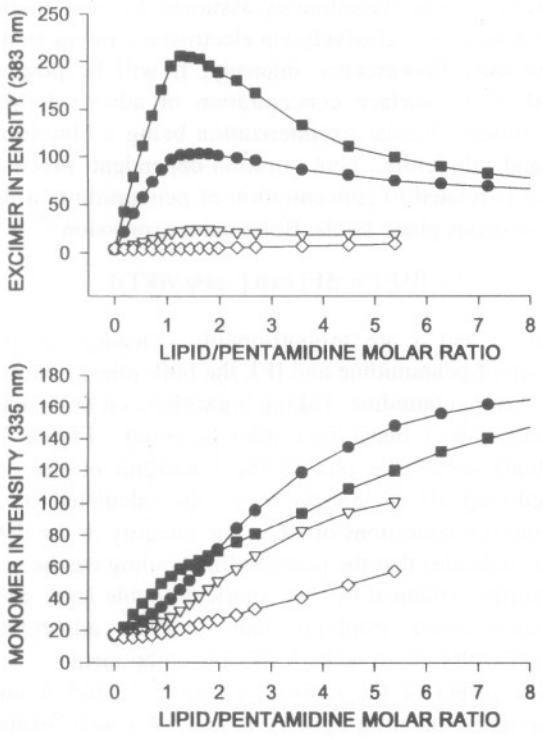

Fig. 9 — Fluorescence titration of pentamidine with lipid A under aqueous conditions. [Pentamidine] = 5 μM. Buffer, 50 mM Tris-HCl, pH 7.4. Effect on excimer intensity at 383 nm (top) and monomer intensity at 335 nm (bottom). Solid circles, monophosphoryl lipid A; solid squares, bis-phosphoryl lipid A; open triangles, phosphatidylserine; open diamonds, dimyristoyl-phosphatidylcholine.

observed also with phosphatidylserine, although it is of a much smaller magnitude with the excimer intensity inflection corresponding to 1:1.6 drug/phospholipid ratio. On the other hand, the monomer fluorescence intensity is essentially nonsaturable within the concentration ranges used, and indicates a partitioning of the drug into nonpolar regions of the lipid assemblies (Fig. 9 bottom).

Since under aqueous conditions similar to those employed in these studies, lipid A exists as lamellar aggregates as examined by electron microscopy (data not shown), pentamidine binds to lipid A superstructures, and not to the monomeric toxin. In order to verify if this could be a reason for the unexpectedly similar drug/lipid A stoichiometry observed for both mono- and bis-phosphoryl lipid A, we sought to examine the interaction of the drug to lipid A in a highly disaggregated form. Titration experiments were therefore carried out with lipid A in chloroform/methanol (1:1 v/v) mixtures. Small aliquots of concentrated stock solutions of lipid A in chloroform were added to pentamidine in 1:1 chloroform/methanol mixture such that the change in methanol concentration at the end of the titration was not more than 0.5%. The results, depicted in Figure 10, show specific binding of pentamidine to bis-phosphoryl lipid A, as evidenced by a sharp inflection of the excimer intensity at 383 nm at a drug:lipid A molar ratio of 1:1. In the case of monophosphoryl lipid A, the increase in excimer intensity is of a smaller magnitude and shows no inflection.

The choice of pentamidine as a potential lipid A-binding molecule was made on the premise that the strongly basic amidinium groups would facilitate favorable electrostatic interactions with the phosphates on lipid A and it was, therefore, of interest to evaluate the contributions of such interactions to the observed affinity of the drug. Ordered assemblies of charged amphiphiles such as lipid A are characterized by the presence of an 'electrical double layer' at the interface between the plane of the charges and the solvent continuum.[27] The binding of ligands which interact with such charged surfaces predominantly through electrostatic interactions are progressively retarded under conditions of increasing ionic strength as a consequence of 'screening' of the electrostatic potential,[28] in contrast to neutral molecules whose partitioning into the lipid phase is primarily a function of its hydrophobicity and is essentially independent of the electrostatic potential. We, therefore, examined the binding of pentamidine to bis-phosphoryl lipid A under conditions of varying ionic strength. The effect of the addition of monovalent and divalent cations on the excimer intensity of pentamidine at 1:1 drug/lipid A ratio is shown in Figure 11. Both mono- and divalent cations quench pentamidine excimer fluorescence, suggesting the primarily electrostatic nature of pentamidine binding. The quenching efficiency of the divalent cations is greater than that of the monovalent species by approximately three orders of magnitude, consistent with previous reports on the adsorption of cations to anionic membranes.[29] We have attempted to quantitatively examine the excimer intensity

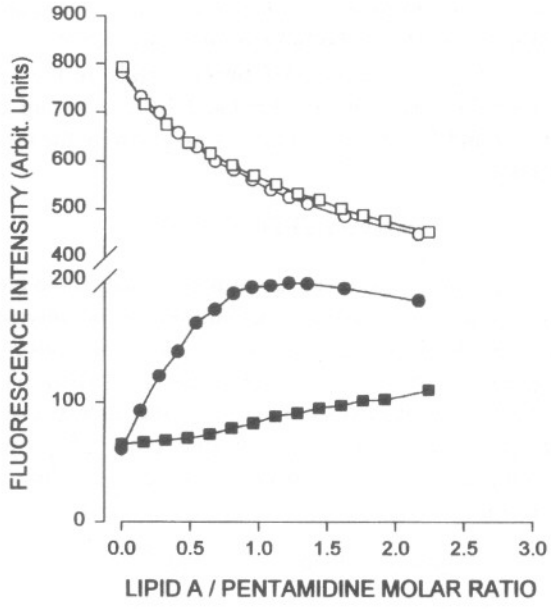

Fig. 10 — Fluorescence titration of pentamidine with lipid A in $CHCl_3/CH_3OH$ (1:1, v/v). [Pentamidine] = 5 μM. Open symbols, monomer intensity at 335 nm; closed symbols, excimer intensity at 383 nm; squares, monophosphoryl lipid A; circles, bis-phosphoryl lipid A.

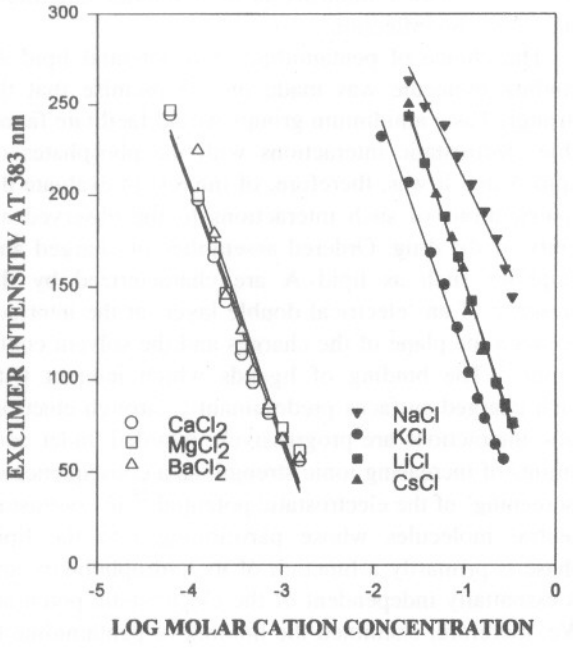

Fig. 11 — Effect of added monovalent (closed symbols) and divalent cations (open symbols) on bis-phosphoryl lipid A-bound pentamidine excimer fluorescence. Pentamidine = 5 μM; lipid A = 8.5 μM.

attenuation using only the KCl data as a representative monovalent cation, disregarding the data obtained for the divalent cations for the sake of numerical tractability. We employ the classic Gouy-Chapman theory of the diffuse double layer which ignores finite size effects and activity coefficients,[28] and bond dissociation energies of the waters of hydration[30] of the ions. These restrictions in the theoretical treatment of data have not posed serious problems in analyzing monovalent cation binding to anionic phospholipid preparations.[28,31]

The electrostatic potential at the surface of the lipid A assembly, ψ_0 can be calculated by combining the Poisson and Boltzmann equations as given by the Gouy equation:[28]

$$\sinh [e\psi_0/(2kT)] = A\sigma/(C)^{0.5}$$

where σ is the charge density at the surface, e, the electronic charge, C is molar concentration of the univalent ion, k is the Boltzmann constant, and T, the absolute temperature. A is a constant equal to $1/(8N\varepsilon_r\varepsilon_0 kT)$ where N is Avogadro's number, ε_r is the dielectric constant, ε_0 is the permittivity of free space. The operational equation at 25°C for monovalent ions reduces to the form:[32,33]

$$\psi_0 = 51.4 \sinh^{-1} [135/(AC^{1/2})]$$

where C is the molar concentration of the univalent cation and A is the area in $Å^2$ per unit charge at the surface. Assuming a cross-sectional area of 1.10 nm^2 for lipid A,[27] and 2 charges per molecule, A is calculated to be 55 $Å^2$/charge. Figure 12 (top) shows the calculated ψ_0 for lipid A as a function of increasing KCl concentration. If pentamidine is assumed to bind to the lipid A surface exclusively via electrostatic interactions, its excimer fluorescence intensity, f, will be proportional to the surface concentration of adsorbed drug {F}, (intermolecular excimerization being a bimolecular and, therefore, concentration-dependent process) which is related to concentration of pentamidine in the bulk aqueous phase by the Boltzmann expression:[28]

$$f = \beta\{F\} = \gamma[F] \exp [-ze\psi_0/(kT)]$$

where β and γ are proportionality constants, z, the valence of pentamidine and [F], the bulk-phase concentration of pentamidine. Taking logarithms on both sides reduces it to a linear first-order equation. Figure 12 (bottom) shows the plot of the logarithm of the observed intensity at 383 nm versus the calculated ψ_0 at varying concentrations of KCl. The linearity of the data points indicates that the pentamidine binding can be satisfactorily explained by the electrical double layer phenomenon alone, implying that it is an adsorption process with little or no hydrophobic component.

The effect of the ionization states of lipid A and pentamidine on drug-lipid A interactions were examined in pH titration experiments, the results of which are shown in Figure 13. Firstly, the excimer intensity of lipid A-bound pentamidine is markedly diminished at either end of the pH range, a consequence of the oppos-

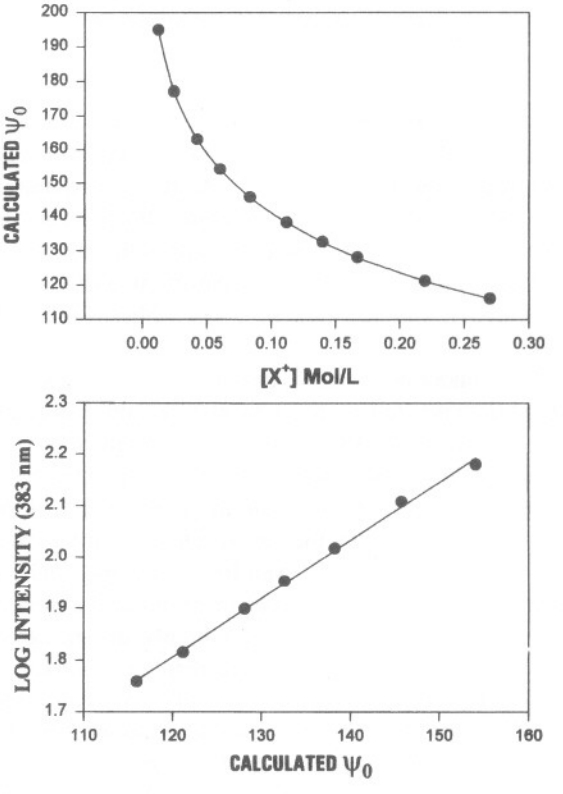

Fig. 12 — Calculated electrostatic potential (mV) of bis-phosphoryl lipid A bilayers as a function of added monovalent cation concentration (top). Plot of the logarithm of pentamidine excimer intensity (383 nm) versus the calculated ψ_0 at varying concentrations for KCl (bottom). Pentamidine = 5 μM; lipid A = 8.5 μM.

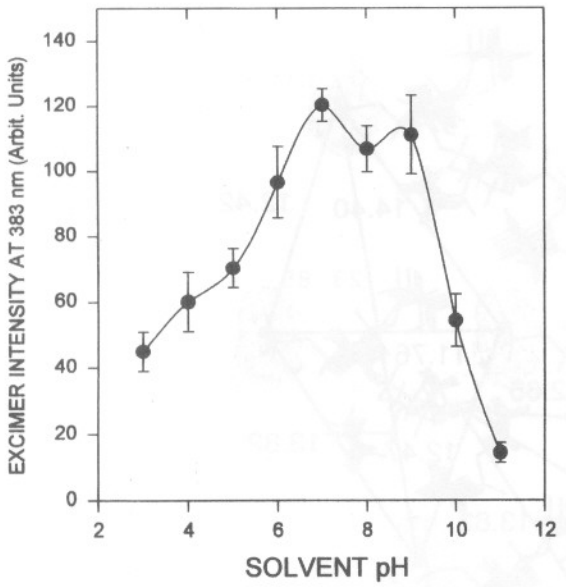

Fig. 13 — Effect of bulk solvent pH on the excimer intensity of bisphosphoryl lipid A-bound pentamidine. Error bars correspond to standard deviations calculated on 3 separate determinations. 50 mM Tris-HCl/NaOH was used. Pentamidine = 5 μM; lipid A = 8.5 μM.

ing effects of the ionization behavior of the two substances. At pH values lower than 5, the phosphate groups on lipid A are protonated and consequently, are predominantly uncharged; conversely, at high pH values, the amidinium groups on pentamidine become deprotonated and uncharged. Secondly, the fluorescence intensities of pentamidine at intermediate pH values suggest the presence of two maxima at about pH 7 and pH 9. While the large error factors preclude an unambiguous interpretation, these results would be consistent with earlier observations in the literature concerning the ionization states of the phosphate groups of lipid A. The microscopic pKs for the second dissociation of the two phosphates of lipid A have been calculated to be 6.93 and 7.76; however, the measured second dissociation constant of the 1-phosphate in Re LPS was determined to be 8.58 in by these authors.[33] The intensities of the two maxima at pH 7 and 9 are of near equal magnitude which, at first sight, may suggest that pentamidine binds as well to the tri-ionic form of lipid A as to the tetra-ionic form at the higher pH of about 8.9. However, it must be noted that the fraction of charged species of pentamidine at 8.9 is less than at pH 6.9 by roughly two orders of magnitude, assuming a pK of 12.2 for the amidinium group (Dr Alan Hill, Wellcome Laboratories, personal communication) and the excimer intensity corrected for the effective concentration of protonated drug would, therefore, be higher.

DISCUSSION

In our earlier work in screening cationic amphiphilic drugs for lipid A binding and antagonistic properties, we had observed that dibasic molecules, such as chlorhexidine and pentamidine, exhibit greater affinity toward lipid A.[7] The results presented in this paper firstly extend our preliminary observations in seeking to evaluate the requirement of two cationic functions in linear molecules, the nature of the cationic groups, and the distance between them as structural correlates of affinity toward lipid A. Secondly, we have characterized in detail, the binding of pentamidine, a prototype dicationic drug, to lipid A, employing its relatively unusual fluorescence properties.

The markedly lower affinity of the acetylated derivatives of spermidine clearly indicates that the presence of two terminal cationic groups correspond to enhanced affinity. The lower affinity of N^4-benzylspermidine can either be a consequence of the replacement of the proton on the imino nitrogen, or due to steric hindrance imposed by the bulky hydrophobic moiety, retarding its apposition on the lipid A assembly. Since the dansylcadaverine displacement potencies of spermidine and diaminooctane (which are of near-identical linear dimensions) are very similar,[7] steric effects, rather than difference in charge, are probably responsible for the lowered affinity. We specifically wished to examine the diguanido derivative of spermidine since the guanido group with its multiple H-bond donor atoms, favorable planar geometry, and charge delocalization is thought to be an ideal phosphate recognizing function and the arrangement of its basic charge-delocalized nitrogen atoms permits simultaneous interaction with two phosphate groups.[34] Guanido groups have been exploited successfully in the design of synthetic receptors for phosphodiesters, and arginine containing peptides bind to anionic phospholipids with greater affinity than do lysine containing homologs.[35] We found, however, that the affinities of spermidine and N^1,N^8-diguanidospermidine are indistinguishable within the limits of experimental error. The affinities toward lipid A of the polyamine, spermidine, and of the bisamidines, berenil, propamidine and pentamidine are also very similar (Fig. 4), and the fluorescent probe displacement curves of pentamidine and of its methoxy and imidazolino derivatives are virtually superimposable (Fig. 3). Furthermore, we have observed that polymyxin B derivatives with the primary γ-amino groups of 5 of its 6 diaminobutyric acid residues guanidated shows no greater affinity than native polymyxin B (S.A. David, unpublished observations). The possible reasons for this unexpected observation may either be related to the ionization behavior of the phosphates, or their spatial disposition on the lipid A superstructures. A simulated two-dimensional array of lipid A molecules is shown in Figure 14. The interphosphate distances in such a lamellar array range from 11.7–23 Å (Fig. 14). The ideal geometry for the interaction of a guanidium group requires that the two phosphate groups be positioned about 7 Å apart, a proviso that is probably not achieved in lipid A assemblies, even under conditions of high-density packing, as represented in Figure 14, which may be contributory to the lack of augmented affinity of the guanidinium compounds. Although the above data imply a functional

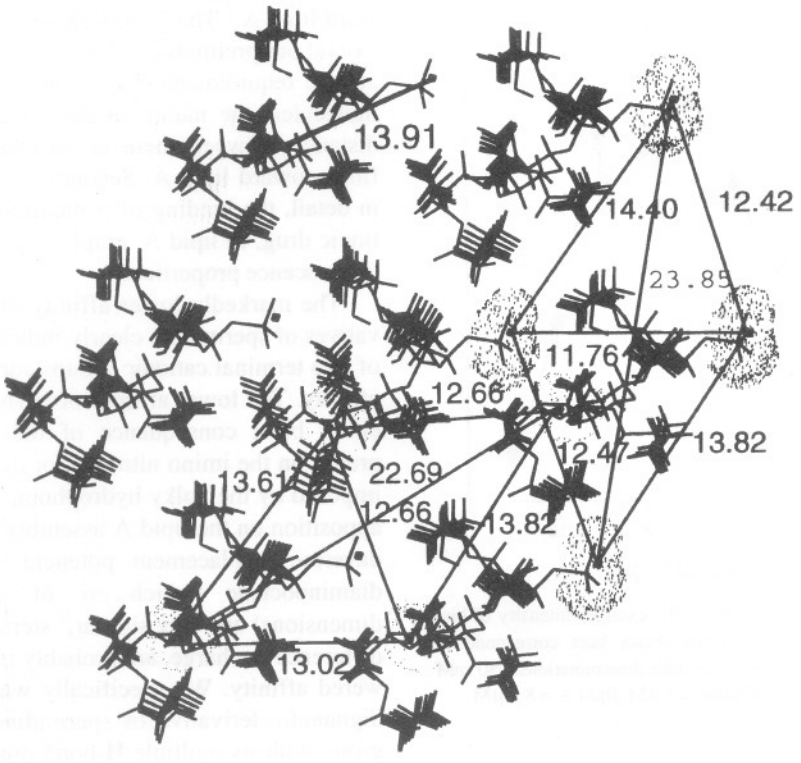

Fig. 14 — A simulated two-dimensional array of bis-phosphoryl lipid A molecules showing interphosphate distances. This array has been generated such that individual lipid A units are so juxtaposed as to be just free of intermolecular short-contacts, and the geometry of the lipid assembly corresponds to the minimal surface area. The glycosidic 1-phosphate is represented by a complete (P and O atoms) van der Waals surface, and the ester-linked 4′ phosphate by partial (P atom only) van der Waals surface. The projection of the figure is such that the acyl chains point toward the back of the page (a parallax of about 10° has been provided to facilitate visualization of the acyl chains).

equivalence of diverse cationic groups, we have observed, in the course of screening polycationic compounds, that amidinium dyes such as Alcian Blue and Alcian Yellow whose amidinium nitrogen atoms, although charged, are quarternized and are therefore nonprotonatable, do not bind lipid A (S.A. David, unpublished observations). In marshaling all of these data, it would appear that the presence of protonatable basic functions is an obligatory requirement in lipid A-binding ligands while the nature and basicity of such groups are unimportant, and a primary amino group would be as efficacious as an amidinium or guanidium moiety. The dependence of such interactions on the presence of protonatable basic functions suggests the involvement of ionic hydrogen bonds (salt bridges) at the lipid A phosphates. The lack of differences in the fluorescent probe displacement potencies between compounds of similar end-to-end dimensions but with varying terminal cationic functions (spermidine / diguanidospermidine and pentamidine / methoxy- / imidazolino-pentamidine) justified the representation of affinity only as a function of internitrogen distance, ignoring all other parameters (Fig. 4). An inspection of the model lipid A lamellar assembly (Fig. 14) suggests that several pairs of phosphate groups positioned 12–24 Å apart are available for recognition by the cationic termini of linear dibasic molecules. The distances between immediately adjacent phosphate groups range roughly between 12 Å and 14 Å, consistent with the sharp increase in affinity between spermidine and berenil (Fig. 4). Dicationic compounds may be envisaged to bind to both phosphates on the same lipid A molecule (13.9 Å), or, crosslink adjacent lipid A units, the anticipated stoichiometries being either 1:1 for the former, or asymptotically approaching 1:1 for the latter model, providing a large matrix of phosphates are considered. The intrinsic fluorescence properties of pentamidine permitted the stoichiometry of the drug-lipid A complex to be examined experimentally in aggregated as well as highly dispersed states of lipid A. Under aqueous conditions, we obtained evidence for 1:1 complexes for both bis-phosphoryl lipid A and monophosphoryl lipid A (Fig. 9). This apparently paradoxical result indicates that the concept of ligand binding within the framework of static lamellar lipid A assemblies is unacceptably simplistic and a rigorous interpretation will have to take into account possible lamellar-to-nonlamellar phase transitions concomitant with drug binding as has been observed with divalent metal binding.[37,38] We feel that a more definitive understanding of the modes of ligand binding to lipid A aggregates will have to await experimental verification using such techniques as small-angle X-ray scattering and is probably not amenable to theoretical analysis by model building studies. The results of the experiments performed in organic solvent mixtures, in which lipid A

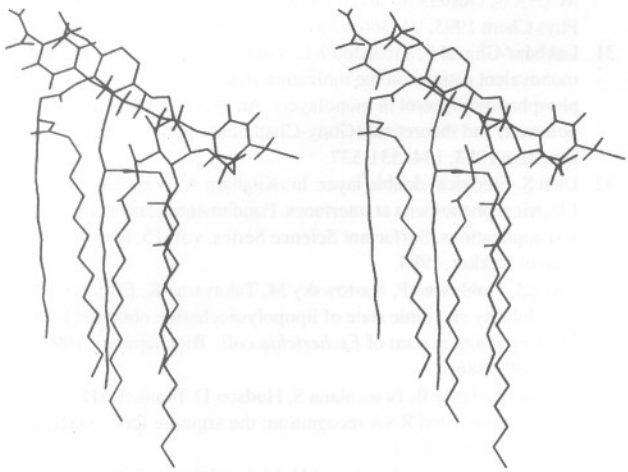

Fig. 15 — A stereo representation of an energy-minimized model of pentamidine docked on monomeric bis-phosphoryl lipid A. Hydrogen atoms are not shown for lipid A. The amidinium protons are within H-bonding distances of the lipid A phosphate O atoms.

is expected to be highly disaggregated (Fig. 10), is more straightforward having eliminated the problem of large superstructures. Pentamidine exhibits specific binding to bis-phosphoryl lipid A with a 1:1 stoichiometry. No evidence for a specific interaction with monophosphoryl lipid A could be obtained (Fig. 10), since we did not observe saturation in fluorescence intensities up to 6:1 drug/ monophosphoryl lipid A molar ratios (data not shown), presenting a strong case for the terminal amidine groups of the drug simultaneously interacting with the two phosphates on lipid A. The nature of the drug binding appears to be driven primarily by electrostatic forces, since the binding isotherms in the presence of extraneous cations yield satisfactorily to analysis on the basis of the electrical double layer theory alone. Based on these observations, we propose a model for a single unit of the pentamidine-lipid A complex as shown in Figure 15. In this model, the phenyl rings of the drug overlie the glycosidic backbone of lipid A and the amidinium protons form H-bonds of good geometry with the lipid A phosphate oxygen atoms as adjudged by N–O distances and H–N–O angles, consistent with the formation of salt bridges. While the model itself is of little intrinsic merit, given the complexity of the state(s) of lipid A under aqueous conditions, it facilitates the consideration of possible sites of introducing suitable hydrophobic groups on scaffolds similar to that of pentamidine. Appropriately positioned hydrophobic moieties which are sterically favorable should considerably enhance the entropic contributions of the free energies of interaction, thereby overcoming the expected instability of pentamidine-lipid A complex under physiologic conditions.

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