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The Effect of Gentamicin on the Biophysical Properties of Phosphatidic Acid Liposomes Is Influenced by the O–C=O Group of the Lipid†

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ABSTRACT: We previously reported that gentamicin binds to liposomes composed of anionic phospholipids and depresses glycerol permeability and raises the activation energy for glycerol permeation in these liposomes. We postulated that these changes in the glycerol permeability and in the activation energy (E_a) for glycerol permeation were due to hydrogen bonding between O—C=O groups in the hydrogen belt and one or more amino groups of gentamicin. To test this hypothesis, we examined the effects of gentamicin on the membrane surface potential, the glycerol permeability coefficient ($\tilde{p}$), the E_a for glycerol permeation, and the aggregation of liposomes composed of 1:1 phosphatidylcholine (PC) and phosphatidic acid with the acyl chains of phosphatidic acid in either an ester (PA) or an ether (PA*) linkage. Gentamicin depressed the membrane surface electrostatic potential, measured by the partitioning of methylene blue between the bulk solution and the liposomal membrane, to an equivalent degree in PC–PA and PC–PA* liposomes, which indicates that substitution of the ether for the ester linkage did not interfere with the electrostatic interaction between the cationic drug and the negatively charged phosphate head group. Gentamicin caused a temperature-dependent decrease of $\tilde{p}$ and raised E_a for glycerol permeation from 17.7 ± 0.3 to 21.6 ± 0.4 kcal/mol in PC–PA liposomes but had little or no effect on these parameters in PC–PA* liposomes. In contrast, gentamicin induced a significantly greater degree of aggregation of PC–PA* liposomes compared to that of PC–PA liposomes. These data support the conclusion that gentamicin interacts with the carbonyl ester groups linking the acyl chains to the glycerol backbone of PA and that this interaction is entirely consistent with hydrogen bonding. These results provide new information about the molecular binding of gentamicin to anionic phospholipids and the effects of such binding on the biophysical properties of model membranes.

Aminoglycoside antibiotics are known to cause nephrotoxicity (Humes et al., 1982; Kahlmeter & Dalhlager, 1984) and ototoxicity (Brummett, 1980) in man and in experimental animals. Toxicity in the kidney is confined to proximal tubular cells that have been shown to transport and accumulate these drugs in high concentration (Kaloyanides, 1984a). Transport and accumulation of aminoglycoside antibiotics by hair cells also underlie the ototoxicity of these drugs (Tran Ba Huy et al., 1986). A growing body of evidence supports the view that the toxicity of these agents resides in their potential to bind to and alter the function of plasma and subcellular membranes (Kaloyanides, 1984b). Investigators have sought to elucidate the mechanism of binding of these drugs to membranes. Studies of biological (Kirschbaum, 1984) and model membranes (Schacht et al., 1978; Lullmann & Vollmer, 1982; Brasseur et al., 1984; Wang et al., 1984; Chung et al., 1985; Au et al., 1986, 1987) have demonstrated that these polycationic drugs bind to anionic phospholipids, especially phosphatidylinositol 4,5-bisphosphate (Schacht et al., 1978; Wang et al., 1984; Au et al., 1986, 1987), whereas little or no binding occurs to anionic nonlipid compounds such as melanin, gangliosides, or chondroitin sulfate (Williams & Schacht, 1986). This has led to the hypothesis that anionic phospholipids, especially the phosphoinositides, function as membrane receptors for these drugs (Schacht, 1979; Sastrasinh et al., 1982).

Chung et al. (1985) examined the binding of gentamicin and spermine to liposomes containing negatively charged phospholipids using the technique of microelectrophoresis. Gentamicin and spermine have similar net charges (about +3.5 at pH 7.4), and both agents depressed the membrane surface

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electrostatic potential to a similar extent. The authors concluded that the binding of these agents followed the prediction of the Gouy–Chapman–Stern theory and, thus, could be explained by an electrostatic interaction between the polycationic drugs and the negatively charged phosphate head groups such that the polycations adsorbed to the membrane and reduced the negative surface electrostatic potential. In addition, Chung et al. (1985) postulated a model in which gentamicin and spermine lie flat on the surface when they bind to the negatively charged membranes. Brasseur et al. (1984) have also proposed an electrostatic interaction between the cationic aminoglycoside antibiotics and anionic phospholipids; however, in their model the drugs penetrate into the hydrophobic core of the membrane.

Recently we examined the interaction of aminoglycosides and spermine with phosphatidylinositol-containing liposomes and observed that the antibiotics decreased the permeability of the liposomal membrane to the nonelectrolyte glycerol and raised the energy of activation for glycerol permeation, whereas spermine was without effect (Ramsammy & Kaloyanides, 1986, 1987). These observations implied that the interaction of aminoglycosides with the liposomal membrane was qualitatively different from that of spermine and involved more than neutralization of surface charge. We postulated a model in which the positively charged amino groups of the antibiotic engaged in hydrogen bonding with carbonyl groups of the glycerol backbone of the lipid.

In the present study we sought to test our model by examining the effects of gentamicin on glycerol permeability and the activation energy for glycerol permeation in liposomes composed of an anionic phospholipid with an ether rather than an ester linkage to glycerol. Our model predicts that the absence of the carbonyl group would greatly attenuate the influence of gentamicin on glycerol permeation by preventing hydrogen bonding.

Materials and Methods

Egg phosphatidylcholine (PC)[1] and 1-palmitoyl-2-oleoyl-*sn*-glycerol 3 phosphate [diester phosphatidic acid (PA)] were obtained from Avanti Polar Lipids (Birmingham, AL). Gentamicin sulfate was a gift from the Schering Corp. (Bloomfield, NJ). The ether form of PA (PA*) was synthesized as described previously (Brockerhoff & Ayengar, 1978) from 1-palmityl-*sn*-glycerol (chimyl alcohol) → 1-palmityl-3-trityl-*sn*-glycerol → 1-palmityl-2-oleyl-3-trityl-*sn*-glycerol → 1-palmityl-2-oleyl-*sn*-glycerol → 1-palmityl-2-oleyl-*sn*-glycerol 3-phosphate (i.e., diether PA*).

Surface Electrostatic Potential. Unilamellar vesicles consisting of PC and PA or PC and PA* in molar ratios of 9:1 and 1:1 were prepared in a solution composed of 5 mM HEPES, 0.2 mM Tris, and 0.2 mM Na_2EDTA, pH 7.0. Dried lipid films were dispersed in buffer by vortexing. The multilamellar vesicles formed were sonicated at 15 °C for 60 s × 2 followed by centrifugation at 100000*g* for 1 h. The supernatant containing the unilamellar vesicles was aspirated and assayed for lipid concentration. Membrane surface potential was calculated from the partitioning of methylene blue (MB) into the bilayer (Nakagaki et al., 1981) as a function of increasing concentrations of anionic phospholipid. Liposomes (0.02–0.3 mg/mL) were incubated with gentamicin (10^{-4} M) for 60 min prior to incubation with MB (2×10^{-5} M) for 30 min at room temperature following which time an absorbance spectrum between 720 and 500 nm was obtained with a Beckman scanning spectrophotometer. The positively charged monomeric MB absorbs maximally at 664 nm. When the dye contacts a membrane with a negative surface potential, it partitions as dimeric MB into the membrane as a function of the strength of the surface potential. The absorbance at 664 nm declines as a consequence of the decrease in the concentration of MB in the bulk solution.

From the partitioning of MB in the membrane the membrane surface electrostatic potential (ψ) can be calculated by using the equation derived by Nakagaki et al. (1981)

$$\psi = 2.30(kT/e)(\log K_0 - \log K)$$

where $2.30(kT/e) = 59.2$ mV at 25 °C and K and K_0 are defined by the partitioning of MB into the membrane as a function of the concentration of acidic phospholipid. The interested reader should consult the original paper of Nakagaki et al. (1981) for specific details about the derivation of this formula.

Glycerol Permeability. Multilamellar liposomes consisting of 1:1 and 9:1 molar ratios of PC–PA and PC–PA* were prepared as previously described (Ramsammy & Brockerhoff, 1982) in 0.15 M KCl and 10 mM Tris, pH 7.0, and incubated with and without gentamicin (10^{-4} M) at 50 °C for 1 h. Twenty microliters of liposomes was added to 1 mL of 0.3 M glycerol, and the change in absorbance at 450 nm due to liposome swelling was monitored. The relative permeability coefficient $\tilde{p}$ was calculated from the formula

$$\tilde{p} = (dA/A_0^2)t^{-1}$$

where dA is the change of absorbance over time, A_0 is the initial absorbance, and t is time in minutes (De Gier et al., 1968; Blok et al., 1976). Measurements were made at temperatures ranging from 24 to 37 °C.

Aggregation. Aggregation was measured by the turbidity method according to published procedures (Ohyashiki et al., 1984). The effect of gentamicin (10^{-3} M) on the aggregation of unilamellar liposomes was monitored continuously at 25 °C in a Beckman recording spectrophotometer at a wavelength of 450 nm. In the absence of liposomes, gentamicin did not influence absorbance at 450 nm.

The data in the text, tables, and figures are expressed as the mean ± SE. Analysis of variance and the Duncan new multiple range test were used to assess statistical significance of differences within each group, and the Student *t*-test for group data was used to assess statistical significance of differences between groups. A P value ≤ 0.05 was considered significant. Linear regression was performed by the method of least-squares.

Results

Figure 1 illustrates a representative absorption spectrum for MB incubated with varying quantities of PC–PA liposomes. As the quantity of PA in the incubation mixture was raised, increasing amounts of MB partitioned from the bulk phase into the liposomal membrane and resulted in a proportional reduction of MB absorbance. Table I summarizes the effect of incubating MB with liposomes containing increasing concentrations of PA or PA* on the MB absorbance ratio (A/A_0), where A and A_0 represent absorbance in the presence and absence of membranes. A ratio of one indicates no partitioning, whereas a ratio of zero signifies complete partitioning of MB into the membrane. A/A_0 progressively declined as the concentration of PA or PA* in the liposomal membrane was increased. Of particular importance is the observation

[1] Abbreviations: E_a, energy of activation; MB, methylene blue; PA, phosphatidic acid with acyl chains in ester linkage; PA*, phosphatidic acid with alkyl chains in ether linkage; PC, phosphatidylcholine.

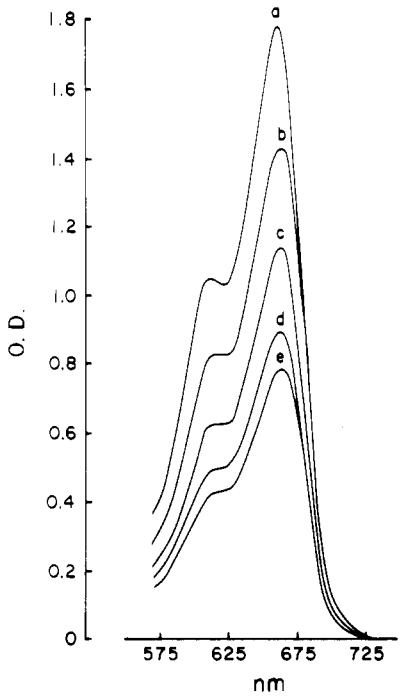

FIGURE 1: Influence of increasing quantities of PC–PA (9:1) liposomes on the absorption of MB: (a) Pure MB; (B) 0.10 mg of phospholipid/mL; (c) 0.15 mg of phospholipid/mL; (d) 0.20 mg of phospholipid/mL; (e) 0.25 mg of phospholipid/mL.

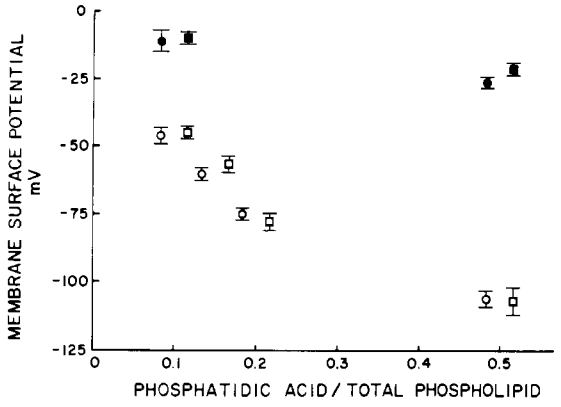

FIGURE 2: Effect of increasing concentration of PA (circles) or PA* (squares) in the absence (open symbols) and presence (solid symbols) of gentamicin on the liposomal membrane surface electrostatic potential.

Table I: Influence of Liposomal Concentration of Phosphatidic Acid on the Partitioning of Methylene Blue into the Membrane As Assessed by the Change in Absorbance Ratio, A/A_0[a]

phosphatidic acid/total phospholipid	A/A_0	
	PA	PA*
0.01	0.819 ± 0.012	0.854 ± 0.025
0.15	0.447 ± 0.014	0.443 ± 0.017
0.20	0.295 ± 0.006	0.274 ± 0.010
0.50	0.226[b] ± 0.006	0.243[b] ± 0.010

[a] A is the absorbance at 450 nm of methylene blue (2×10^{-5} M) in the presence of liposomes (0.1 mg of phospholipid/mL). A_0 is the absorbance of methylene blue in the absence of liposomes. Data represent mean ± SE, $N = 5$. [b] Lipid concentration is 0.04 mg/mL.

that PA*-containing liposomes affected A/A_0 to the same extent as liposomes containing identical concentrations of PA. From these data the liposomal membrane surface electrostatic potential was calculated.

Figure 2 illustrates that the membrane surface electrostatic potential became progressively more negative as the concentration of PA or PA* in the liposome was increased. The values obtained for the surface potentials are comparable to published values for liposomes containing negatively charged lipids obtained by using different methods (Nakagaki et al., 1981; Castle & Hubbel, 1983; Chung et al, 1985). Interestingly, liposomes containing identical concentrations of PA and PA* had similar membrane surface electrostatic potentials.

When PA- and PA*-containing liposomes were incubated with gentamicin (10^{-4} M), the membrane surface electrostatic potentials were depressed to the same extent (Figure 2), which indicates similar electrostatic interactions between the polycationic gentamicin and the negatively charged phosphate head groups of PA and PA*. Although gentamicin depressed the liposomal membrane surface electrostatic potentials to the same extent, gentamicin induced a significantly greater degree of aggregation of PC–PA* liposomes compared to that of PC–PA liposomes (Figure 3).

Gentamicin caused a temperature-dependent depression of glycerol permeability in PC–PA liposomes. The glycerol permeability of PC–PA* liposomes was significantly lower than that of PC–PA liposomes, and gentamicin exerted only

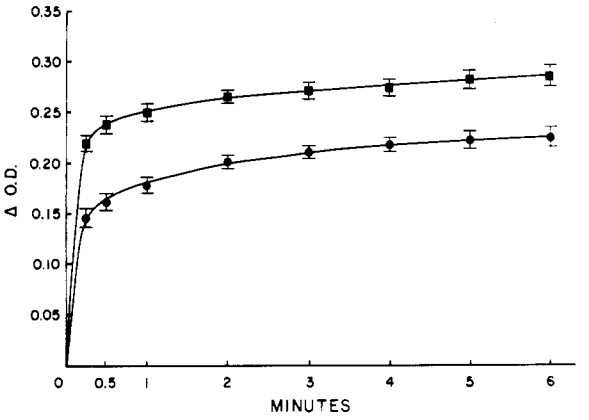

FIGURE 3: Effect of gentamicin on the aggregation of unilamellar PC–PA (1:1) (circles) and PC–PA* (1:1) (squares) liposomes as assessed by the change in absorbance at 450 nm.

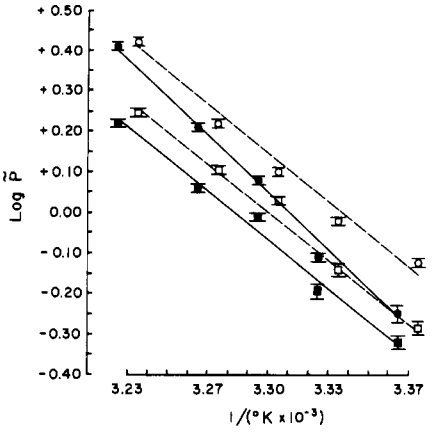

FIGURE 4: Arrhenius plot of the relative permeability coefficient ($\tilde{p}$) of liposomes comprised of PC–PA (1:1) (circles) and PC–PA* (1:1) (squares) in the absence (open symbols) and presence (solid symbols) of gentamicin (10^{-4} M). Data depict mean ± SE, $N = 5$. PA, diester phosphatidic acid; PA*, diether phosphatidic acid; G, gentamicin. The equations for the regression lines are

PA $Y = -3.86X + 12.86$, $r^2 = 0.98$

PA + G $Y = -4.73X + 15.69$, $r^2 = 0.99$

PA* $Y = -3.67X + 12.08$, $r^2 = 0.99$

PA* + G $Y = -3.91X + 12.87$, $r^2 = 0.99$

a trivial and inconsistent effect on glycerol permeability of these liposomes. The Arrhenius plot of the permeability data (Figure 4) demonstrates a significant difference between the slope of the regression line describing the data of PC–PA liposomes incubated with gentamicin and the slopes of the regression lines of the other three groups. Calculation of the activation energy (E_a) for glycerol permeation revealed that E_a was significantly increased from 17.7 ± 0.3 to 21.6 ± 0.4 kcal/mol ($P < 0.01$) in PC–PA liposomes incubated with gentamicin but was not altered (16.8 ± 0.5 to 17.8 ± 0.5 kcal/mole, $P > 0.2$) in PC–PA* liposomes incubated with gentamicin.

Discussion

In this study we have shown that substitution of an ether group (O—CH_2) for the carboxyl ester group (O—C=O) of the acyl chains of phosphatidic acid did not alter the magnitude of the membrane surface electrostatic potential and did not prevent gentamicin from interacting electrostatically with the anionic phosphate head group of phosphatidic acid. The latter conclusion is based on the observation that gentamicin depressed the membrane surface electrostatic potentials of PA and PA* liposomes to the same degree.

It should be noted that in phospholipid monolayers the C=O group has been shown to play a significant role in determining the membrane potential (ΔV) as evident by the finding that phospholipid monolayers composed of PC in ether linkage have membrane potentials 30–200 mV lower than those of the corresponding phospholipid monolayers in ester linkage (Paultauf et al., 1971; Shah & Schulman, 1965). The MB method like the electrophoretic mobility method (McLaughlin & Harary, 1976) does not sense the potential within phospholipid bilayers but measures only the surface electrostatic potential (diffuse double layer). Thus, in PC liposomes where the surface charge has been estimated to be zero by the electrophoretic mobility technique (Chung et al., 1985; McLaughlin, 1977), we detected no measurable partitioning of MB into the bilayer. Although the implicit neglect of interfacial structure and surface polarity effects is recognized as a fundamental shortcoming of the diffuse double layer model, this limitation does not invalidate our conclusion that the ether/ester substitution in phosphatidic acid did not alter the magnitude of the membrane surface electrostatic potential and did not prevent gentamicin from interacting electrostatically with the anionic head group of phosphatidic acid.

Whereas the ether/ester substitution in phosphatidic acid did not affect the membrane surface electrostatic potential, it did affect other biophysical properties of the liposomal membrane. The glycerol permeability coefficient of PA* liposomes was significantly reduced below that of PA liposomes. The mechanism underlying this change in permeability is not clear. We considered the possibility that it might be related to an increase in the effective hydrophobic length of the alkyl chains of PA* liposomes because the permeability of a phospholipid bilayer has been shown to be influenced by the effective hydrophobic length of the acyl chains (De Gier et al., 1968; Blok et al., 1975; Tirri et al., 1977; Brockerhoff, 1977), and substitution of the ether for the ester linkage increases the effective hydrophobic length of the alkyl chains by one carbon (Tanford, 1973). In our experiments the acyl chains of PA liposomes and the alkyl chains of PA* liposomes were identical. Therefore, the effective hydrophobic length of the alkyl chains of PA* liposomes should have increased by one CH_2 group as a consequence of the ether/ester substitution (Tanford, 1973). Since an increase of the effective hydrophobic length by two CH_2 groups reduces the glycerol permeability of liposomes by approximately 50% (De Gier et al., 1968), it seemed reasonable to postulate that an increase of the effective hydrophobic length by one CH_2 group could explain the decrease in glycerol permeability of PA* liposomes observed in our study. However, Clejan et al. (1979), using a similar experimental design, found that the substitution of the ether for the ester linkage had no significant effect on glycerol permeation. We discerned no obvious explanation for the different results of the two studies. In light of this difference, the reason for the decreased glycerol permeability of PA* liposomes must be viewed as uncertain at this time.

Whereas gentamicin depressed glycerol permeability and raised E_a for glycerol permeation in PA liposomes, gentamicin had only a trivial effect on glycerol permeation of PA* liposomes and no effect on E_a for glycerol permeation despite the fact that gentamicin reduced the membrane surface potentials of both types of liposomes to the same extent. From these observations we conclude that gentamicin interacted with the carboxyl ester groups of phosphatidic acid and that this interaction was responsible for the observed changes in glycerol permeation and E_a for glycerol permeation. We considered the possibility that gentamicin's ability to reduce glycerol permeability in PA but not PA* liposomes might be due to the fact that gentamicin condenses bilayers of PA but not of PA* liposomes because the latter are a priori in a more condensed state. We discounted this explanation because it has been shown that molecular packing in ether lipid liposomes is not different from that of ester lipid liposomes (Fong et al., 1977; Paltauf et al., 1971). In addition, it has been shown that diester and diether lipids form multilamellar and unilamellar vesicles of similar size (Tirri et al., 1977; Ramsammy et al., 1983).

Although we did not investigate directly the nature of the interaction between gentamicin and the carbonyl groups of phosphatidic acid, previous studies provide support for the hypothesis that this interaction involves hydrogen bonding. Glycerol permeation of model membranes has been shown to be influenced by the fluidity of the membrane and the membrane's capacity to dehydrate glycerol (Stein, 1967; Cohen, 1975; Ramsammy & Brockerhoff, 1982). E_a for glycerol permeation is independent of membrane fluidity. Rather, it correlates with the ability of the membrane to dehydrate glycerol (De Gier et al., 1971; Wright & Bindslev, 1976; Ramsammy & Brockerhoff, 1982), and the carboxyl ester linking the fatty acid to the glycerol back bone has been implicated in this dehydration process (Tirri et al., 1977; Ramsammy & Brockerhoff, 1982; Ramsammy et al., 1983). This region of the membrane has been termed the hydrogen belt because the carboxyl ester groups can form hydrogen bonds with groups such as OH^- of cholesterol and water and with amino groups of protein (Brockerhoff, 1977; Ramsammy & Brockerhoff, 1982; Ramsammy et al., 1983). Previous studies have shown that enhancement of hydrogen bonding within the hydrogen belt augments E_a for nonelectrolyte permeation (Ramsammy & Brockerhoff, 1982; Ramsammy et al., 1983). In PC–PA liposomes incubated with gentamicin the E_a for glycerol permeation was raised from 17.7 ± 0.3 to 21.6 ± 0.4 kcal/mol, which is consistent with the energy required to break approximately two hydrogen bonds (Tanford, 1973; Cohen, 1975). Substitution of the ether for the carboxyl ester linkage is known to reduce the capacity of the hydrogen belt to participate in hydrogen bonding (Tirri et al., 1977; Ramsammy et al., 1983). This could account for the failure of gemtamicin to influence the E_a for glycerol permeation in PC–PA* liposomes.

Substitution of the ether for the carboxyl ester linkage also facilitated aggregation of PC–PA* liposomes by gentamicin. In order for membrane aggregation to occur, the electrostatic repulsive force generated by the membrane surface charge must be neutralized (Poste & Allison, 1973; Papahadjopoulos et al., 1979; Schuber et al., 1983). In addition to electrostatic repulsion, the major force restricting membrane aggregation, the repulsive force of hydration, also limits membrane aggregation (Cowley et al., 1978; Wilschut et al., 1981; Ohyashiki et al., 1984). Water is known to hydrogen bond to carboxyl ester groups of lipids (Brockerhoff, 1977; Smaby et al., 1984). To aggregate PC–PA liposomes, gentamicin had to overcome the major repulsive force generated by the negative membrane surface charge and the minor hydration force generated by hydrogen bonding of water to the carboxyl ester groups. In PC–PA* liposomes substitution of the ether for the ester linkage in PA* limited hydrogen bonding between water and the carboxyl ester groups. We postulate that the reduced force of hydration in PC–PA* liposomes accounts for the difference between the extent of aggregation of PC–PA* liposomes and of PC–PA liposomes induced by gentamicin. One might argue that the postulated interaction between gentamicin and the C=O group should reduce the hydration force and, therefore, the rates of aggregation should be equivalent. However, this interaction does not necessarily cause a complete reduction of the hydration force. It is also possible that the hydration force is stabilized rather than reduced by the interaction.

It should be emphasized that hydrogen bonding requires precise spatial orientation of donor groups and acceptor groups. In the case of aminoglycoside antibiotics, we postulate that hydrogen bonding occurs between amino groups and the carboxyl ester groups of PA. In previous studies we observed that the rank order of aminoglycoside antibiotics with respect to the magnitude of their effects on E_a for glycerol permeation (Ramsammy & Kaloyanides, 1986) as well as their potency in promoting aggregation of PC–PI liposomes (Ramsammy et al., 1987) did not correlate with the net charges of these agents. These findings are not unexpected and serve to emphasize that spatial orientation rather then net charge is the critical determinant of hydrogen bonding. Thus, the failure of spermine, with a net charge equal to that of gentamicin (Josepovitz et al., 1982), to influence the E_a for glycerol permeation (Ramsammy & Kaloyanides, 1986) and its weak effect on liposomal aggregation (Ramsammy et al., 1987), despite the fact that it interacted electrostatically and decreased the surface charge of liposomes to the same extent as gentamicin (Chung et al., 1985), probably reflect its linear molecular structure that precludes hydrogen bonding with components of the hydrogen belt when the molecule is oriented parallel to the membrane surface.

It is also noteworthy that the rank order of aminoglycosides with respect to the magnitude of their effects on E_a for glycerol permeation (Ramsammy & Kaloyanides, 1986) and aggregation of PC–PI liposomes (Ramsammy et al., 1987) correlated with the established nephrotoxicity potentials of these agents (Kaloyanides & Pastoriza, 1980). Derangement of membrane function consequent to the binding of aminoglycosides to plasma and subcellular membranes has been advanced as the fundamental pathogenic mechanism of aminoglycoside toxicity (Kaloyanides, 1984b). Knowledge about the binding of aminoglycosides to model membranes gained from the present experiments may provide new insight into possible molecular mechanisms underlying these derangements of membrane function.

Registry No. Gentamicin, 1403-66-3.

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Conversion of Cu_A to a Type II Copper in Cytochrome *c* Oxidase†

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ABSTRACT: When cytochrome *c* oxidase is incubated at 43 °C for ~75 min in a solution containing the zwitterionic detergent sulfobetaine 12, the Cu_A site is converted into a type II copper as judged by changes in the 830-nm absorption band and the EPR spectrum of the enzyme. SDS-PAGE and sucrose gradient ultracentrifugation indicate concomitant loss of subunit III and monomerization of the enzyme during the heat treatment. Comparison of the optical and resonance Raman spectra of the heat-treated and native protein shows that the heme chromophores are not significantly perturbed; the resonance Raman data indicate that the small heme perturbations observed are limited to the cytochrome a_3 site. Proton pumping measurements, conducted on the modified enzyme reconstituted into phospholipid vesicles, indicate that these vesicles are unusually permeable toward protons during turnover, as previously reported for the *p*-(hydroxymercuri)benzoate-modified oxidase and the modified enzyme obtained by heat treatment in lauryl maltoside. The sulfobetaine 12 modified enzyme is no longer capable of undergoing the recently reported conformational transition in which the tryptophan fluorescence changes upon reduction of the low-potential metal centers. Control studies on the monomeric and subunit III dissociated enzymes suggest that the disruption of this conformational change in the heat-treated oxidase is most likely associated with perturbation of the Cu_A site. These results lend support to the suggestion that the fluorescence-monitored conformational change of the native enzyme is initiated by reduction of the Cu_A site [Copeland et al. (1987) *Biochemistry 26*, 7311].

As a redox-linked proton pump, cytochrome *c* oxidase is expected to be an allosteric enzyme. Evidence for allosteric behavior has recently been put forth for the mitochondrial protein (Scholes & Malmström, 1986; Copeland et al., 1987, and references cited therein). Scholes and Malmström (1986) reported that electron input into the low-potential centers, namely, cytochrome *a* and Cu_A, greatly accelerates the rate of inhibitory cyanide binding at the dioxygen binding site, i.e., cytochrome a_3/Cu_B. These workers suggested that reduction of the two low-potential metal centers induces a conformational transition of the protein, opening up the dioxygen binding pocket to the ligation of exogenous ligands. This phenomenon accordingly has been termed the "open-closed transition". More direct evidence for such a protein allosteric effect has recently been provided by intrinsic tryptophan fluorescence spectroscopy (Copeland et al., 1987). These workers noted that a small number of otherwise buried tryptophan residues in the beef heart enzyme become exposed to the solvent upon reduction of Cu_A and that the fluorescence yield of the exposed tryptophans appears to be correlated with the inhibitory cyanide-binding kinetics reported by Scholes and Malmström (1986). On the basis of a detailed analysis of both the fluorescence and kinetic data, it was argued that the open-closed transition and the fluorescence-monitored transition are more likely linked to the reduction of Cu_A alone rather than

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