Drug–Biomolecule Interactions: Interaction of Gentamicin with Lipid Monomolecular Films

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Abstract □ The interaction of gentamicin with monomolecular films of a series of biologically important lipids spread on an aqueous buffered subphase was studied. The surface pressure, π, of these films was determined by the Wilhelmy plate method as a function of surface area, A, and π–A curves were constructed. Changes in the π–A characteristics in the presence of gentamicin were used as a measure of antibiotic–film interaction. No interaction was observed between gentamicin and films of cholesterol, egg lecithin, dipalmitoyl lecithin, phosphatidyl ethanolamine, stearyl alcohol, and bovine ceramides at all pH values studied. Stearic acid films showed no interaction with gentamicin at pH 5. At pH 7 and 8, a small increase in pressure (~3 dynes/cm) was noted. A dramatic increase in surface pressure was observed in the presence of stearyl aldehyde films ranging from approximately 9 dynes/cm at pH 7.2 to 23 dynes/cm at pH 8.4. This effect was attributed to a Schiff-base reaction between the nonprotonated primary amino groups on the gentamicin molecule and the stearyl aldehyde. Further evidence was provided by the fact that the addition of glucose (which has been reported to participate in Schiff-base formation with amines) to the subphase inhibited the stearyl aldehyde–gentamicin interaction. Sucrose did not show a corresponding effect. The addition of sodium bisulfite, which reacts with aldehydes to form α-hydroxysulfonic acid, also inhibited the gentamicin–stearyl aldehyde interaction. It is postulated that Schiff-base formation is a step in the *in vivo* transport of gentamicin across the membrane of sensitive organisms.

Keyphrases □ Gentamicin—interaction with lipid monomolecular films, surface pressure, π–A curves □ Monomolecular films—interaction of biologically important lipids with gentamicin, surface pressure, π–A curves □ Films, monomolecular—interaction of gentamicin with lipids □ Drug–biomolecule interactions—gentamicin with monomolecular films □ Interactions—drugs with biomolecules, symposium

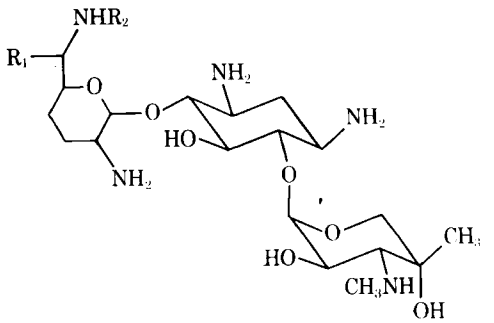
Gentamicin is a water-soluble aminoglycoside antibiotic derived from the micromonospora family and is active against both Gram-negative and Gram-positive bacteria. The gross structures of the gentamicin components (I) were recently reported (1). The biological activities of the three gentamicin components are essentially identical (2).

Gentamicin, in common with the other members of the kanamycin family, exhibits some interesting biophysical properties. It does not transport to any significant degree across the membranes of the GI tract, as evidenced by the fact that less than 2% absorption has been observed following oral administration[1]. In contrast, gentamicin does cross some specialized membranes; *e.g.*, it penetrates the aqueous humor of the eye (3), passes the placental barrier and into the amniotic fluid (4), and transports across the otic membrane (5). Furthermore, since the site of gentamicin antibacterial action is at the ribosomal level (6), it clearly must penetrate the bacterial membrane.

The mechanism by which gentamicin transports across bacterial and other membranes, however, has not been firmly established. Its solubility characteristics and its negligible oil–water partition coefficient (7) preclude dissolution or partitioning into lipoidal membranes. Its relatively large molecular size precludes transport through water-filled pores or channels, as has been postulated for some simple cations and anions. Therefore, it appears that its transport must involve, at least as a first step, some specific interaction with a cell membrane component.

Studies with other aminoglycoside antibiotics in which an interaction with bacterial cell membranes has been demonstrated (8) support this postulation. However, the specific sites on the membrane involved in the antibiotic–cell membrane interaction have not been identified, nor has the role of specific membrane lipids in this process been established.

This investigation examined the potential participation of membrane lipids in the transport of gentamicin across cell membranes. Monomolecular films were selected as the membrane model, because they provide a relatively simple, well-defined, oriented structure with some similarity to biomembranes. In addition, they allow for modification of composition of both the "membrane" (*i.e.*, the film) and the subphase on which the film is spread. Furthermore, numerous studies have demonstrated that there is a correlation between the interaction of many drugs with monomolecular films and their biological activity (9).



I

C_1: $R_1 = R_2 = CH_3$
C_{1A}: $R_1 = R_2 = H$
C_2: $R_1 = CH_3$, $R_2 = H$

[1] D. Cooper, Schering Corp., Bloomfield, N.J., personal communication, 1970.

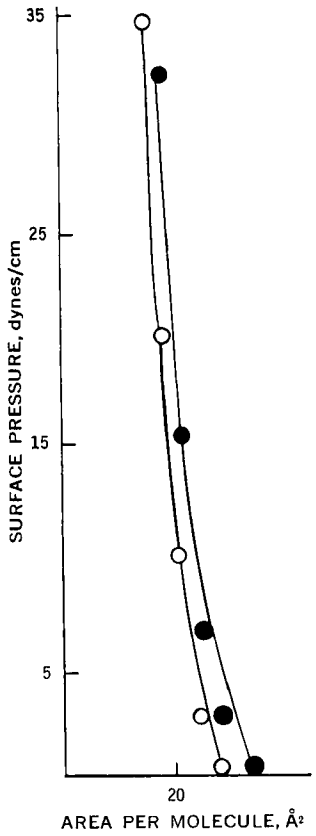


Figure 1—*Surface pressure–surface area curves of stearic acid at pH 7.0, alone* (○) *and in the presence of 1* × 10^{-3} M *gentamicin* (●).

EXPERIMENTAL

Gentamicin base[2] was obtained as a mixture of active components C_1, C_{1A}, and C_2. Stearyl aldehyde[3] (purity >99%) was obtained as a solution in carbon disulfide. The stearic acid[4], stearyl alcohol[4], cholesterol[3], L-α-dipalmitoyl lecithin[4], and egg lecithin[5] were obtained chromatographically pure. Cerebrosides[6], ceramides[6], and phosphatidyl ethanolamine[3], extracted from bovine brain, and sucrose dipalmitate[7] were used as obtained. Dextrose USP and sucrose USP were crystallized from boiling water. All inorganic chemicals used were reagent grade, and all organic solvents were of spectrophotometric grade. The water used was prepared by double distillation from an all-glass apparatus.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the films was described previously (10).

The temperature of the subphase was maintained at 25 ± 0.1° by circulating water from a constant-temperature bath through a water jacket around the trough.

The lipids were placed on the surface of the subphase, using a micrometer syringe[8] capable of delivering volumes as small as 0.001 ml. The spreading solvent for the lipids was *n*-hexane, except that benzene was the spreading solvent for sucrose dipalmitate, cerebrosides, and ceramides. To allow for complete evaporation of the spreading solvent, 5 min was allowed before manual compression was initiated. After each compression, 15 sec was allowed to elapse before the next surface pressure measurement was taken.

Mixed monomolecular films of various mole fractions of egg lecithin and stearyl aldehyde were prepared by adding the appropriate quantities of each component to the surface. The number of film molecules was kept constant.

The interaction of gentamicin with the films was studied by the following procedures. Gentamicin (10^{-3} or 10^{-4} *M* concentration) was dissolved in the subphase (0.01 *M* phosphate buffer at constant ionic strength and various pH values), and the various films were spread over this subphase. Differences in the π–A characteristics of pure films and films in the presence of drug were determined and used as a measure of drug–film interactions.

[2] Schering Corp., Bloomfield, N.J.
[3] Applied Science Laboratories, State College, Pa.
[4] Mann Research Laboratories, New York, N.Y.
[5] Sylvana Laboratories, Millburn, N.J.
[6] P-L Biochemicals, Milwaukee, Wis.
[7] Pfaltz & Bauer, Flushing, N.Y.
[8] Agla.

RESULTS AND DISCUSSION

Cholesterol and the phospholipids, egg lecithin, L-α-dipalmitoyl lecithin, and phosphatidyl ethanolamine, represent components that constitute a major fraction of the lipid constituents of cell membranes. None of these materials, when spread as monomolecular films, showed any significant interaction with gentamicin (1 × 10^{-4} or 1 × 10^{-3} *M* concentration) over a pH range of 4–8.4. Bovine ceramides and stearyl alcohol also exhibited no interaction with the antibiotic under similar conditions.

These data demonstrate that gentamicin does not interact nonspecifically with lipid films and support the contention that gentamicin does not transport into bacterial membranes *via* a partitioning mechanism.

Since gentamicin is a base, the interaction with an acidic film was explored next. Stearic acid was selected as the model system. At a gentamicin concentration of 1 × 10^{-4} *M*, no interaction was observed with the stearic acid film over a pH range of 5–8. At 1 × 10^{-3} *M*, an increase of about 3 dynes/cm at all areas per molecule was observed at pH 7 (Fig. 1) and similarly at pH 8. No interaction was observed at lower pH values. Thus, it appears that, as the ionization of gentamicin is partially depressed within the physiological pH range, some film–antibiotic interaction does occur.

The natural cerebrosides, which are mixtures of galactolipids, also exhibited an interaction with 1 × 10^{-3} *M* gentamicin (Fig. 2) of about the same magnitude as was observed with the stearic acid film. Since the cerebrosides differ from the previously studied ceramides (which showed no interaction) only by the addition of a galactose sugar moiety, it appears that the observed interaction is specific and is dependent on the presence of this sugar residue.

However, when sucrose dipalmitate was spread, no interaction with gentamicin was observed. This finding indicates that the free aldehydic function of the galactose residue is essential for the interaction.

To investigate this possibility further, the interaction of gentamicin with stearyl aldehyde films was studied. The π–A curves of stearyl aldehyde films spread on a subphase in the absence of gentamicin were identical throughout the pH range of 6–8.4. In the presence of 1 × 10^{-3} *M* gentamicin, a dramatic interaction was observed, and the extent of this interaction increased regularly with increasing pH (Fig. 3). A similar, but reduced, interaction was observed at 1 × 10^{-4} *M*.

Thus, it is evident that the reaction not only is dependent on the

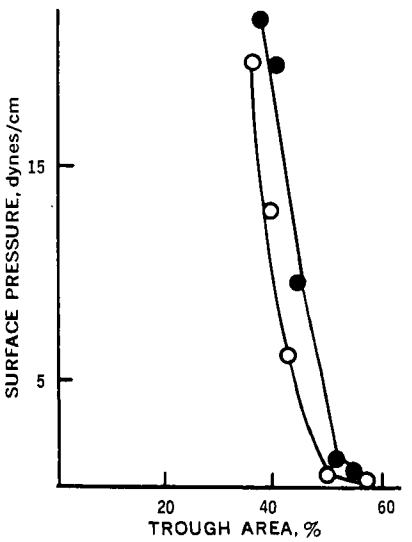


Figure 2—*Surface pressure–percent trough area curves of cerebrosides at pH 7.0, alone* (○) *and in the presence of 1* × 10^{-3} M *gentamicin* (●).

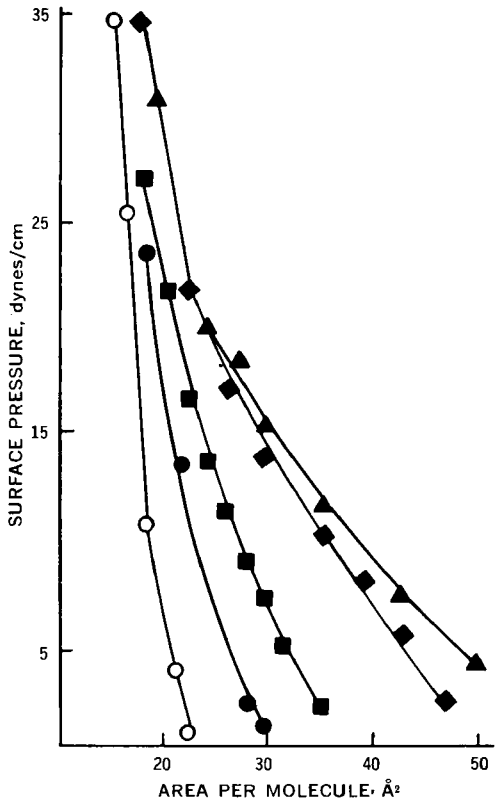


Figure 3—*Surface pressure–surface area curves of stearyl aldehyde, alone at pH values of 6.0–8.4* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH values of 7.2* (●), *7.6* (■), *8.0* (◆), *and 8.4* (▲).

presence of an aldehydic function in the film but also on the degree of protonation of the primary amino groups on the gentamicin and that the observed film–antibiotic interaction is the result of a Schiff-base interaction between these two functional groups.

The addition of glucose (1×10^{-4} *M*) into the subphase partially inhibited the film–antibiotic interaction while the addition of sucrose did not. This effect apparently was the result of a competition between the stearyl aldehyde and the aldehydic function of the glucose for the dissolved gentamicin. Sucrose, which does not have an available aldehydic function, cannot participate in this competition.

Sodium bisulfite, when added to the subphase, also inhibited the gentamicin–film interaction, apparently because of its ability to interact with the stearyl aldehyde *via* the formation of an α-hydroxysulfonic acid.

While free fatty aldehydes have been identified as natural components of various tissues (11–14), it is likely that they constitute only a small fraction of the lipid portion of cell membranes. Therefore, the effect of the addition of an "inert" lipid to the monomolecular film on the gentamicin–aldehyde interaction was determined. Egg lecithin was selected since it had shown no interaction with gentamicin and was representative of the phospholipid fraction of biomembranes. Mixed films of stearyl aldehyde and egg lecithin (mole fraction ratios of 3:1, 1:1, and 1:3) were spread, and the interaction with gentamicin was determined.

It can be seen in Fig. 4 that at a mole fraction ratio of 3:1 the interaction of gentamicin appears to be reduced over that observed with the stearyl aldehyde alone, as evidenced by the relatively small expansion of the π–A curve at low pressures. At high pressures, a condensation effect can be seen, suggesting that material is being lost from the interface. At a 1:1 mole fraction ratio, some expansion is also observed at low pressure, although less than that observed for the 3:1 system. At high pressures, however, the condensation effect is more pronounced.

At a 1:3 mole fraction ratio (Fig. 5), the condensation effect is still more pronounced and occurs at all surface pressures. Furthermore, it is most evident at the higher pH value (pH 8.0), a condition under which the gentamicin–stearyl aldehyde interaction approaches a maximum.

Thus, it appears that egg lecithin does not inhibit the gentamicin–aldehyde interaction but rather influences the ability of the resultant Schiff base to remain at the interface. This effect may be the result of the difference in the comparative strength of the interaction between neighboring stearyl aldehyde molecules and between neighboring stearyl aldehyde and egg lecithin molecules. Thus, if the latter interaction is weaker than the stearyl aldehyde–stearyl aldehyde interaction, the increase in hydrophilicity of the aldehyde as the result of its reaction with gentamicin may lead to the loss of the product (Schiff base) from an area-determining position at the interface.

CONCLUSIONS

The data demonstrate that gentamicin does not interact nonspecifically with cholesterol and phospholipid films and that it does interact with aldehydic films *via* Schiff-base formation. In a membrane composed essentially of cholesterol and phospholipids and interspersed with small amounts of fatty aldehydes, these latter compounds would serve as a site at which a gentamicin–membrane interaction might occur. Under such conditions, the increased hydrophilicity of the product (Schiff base) and the relatively weak interaction between the aldehyde and other membrane lipids would lead to desorption of the Schiff base from the membrane. This would result in the formation of pores through which intracellular material could leak out and unreacted gentamicin could enter and interact with the ribosomes within the cell.

While it is recognized that a physical model in itself cannot unequivocally elucidate the mechanism of action in a biological system, supporting data for this Schiff-base mechanism can be found in the literature. For example, alkalinization of urine promotes the activity of gentamicin (15), while *n*-alkylation of aminoglycoside

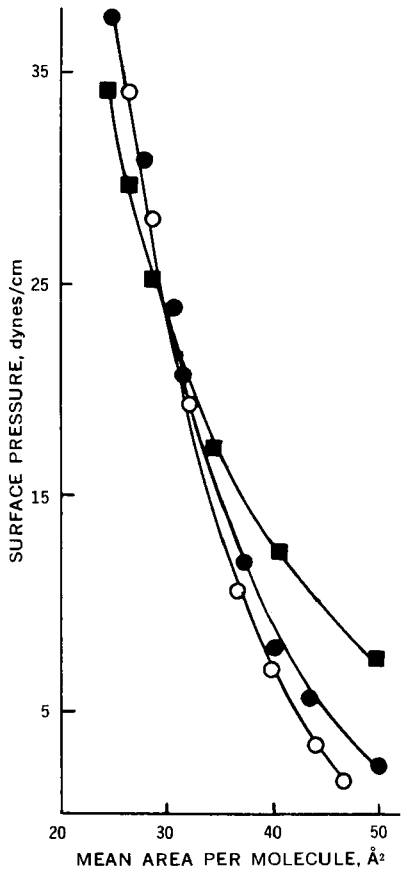


Figure 4—*Surface pressure–mean area per molecule of stearyl aldehyde plus egg lecithin at a 3:1 mole fraction ratio, alone* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH 7.0* (●) *and 8.0* (■).

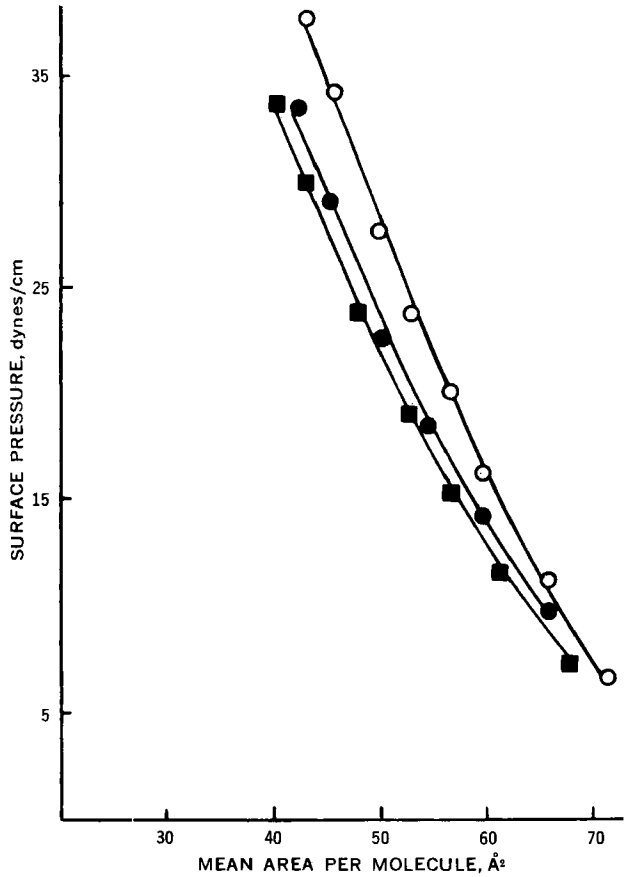


Figure 5—*Surface pressure–mean area per molecule of stearyl aldehyde plus egg lecithin at a 1:3 mole fraction ratio, alone* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH 7.0* (●) *and 8.0* (■).

antibiotics dramatically reduces their activity (16). Both of these observations correspond with the fact that only an undissociated primary amino group can participate in a Schiff-base reaction and with the postulation that such a reaction is necessary for gentamicin activity.

In addition, recent work in these laboratories (17) demonstrated that the addition of a small amount of sodium bisulfite to the growth media of *Escherichia coli* reduces the sensitivity of these organisms to gentamicin. It was postulated that this effect was the result of an interaction between the bisulfite and membrane aldehydes, which made these aldehydes less available to attack by gentamicin.

REFERENCES

(1) D. Cooper, P. Daniels, M. Yudis, and H. Marigliano, *J. Chem. Soc. (C)*, **1971**, 3126.

(2) M. J. Weinstein, G. Wagman, E. Oden, and J. Marquez, *J. Bacteriol.*, **94**, 789(1967).

(3) F. P. Furgiuele, *Amer. J. Ophthalmol.*, **64**, 425(1964).

(4) D. Von Kobletzki, "Experiments on the Placental Passage of Gentamycin," presented at the 5th International Congress of Chemotherapy, Vienna, Austria, July 1, 1967, p. 27.

(5) M. J. Weinstein, E. Oden, W. Zeman, and G. Wagman, *Antimicrob. Ag. Chemother.*, **1965**, 239.

(6) F. E. Hahn and S. G. Sarre, *J. Infect. Dis.*, **119**, 364(1970).

(7) D. E. Auslander, Ph.D. thesis, Rutgers—The State University, New Brunswick, N.J., 1972.

(8) D. S. Feingold and B. D. Davis, *Biochim. Biophys. Acta*, **55**, 787(1962).

(9) A. Felmeister, *J. Pharm. Sci.*, **61**, 151(1972).

(10) A. Felmeister, M. Amanat, and N. D. Weiner, *Environ. Sci. Technol.*, **2**, 40(1968).

(11) V. Winterfeld and H. Debuch, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 903(1968).

(12) R. Davis and R. Janis, *Nature*, **210**, 318(1966).

(13) J. Gilbertson, W. Ferrell, and R. Gelman, *J. Lipid Res.*, **11**, 486(1970).

(14) M. Eley and M. J. Cormier, *Biochem. Biophys. Res. Commun.*, **32**, 454(1968).

(15) A. Kennedy, M. Allison, J. Briggs, and J. McGeachie, *Scot. Med. J.*, **14**, 71(1969).

(16) A. Fujii, K. Maeda, and H. Umezawa, *J. Antibiot.*, **21**, 340(1968).

(17) D. E. Auslander, A. Felmeister, B. J. Sciarrone, and A. Segelman, *J. Pharm. Sci.*, **62**, 167(1973).

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