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# BINDING OF PROPRANOLOL AND GENTAMICIN TO SMALL UNILAMELLAR PHOSPHOLIPID VESICLES

## CONTRIBUTION OF IONIC AND HYDROPHOBIC FORCES

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**Abstract**—Binding of propranolol and gentamicin to small unilamellar phospholipid vesicles having different surface charges was studied at pH 4.4 using an ultra-centrifugation method, and the results were analyzed by an equation describing the Langmuir adsorption isotherms. Gentamicin, a polycationic drug, bound to negatively-charged small unilamellar vesicles composed of 60% phosphatidylcholine and 40% of either phosphatidylinositol, phosphatidylglycerol or phosphatidylserine in a manner consistent with a single class of binding sites but did not bind at all to small unilamellar vesicles of phosphatidylcholine alone. In contrast, propranolol bound readily to both neutral and negatively-charged liposomes in a manner consistent with two types of binding sites. Based on the binding parameters calculated from replots, it is suggested that the high-affinity site is probably at the surface of the liposome and that ionic forces are primarily responsible for this binding. The low-affinity, high-capacity binding site for propranolol was demonstrated with both neutral and negatively-charged liposomes and appeared to be independent of the surface charge. Gentamicin, which is not hydrophobic, did not bind to the low-affinity site. It is hypothesized that hydrophobic interactions are the driving force for propranolol binding to the low-affinity site which may be the interior of the lipid bilayer.

Studies on the influence of drugs on biological membranes and their constituent phospholipids were reported by Meyer [1] and Overton [2] in the late nineteenth century to elucidate the pharmacological effects of local anesthetics. These concepts are still of interest in regard to the mechanisms involved in the side-effects of cationic amphiphilic drugs and polycationic aminoglycoside antibiotics where binding to phospholipids has been reported to be a factor in decreased catabolism of membrane lipids [3, 4]. Although a number of articles have reported binding of cationic amphiphilic drugs to subcellular organelle membranes and their constituent phospholipids [5–7], there is disagreement about the mechanisms involved, and both ionic and hydrophobic mechanisms have been suggested. Aminoglycosides are thought to bind primarily by ionic interactions with negatively charged phospholipids, especially the phosphoinositides [8–14].

We have recently developed a method to study the equilibrium binding of drugs to small unilamellar phospholipid vesicles [15] which employs ultracentrifugation to separate free and bound drug. Using this approach, we investigated the binding at pH 4.4 of propranolol and gentamicin to small unilamellar vesicles composed of phospholipids that have different polar head groups bearing either neutral or negative surface charges. The pH was chosen to resemble the intralysosomal pH since we wished to evaluate drug–phospholipid binding under conditions similar to those involved in drug-induced phospholipidosis. Propranolol is shown to bind to two distinct types of sites on both neutral and negatively-charged vesicles. Gentamicin binds to a single type of site which is present only with negatively-charged vesicles.

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### MATERIALS AND METHODS

*Preparation of liposomes.* Four types of small unilamellar liposomes were made for the drug-binding studies: phosphatidylcholine alone (PC), 60% phosphatidylcholine and 40% of either phosphatidylinositol (PC/PI), phosphatidylglycerol (PC/PG) or phosphatidylserine (PC/PS). The phospholipid preparations were dissolved in chloroform/methanol (2/1, v/v) and placed in a thick-wall glass tube, and the solvent was removed with a nitrogen stream. Two milliliters of 2 mM Tris–HCl (pH 7.4) containing 20 mM NaCl was added to the dried lipid film (8.8 μmoles of total phospholipids), and the tube was vortexed gently for 1 min. This was repeated fifteen times with an interval of 1 min standing on ice for swelling. The lipid suspension was sonicated for 30 min with the cuphorn of a Heat Systems Sonicator (model W-225R) at maximum output. The clear solution was centrifuged at 15° at 100,000 *g* for 1 hr in a 50 Ti rotor (Beckman Instruments) to sediment multilamellar liposomes. Small unilamellar vesicles (SUV) that remained in the supernatant fraction were used in the drug-binding studies. The total recovery of phospholipids in the SUV fraction ranged from 60 to 80%.

*Binding of drugs to liposomes.* The principle used to determine drug binding to liposomes is that the radioactively-labeled drug which binds to the SUV will sediment with these vesicles by centrifugation. Thus, the amount of decrease of radioactivity in the supernatant fraction represents the amount of drug bound to liposomes. The system for studying binding contained 50 mM sodium acetate (pH 4.4), 0.8 mM SUV of phospholipid (four types of liposomes) and 0.01 to 10 mM propranolol or gentamicin with approximately 11 nCi of L-[4-$^3$H]propranolol or [$^{14}$C]gentamicin, respectively, in a total volume of 250 $\mu$l. Each experiment was done in duplicate. A 50-$\mu$l aliquot was removed from the mixture for liquid scintillation counting to measure the initial radioactivity which represents the total drug concentration. Subsequently, the remaining mixture was centrifuged at 15° at 209,000 *g* for 15 hr in 7X20 mm cellulose proprionate tubes (Beckman, No. 3423303) in a Beckman 42.2 Ti rotor. After centrifugation, a 50-$\mu$l aliquot was taken from the top quarter to measure the amount of drug not bound to phospholipid vesicles. These conditions were verified previously using SUV of di[1-$^{14}$C]oleoylphosphatidylcholine ([1-$^{14}$C]DOPC) [15]. The two drugs were also subjected to centrifugation over the same concentration range in the absence of liposomes to obtain control values which were used to correct the observed binding data (see results in Fig. 1). The weight changes of tubes before and after centrifugation were also measured to adjust for evaporation during the 16-hr spin; although the decrease in volume were small, 2.9 ± 3.1% of initial fluid weight, the data were also corrected for this factor.

*Materials.* Phosphatidylcholine (egg), phosphatidylinositol (bovine liver), phosphatidylglycerol (egg) and phosphatidylserine (bovine brain) were from Avanti Polar Lipids, Birmingham, AL. L-[4-$^3$H]Propranolol was from New England Nuclear, Boston, MA, and *d,l*-propranolol was a gift from Ayerst Laboratories Inc., New York, NY. [$^{14}$C]Gentamicin and unlabeled gentamicin were provided by the Schering Corp., Bloomfield, NJ.

## RESULTS

*Evaluation of the binding system.* Ultracentrifugation for 16 hr at 209,000 *g* sedimented 94.5% of [1-$^{14}$C]DOPC to the bottom half of the tube. The top quarter (50 $\mu$l) contained only 0.1% of the total and the second quarter (50 $\mu$l) contained 5.4%, demonstrating that the conditions for ultracentrifugation and sampling were appropriate. Drugs that were centrifuged without the addition of liposomes also sedimented to some extent, as shown in Fig. 1. Five to eight percent of propranolol and thirteen to twenty-four percent of gentamicin sedimented. The binding data were corrected by subtracting these numbers from the observed amount of bound drug.

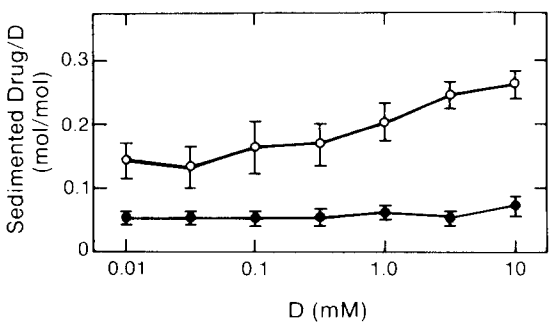


Fig. 1. Sedimented drugs with no liposome addition. Symbols: (●) propranolol, and (○): gentamicin. Data shown are the mean of four separate experiments ± standard deviation.

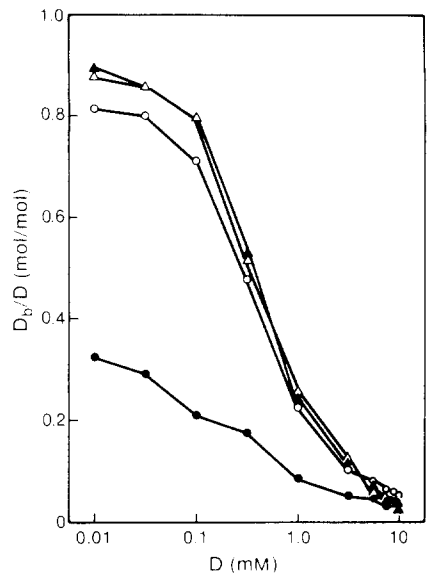


Fig. 2. Binding of propranolol to small unilamellar vesicles made of four kinds of different phospholipids. Symbols: (▲) 60% of phosphatidylcholine and 40% of phosphatidylinisotol, (△) 60% of phosphatidylcholine and 40% of phosphatidylglycerol, (○) 60% of phosphatidylcholine and 40% of phosphatidylserine, and (●) phosphatidylcholine. Data shown are the mean of duplicate determinations.

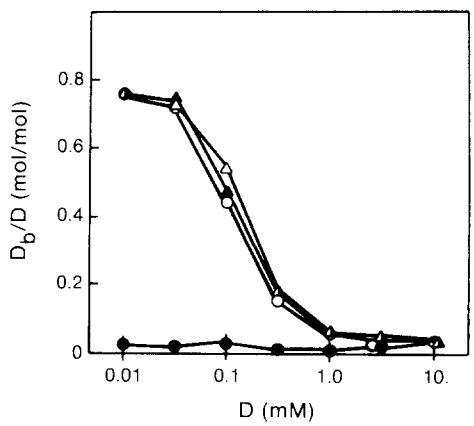


Fig. 3. Binding of gentamicin to small unilamellar vesicles made of four kind of different phospholipids. Symbols: same as for Fig. 2. Data shown are the mean of duplicate determinations.

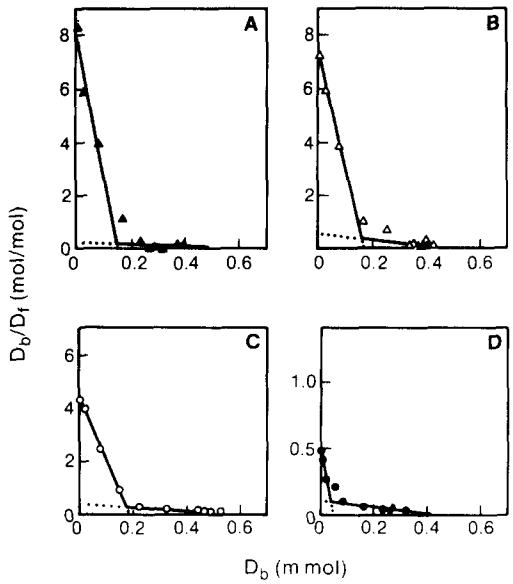


Fig. 4. Replots of binding data of propranolol shown in Fig. 2. (A) PC/PI, (B) PC/PG, (C) PC/PS, and (D) PC. Symbols: same as in Fig. 2. Data shown are the mean of duplicate determinations. Solid lines on the steep part of the hyperbolic curve represent the apparent high-affinity binding site and the other solid lines represent the low-affinity binding site.

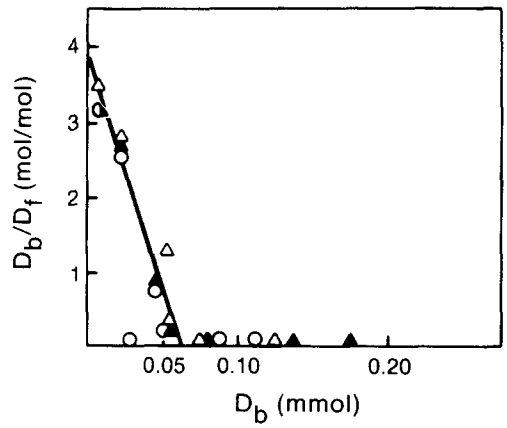


Fig. 5. Replots of binding data of gentamicin shown in Fig. 3. Symbols: same as in Fig. 2. There are no data for phosphatidylcholine.

*Binding of propranolol and gentamicin.* The binding of propranolol and gentamicin to phospholipid SUV is shown in Figs. 2 and 3 respectively. Propranolol bound to PC/PI, PC/PG and PC/PS to almost identical degrees throughout the whole range of drug concentrations, whereas it bound to PC to a lower degree (Fig. 2). Gentamicin also bound to PC/PI, PC/PG and PC/PS to almost identical degrees up to a drug concentration of 1 mM, but it did not bind to PC at all throughout the entire concentration range (Fig. 3). In the case of vesicles of PC/PI, PC/PG and PC/PS, the binding of propranolol was greater than that of gentamicin, and increasing amounts of propranolol were bound up to 10 mM whereas additional gentamicin binding was not noted between 3 and 10 mM.

*Replots of binding data.* For further analysis of binding modes, we used the Langmuir adsorption isotherms, and the equation which follows:

$$\frac{(N \cdot PL - D_b) \cdot D_f}{D_b} = \frac{1}{K_A} \quad (1)$$

where $K_A$ is the association constant of drug to liposomes, and $PL$, $D_b$ and $D_f$ are the concentrations of total phospholipid, drug bound to liposomes, and free drug, respectively, while $N$ is the saturation number of drug with respect to phospholipid in moles per drug per mole of PL. Equation (1) can be rearranged to the form below:

$$\frac{D_b}{D_f} = N \cdot K_A \cdot PL - K_A \cdot D_b \quad (2)$$

Equation (2) gives $N \cdot PL$ as the X intercept and $-$(slope) gives $K_A$ when $D_b$ is plotted on the X-axis and $D_b/D_f$ on the Y-axis. Figure 4 shows replots of binding data for propranolol. Propranolol binding was hyperbolic in each case, indicating that the binding occurs in a cooperative manner. We regarded these curves as being composed of two straight lines, one representing a high-affinity, low-capacity bind-

Table 1. Binding parameters of propranolol and gentamicin to several kinds of liposomes

| Liposomes | Affinity of binding site | Propranolol | | Gentamicin | |
|---|---|---|---|---|---|
| | | $K_A$ | $N$ | $K_A$ | $N$ |
| 60% Egg PC | High | 54,900 ± 24,200 | 0.186 ± 0.017 | 62,100 ± 11,500 | 0.077 ± 0.005 |
| 40% PI | Low | 402 ± 1,673 | 0.728 ± 0.340 | — | — |
| 60% Egg PC | High | 44,400 ± 8,230 | 0.208 ± 0.007 | 56,600 ± 22,500 | 0.085 ± 0.010 |
| 40% PG | Low | 1,140 ± 1,620 | 0.572 ± 0.106 | — | — |
| 60% Egg PC | High | 24,100 ± 3,600 | 0.231 ± 0.010 | 70,100 ± 11,600 | 0.069 ± 0.004 |
| 40% PS | Low | 645 ± 410 | 0.740 ± 0.087 | — | — |
| 100% Egg PC | High | 11,900 ± 2,300 | 0.054 ± 0.002 | — | — |
| | Low | 225 ± 180 | 0.553 ± 0.080 | — | — |

* Values are mean ± S.D. Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; $K_A$, observed association constant; and $N$, saturation number, mole drug/mole phospholipid.

ing site (an apparent high-affinity binding site) and the other representing a low-affinity, high-capacity binding site (a low-affinity binding site). Figure 5 shows replots of binding data for gentamicin. Binding of gentamicin is represented only by the steep part of the hyperbolic curves, which were taken to be a straight line indicating the presence of a single high-affinity binding site.

*Binding parameters.* To fit the hyperbolic curve to two straight lines, we ignored data around the junctional area between two lines and calculated the slopes and the X and Y intercepts by the method of least squares; the results for the respective binding parameters are listed in Table 1. The $K_A$ of propranolol for the high-affinity binding site was highest with liposomes of PC/PI followed by PC/PG and PC/PS; the $K_A$ of propranolol was lowest with PC liposomes. The saturation numbers ($N$) were identical for vesicles of PC/PI, PC/PG and PC/PS, whereas the value of $N$ for vesicles of PC was much lower. In contrast, gentamicin did not bind to vesicles of PC. It bound to PC/PI, PC/PG and PC/PS vesicles with nearly identical values for $K_A$ and $N$. Binding to the negatively charged phospholipid vesicles can be classified as a high-affinity binding on the basis of the association constants. A low-affinity binding site was not detected for gentamicin.

## DISCUSSION

Phosphatidylcholine is neutral, whereas phosphatidylglycerol, phosphatidylinositol and phosphatidylserine each have one net negative charge on their polar head groups. There is little difference in the hydrophobic moieties of the naturally-occurring phospholipids used in these studies. Thus, in these studies at pH 4.4, liposomes made of phosphatidylcholine (PC) had an electrostatically neutral surface but liposomes made of 60% phosphatidylcholine with 40% of either phosphatidylglycerol (PC/PG), phosphatidylinositol (PC/PI) or phosphatidylserine (PC/PS) had a net negative charge on their surface. Propranolol binding to the high-affinity site was greater when a negative surface charge was present ($N$ 0.19–0.23 v 0.054) and $K_A$ was also greater (2.4 to $5.5 \times 10^4$ v $1.2 \times 10^4$). Gentamicin, a hydrophilic polycationic drug, had 5 positive charges at our experimental conditions (pH 4.4) and bound only to liposomes having a net negative charge. Although the polar head groups of the negatively charged liposomes are different, the binding parameters were not significantly different, indicating that the chemical nature of the head group was not important. Gentamicin bound to a single class of binding sites having a high affinity and a low capacity. Since there was no binding to PC vesicles, gentamicin binding appears to be due exclusively to electrostatic interaction between its amino groups (positive) and the polar head groups of phospholipids (negative).

Our observations are in agreement with several previous reports. For example, Auslander *et al.* [4] did not detect binding of gentamicin to monomolecular films of neutral or positively charged phospholipids. Several groups have reported that aminoglycoside antibiotics such as gentamicin bind to negatively charged liposomes made of a mixture of phosphatidylcholine and phosphatidylinositol or other negatively charged phospholipids whereas they do not bind to neutral liposomes made of phosphatidylcholine; they hypothesized that the attraction between positively charged nitrogen groups on the antibiotics and negatively charged groups of acidic phospholipids are predominantly responsible for the interaction [9, 14]. Laurent *et al.* reported the binding of gentamicin to phosphatidylinositol-containing phospholipid vesicles using a gel filtration method [10] and demonstrated that the binding is largely dependent on electrostatic interactions [11]. Several reports have indicated that aminoglycoside antibiotics displace calcium ion from subcellular membrane components or negatively-charged phospholipid vesicles [8, 12, 13]. These observations are in general agreement with the hypothesis that electrostatic interactions are the major determinant of gentamicin binding.

Chung *et al.* recently reported "intrinsic" association constants for gentamicin with PI-containing vesicles of $10\ M^{-1}$ [14], whereas our observed $K_A$ for gentamicin was $6.2 \times 10^4\ M^{-1}$. This lack of agreement is probably due, in part, to differing methodology and to the calculation of "intrinsic" $K_A$ values by the former authors versus our use of observed values for $K_A$. In addition, it has been shown that gentamicin binding to PI-containing vesicles increases as the pH decreases [14] and our experiments were done at pH 4.4 whereas the former studies [14] were done at pH 7.4. We used a direct measurement of binding by observing the sedimentation of [$^{14}$C]gentamicin in the presence or absence of phospholipid vesicles, whereas the studies of Chung *et al.* were done by measuring electrophetic mobility (zeta potential) in the presence of gentamicin and calculating "intrinsic" values for $K_A$ [14]. The reasons for the apparent lack of agreement between "observed" and "intrinsic" $K_A$ values has been extensively discussed previously with regard to other gentamicin binding studies showing a similar discordance in $K_A$ values [14].

There are several previous reports about the binding of cationic amphiphilic drugs to biological membranes and phosopholipid vesicles but the mechanism of binding of cationic amphiphilic drugs to phospholipid vesicles is not yet well understood. Leterrier and Kersanté [16] reported that chlorpromazine and perphenazine are preferentially located in the polar part of egg yolk lecithin multibilayer by a photochemical study, whereas Seydel and Wassermann [17] suggested that increasing lipophilicity of amphiphilic drugs is correlated with an increase in binding to phosphatidylcholine vesicles, based on nuclear magnetic resonance. Lüllmann and Wehling [18] also reported that hydrophobic forces are mainly responsible for the binding of amphilic drugs to phosphatidylcholine vesicles by the equilibrium distribution method. These reports suggest that both electrostatic and hydrophobic interactions are involved in binding of cationic amphiphiles to phospholipids.

We found that propranolol, which is an amphiphilic drug having a single positive charge, bound readily to high-affinity binding sites of PC/PG, PC/

PI and PC/PS vesicles, but it bound less avidly to vesicles composed of PC alone, in agreement with the previous studies of Surewicz and Leyko [19]. These observations suggest that a negative charge on the liposome surface plays an important role in the binding of propranolol to high-affinity binding sites. The saturation number ($N$) for the high-affinity binding sites for propranolol with three kinds of negatively-charged liposomes is approximately three times greater than that observed for gentamicin. However, ionic forces are apparently not the only mechanism involved in propranolol binding to the high-affinity site because this agent also binds to a high-affinity binding site on vesicles of PC alone which have an electrostatically neutral surface.

Propranolol has a low-affinity binding site on all four types of liposomes. Since the low-affinity binding parameters were not significantly different and were independent of surface charge, it appears that this may represent a nonionic interaction. This observation would be most consistent with the hypothesis that the low-affinity site is in the hydrophobic interior of the membrane bilayer. In agreement with this hypothesis, gentamicin, which is hydrophilic, did not bind to the low-affinity site, as shown in Figs. 3 and 5.

Demonstration of the binding of cationic amphiphilic drugs to two types of sites in membranes was first reported by Bickel and Steele [5] who demonstrated binding of chlorpromazine and imipramine to rat tissue subcellular membrane fractions. Di Francesco and Bickel [6] demonstrated two kinds of binding sites for chlorpromazine on sonicated vesicles of egg lecithin. Schwendener and Weder [7] reported a two-phase process for binding of chlorpromazine to single bilayer liposomes of egg yolk lecithin. Recently, Henry *et al.* [20] used circular dichroism methodology to demonstrate two classes of binding sites for adriamycin in SUV consisting of egg phosphatidylcholine and either cardiolipin or phosphatidic acid. The first site is based on ionic interactions alone but the second site involves both ionic and hydrophobic interactions [20]. Although the studies mentioned above were done by other methods, the latter three reports agree, in general, with our results with propranolol.

Our studies differ from most other reports since we measured binding at an acidic pH. This was done in order to evaluate binding of gentamicin to phospholipids under conditions similar to that of the lysosomal interior where gentamicin accumulates causing inhibition of lysosomal phospholipase A [21]. This sequence of events is believed to be involved in the toxicity of aminoglycoside antibiotics such as gentamicin.

In conclusion, our results demonstrate the binding of propranolol to two binding sites on small unilamellar vesicles of PC alone or 60% PC and 40% of either PI, PS or PG. Gentamicin bound to a single binding site present only in the liposomes containing 40% of the acidic phospholipids; it did not bind to PC vesicles alone. Our data suggest that the high-affinity binding site is probably at the surface and that ionic interactions are primarily involved. However, the binding of propranolol to the low-affinity site was unaffected by surface charge, and gentamicin, a hydrophilic drug, did not bind to the low-affinity site at all. The data suggest that hydrophobic forces were responsible for low-affinity binding.

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