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Fluorescence studies of the binding of amphiphilic amines with phospholipids

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Abstract The binding characteristics of several amine drugs with dispersed phospholipids (phosphatidylcholine, phosphatidylserine, and phosphatidylglycerol) have been studied using the fluorometric method and 1-anilino-8-naphthalene sulfonate and 1,6 diphenyl-1,3,5-hexatriene as fluorescence probes. The results show that amphiphilic amines, such as chlorphentermine, interact with phospholipids via both ionic and hydrophobic forces. The ionic interaction, which occurs between the protonated amine group of the drug and the phosphate oxygen of the lipid, changes the amphiphilic characteristics of the lipid by reducing the number of negative charges on the lipid vesicles, and inhibits the Ca^{2+}-dependent lipid hydrolysis by blocking the Ca^{2+} binding sites on the lipid vesicles. The hydrophobic interaction, which involves the nonpolar moieties of the drug and the lipid, is of primary importance to the overall drug-lipid binding stability. Drugs without a strong hydrophobic moiety, such as dopamine, do not interact with phospholipids. – **Ma, J. Y. C., J. K. H. Ma, and K. C. Weber.** Fluorescence studies of the binding of amphiphilic amines with phospholipids. *J. Lipid Res.* 1985. **26:** 735–744.

Supplementary key words fluorometric method • lipidosis • chlorphentermine • surfactant

Cationic amphiphilic drugs such as chlorphentermine and imipramin have been shown to cause phospholipid storage disorders in rat lungs characterized by the accumulation of large, phospholipid-laden alveolar macrophages (1–4). In a study of the pharmacokinetics of phentermine and chlorphentermine in rats, Lüllmann, Rossen, and Seiler (5) demonstrated that the drug-induced lipidosis may be directly related to the tissue concentration of the drug. Their results show that phentermine, which does not induce lipidosis, is more readily eliminated from tissues than is chlorphentermine in rats treated with a single dose. The results also show that in chronically treated rats there is a steady increase in tissue accumulation of chlorphentermine but not phentermine, suggesting that the binding of the amphiphilic drug to the tissue components, or perhaps to phospholipids, may be required in order to produce lipidosis.

In the attempt to further elucidate the mechanism of the drug-induced lipidosis, several studies dealing with drug-lipid interactions have been reported. Lüllmann, Lüllmann-Rauch, and Wassermann (6, 7) proposed that the cationic amphiphilic amines interact with phospholipids via both electrostatic and hydrophobic forces, and that such binding may lead to impaired lipid metabolism by changing the physicochemical properties of the lipids such that they become less readily attacked by phospholipases. Indeed, a study by Gräbner (8) has shown that chlorphentermine inhibits the hydrolysis of phosphatidylcholine by bee venom phospholipase A_2. The importance of the hydrophobic interaction between the drug and lipids has been demonstrated by Seydel and Wassermann (9, 10). They found that several amphiphilic drugs including chlorphentermine, but not phentermine, are capable of binding with phosphatidylcholine and phosphatidylethanolamine via hydrophobic interaction between the nonpolar moieties of the drugs and the lipids. They further showed that drugs such as chlorphentermine do not bind appreciably with pancreatic phospholipase A_2, suggesting that the drug-lipid interaction may be directly responsible for the inhibition of phospholipid catabolism.

To provide more insight as to the nature of the drug-lipid interactions and the mechanism of drug inhibition of phospholipid catabolism, we have carried out binding studies of phospholipids with several amine drugs of varying amphiphilic characteristics by using a fluorometric technique with 1-anilino-8-naphthalenesulfonate (ANS) and 1,6-diphenyl-1,3-5-hexatriene (DPH) as fluorescence probes. Phospholipids are amphiphilic molecules that consist of a hydrophilic phosphate head group and long aliphatic chains that are hydrophobic in nature. The phosphate moiety exhibits a negatively charged

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Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine); LPC, lysophosphatidylcholine (1-palmitoyl-*sn*-glycero-3-phosphocholine); PS, phosphatidylserine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol.

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oxygen group which is capable of interacting with cationic species. Thus, it is not surprising that phospholipids exhibit strong affinity for the highly cationic amphiphilic amines. ANS is an anionic fluorescent agent which has been shown to be a valuable probe for the study of lipid interactions involving the hydrophilic phosphate moiety (11, 12). DPH, which has been shown to bind to the interior of the lipid vesicles (13–15), is used as a probe to characterize the hydrophobic interaction between the cationic amphiphilic amines and phospholipids.

EXPERIMENTAL

Materials

The fluorescence probes, 1-anilino-8-naphthalene sulfonate (ammonium salt; ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH), were obtained from Aldrich Chemical Co. (Milwaukee, WI) and from Molecular Probes, Inc. (Junction City, OR), respectively. Dopamine hydrochloride, ephedrine hydrochloride, and phenylpropanolamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO), and the hydrochloride salts of chlorphentermine and phentermine were kindly supplied by Dr. M. Reasor of the Department of Pharmacology, West Virginia University School of Medicine. Phospholipase A_2 from bee venom was obtained from Sigma Chemical Company. The chloride salts of Ca^{2+}, Ba^{2+}, and Mg^{2+} were obtained from Fisher Scientific Company.

The synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC) were obtained from Calbiochem-Behring Corp. The synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) and phosphatidylserine (PS) from bovine brain were obtained from Avanti Polarlipids, Inc. The phospholipid vesicles dispersed in (0.01 M) Tris buffer (pH 7.0) were prepared by sonication of lipid solutions under nitrogen using a heat system W375 sonifier. Samples of DPPC and DPPG were sonicated for 30 min at 50°C, whereas samples of PS were sonicated for 10 min at 4°C. Small vesicles of these solutions were fractionated from the larger liposomes by centrifugation at 105,000 *g* for 60 min. The concentration of phospholipids was determined by measuring the inorganic phosphorus using the method of Bartlett (16).

Instruments

The fluorescence measurements were carried out using an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD) equipped with a 150 W Xenon lamp, a 1P21 photomultiplier tube, and an X-Y spectral recorder. Standardization of the fluorescence intensity of the instrument was routinely carried out using a solid sample of tetraphenylbutadiene (excitation, 348 nm; emission 422 nm) obtained from Atarna Scientific, Ltd., Swarthmore, PA. Unless otherwise mentioned, the temperature of the fluorometer cell compartment was controlled for all experiments at 25 ± 0.1°C using a circulatory water bath from Forma Scientific, Inc., Marietta, OH.

Fluorometric binding studies

Binding studies of the amphiphilic amines with phospholipids were carried out in 0.01 M Tris buffer (pH 7.0) using a fluorescence probe technique. To characterize the hydrophobic interaction between the amines and phospholipids, DPH was used as a fluorescence probe. The lipid-bound DPH shows intense fluorescence at 440 nm (excitation, 368 nm), while the non-bound DPH does not exhibit fluorescence in aqueous solution. Studies of the drug–lipid interaction using DPH as a probe were based on a competitive binding mechanism in which the quenching of DPH fluorescence by the addition of drug was assumed to be due to the binding of the drug at or near the DPH binding sites on the lipid. For the study of the ionic interaction between the drugs and phospholipids, ANS was used as the fluorescence probe. The relative binding strength of the drugs was evaluated based on the effect of the drugs on the binding of ANS to the lipids.

Binding studies using DPH as a probe. Stock solutions of DPH in tetrahydrofuran (10^{-3} M) were freshly prepared and protected from light exposure. To determine the binding of DPH to DPPC, samples containing a constant concentration of DPPC and various concentrations of DPH in the range of 5×10^{-8}–5×10^{-6} M were incubated at 50°C for 60 min. After the samples were allowed to cool to room temperature, 2 ml was used for measurement of fluorescence intensity. The fluorescence was then plotted as a function of DPH concentration. This experiment was repeated using different lipid concentrations. When the DPPC concentration was 10^{-3} M or above, the fluorescence was found to be linear with DPH concentration, suggesting that all DPH molecules in the solution are in the bound form. These data were used to calculate the bound and free concentrations of DPH in a solution of lower DPPC concentration ($\sim 10^{-5}$ M); i.e., the fraction of bound-DPH in the solution of lower DPPC concentration was taken to be the ratio of its fluorescence with that of the solution containing 10^{-3} M DPPC at the same total concentration of DPH.

The binding constant and the binding capacity between DPH (probe) and DPPC vesicles were determined using equation *1*, assuming equal binding strength for all binding sites (Scatchard equation):

$$\frac{\nu}{[P]} = n K - \nu K \qquad \textit{Eq. 1)}$$

where ν is the number of moles of bound probe per mole

of lipid; [P] is the free concentration of the probe, DPH; n is the maximum value of ν which indicates the binding capacity of the lipid for DPH; and K is the binding constant. For the competitive binding studies, fluorometric titrations of solutions containing 5×10^{-5} M DPPC and a constant concentration of DPH with stock solutions of the amine drugs were performed, and the quenching of the fluorescence was recorded as a function of drug concentration. The fluorescence data were used to calculate the free and bound concentrations of DPH in the presence of the drug. Among the drugs used in this study, only chlorphentermine exhibited a significant effect on the fluorescence of lipid-bound DPH at room temperature.

The binding of chlorphentermine to DPPC was determined using equation *2*. This equation was derived from the competitive binding mechanism assuming that the drug competes for n_1 of the n probe binding sites with an intrinsic binding strength of K_1:

$$\frac{(n - \nu_1)\nu_1}{(n - \nu^* - \nu_1)\,[D]} = n_1K_1 - \nu_1K_1 \,. \qquad Eq.\ 2)$$

In this equation, ν^* is the ratio of bound-probe per total lipid concentration in the presence of drug; ν_1 is the ratio of bound-drug per total lipid concentration; n_1 is the maximum value of ν_1 which indicates the probe binding sites which may be replaced by drug binding; [D] is the free drug concentration; and K_1 is the binding constant for the drug–lipid interaction. The term n has been previously defined. For the calculation of n_1 and K_1 using equation *2*, ν^* was determined from the fluorescence data, whereas ν_1 was calculated from equation *3*, after n and K had been determined using equation *1*:

$$\nu_1 = n - \nu^* - \frac{\nu^*}{[P]^*K} \,. \qquad Eq.\ 3)$$

The term $[P]^*$ represents the free concentration of the probe in the presence of drug. After ν_1 was determined, [D] was taken to be the difference between the total drug concentration and the product of ν_1 and total lipid concentration.

Binding studies using ANS as a probe. ANS exhibits weak fluorescence at 510 nm (excited at 380 nm) in aqueous solution alone, but shows enhanced fluorescence intensity at 480 nm (excited at 380 nm) when bound to DPPC. The fluorescence of the lipid-bound ANS was further increased by the addition of amphiphilic drugs, suggesting the formation of a drug–lipid–ANS complex in the ternary system. To evaluate the binding phenomena, the emission coefficient (A) of the different species of ANS, at 480 nm defined as the fluorescence intensity/species concentration ratio, was determined.

For the binary ANS-lipid system, fluorometric titrations of solutions containing a constant concentration of phospholipid with a stock solution of ANS were carried out at several lipid concentrations. At relatively high concentrations of lipid (1×10^{-3} M) and low concentration of ANS (in the order of 10^{-7}–10^{-6} M), the titration showed a linear relationship between fluorescence intensity and ANS concentration. The slope of the linear plot was taken to be the emission coefficient of the binary ANS complex (A_b). The binding of ANS to phospholipid was determined using solutions containing 2×10^{-5} M lipid and varying concentrations of ANS. The fraction of bound ANS, X_b, in these solutions was calculated according to the following equation:

$$X_b = (F_b - F_o)/(A_b - A_o) \qquad Eq.\ 4)$$

where F_b and F_o are the fluorescence intensities for solutions with and without lipid, while A_b and A_o are the emission coefficients for the lipid-bound and free ANS, respectively. The values of A_o and A_b for DPPC at 480 nm were determined to be 0.5×10^6 and 68×10^6, respectively. From the value of X_b, the concentrations of free and bound ANS were calculated for each solution and the data were treated using equation *1*.

In the ternary system, fluorometric titrations of solutions containing 10^{-2} M drug and constant concentration of phospholipid with a stock solution of ANS were carried out at several lipid concentrations. The drug concentration of 10^{-2} M was found to be sufficient to saturate the lipid's drug-binding sites. At the relatively high concentration of lipid (1×10^{-3} M) and low concentrations of the probe, the plot of fluorescence intensity versus ANS concentration was linear, and the slope of the linear plot was then taken to be the emission coefficient of the ternary ANS complex (A_t). For the quantitative determination of the binding of ANS to lipid saturated with drug binding, a solution containing 2×10^{-5} M lipid and 10^{-2} M drug was titrated with a stock solution of ANS and the fluorescence of each titration increment was measured. The free and bound concentrations of ANS corresponding to each total ANS concentration along the titration curve were calculated using A_t in the same manner as described for the binary system. The binding parameters were then determined using equation *1*. In this system, since the fluorescence change is due to the formation of the drug–lipid–ANS ternary complex, the value of n should also indicate the binding capacity of the lipid for the amphiphilic drug.

RESULTS AND DISCUSSION

The binding nature of amphiphilic amines with phospholipids

ANS, a relatively nonfluorescent molecule, shows intense fluorescence with a blue shift of its emission maximum when bound to macromolecules capable of providing a hydrophobic environment (17). Studies have

shown that ANS binds to phospholipids in such a way that the anionic sulfonate group is oriented toward the hydrophilic head group of the lipid (11, 12, 18). Due to ionic interaction leading to charge neutralization, such binding and the fluorescence enhancement are obviously stronger for lipids possessing a non-hindered positive nitrogen such as DPPC. Phospholipids contain a negative phosphate oxygen group at physiological pH. Thus, further enhancement of the fluorescence of lipid-bound ANS may be brought about by the addition of cationic species capable of binding to the negative oxygen group. The effects of two cationic species, Ca^{2+} and chlorphentermine, on the fluorescence of DPPC-bound ANS are shown in **Fig. 1.** Both Ca^{2+} and chlorphentermine increase the fluorescence of DPPC-bound ANS, suggesting that these ions interact with the negative phosphate oxygen group and provide a more hydrophobic environment in the vicinity of the ANS molecule. The results also show a greater fluorescence enhancement for the monovalent chlorphentermine ion than that for the divalent Ca^{2+}. The negative phosphate oxygen group of the lipid as a binding site for Ca^{2+} has been well established in several studies (11, 12).

The effects of chlorphentermine on the fluorescence of DPPC, DPPG, and PS-bound ANS are shown in **Fig. 2.**

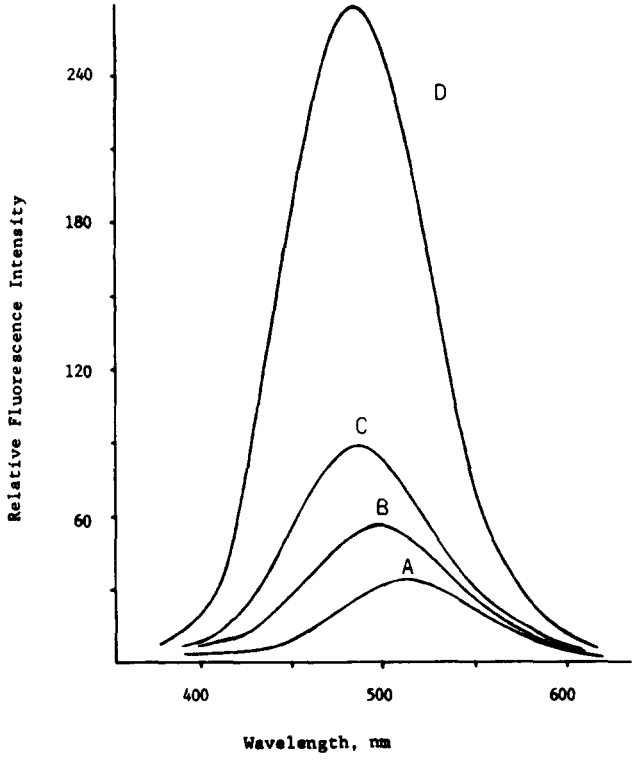


Fig. 1. Fluorescence spectra of solutions containing 5×10^{-5} M ANS (A) and in the presence of B) 9.36×10^{-6} M DPPC, C) 9.36×10^{-6} M DPPC, and 10^{-2} M Ca^{2+}, and D) 9.36×10^{-6} M DPPC and 10^{-2} M chlorphentermine in pH 7.0, 0.01 M Tris buffer. The spectra were obtained with an excitation at 380 nm. In the absence of DPPC, Ca^{2+} or chlorphentermine did not show significant effects on the ANS spectrum.

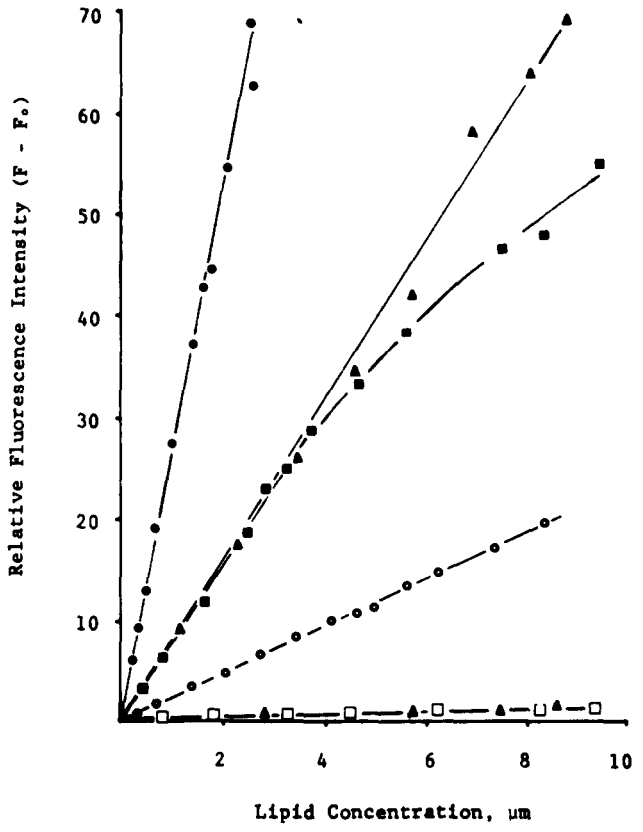


Fig. 2. Plots of fluorescence intensity, F-Fo, as a function of lipid concentration for solutions containing 5×10^{-5} M ANS and varying concentrations of lipids (circles, DPPC; triangles, PS; squares, DPPG) in the absence (open symbols) and presence (filled symbols) of 10^{-2} M chlorphentermine. F-Fo is the difference in fluorescence between the above solutions and the solution of 5×10^{-5} M ANS. The drug and ANS concentrations used are sufficiently high to saturate the lipid binding sites, so that the value, F-Fo, is proportional to the concentration of lipid-bound ANS. The fluorescence intensity was measured at 480 nm with excitation at 380 nm.

The binding of ANS with DPPG and PS did not result in a significant increase of ANS fluorescence probably due to the lack of a non-hindered positive nitrogen group in these lipids. However, strong fluorescence enhancement was observed when the system contained chlorphentermine. The results further suggest that chlorphentermine interacts with the negative oxygen group of the lipid's phosphate moiety.

The ionic interaction of phospholipids is of significant consequence to the lipid's solubility and surface activity. At high concentrations of lipid and ANS, addition of chlorphentermine results in a precipitation of the lipid due to charge neutralization and the subsequent loss of its amphiphilic characteristics.

DPH is a nonfluorescent molecule in aqueous solution, but is known to bind to the interior of lipid vesicles and gives intense fluorescence at 440 nm (13-15). The effect of chlorphentermine on the binding of DPH to DPPC vesicles at room temperature and at 45°C is shown in

Fig. 3. The addition of chlorphentermine quenches the fluorescence of the lipid-DPH solution, probably indicating a binding displacement of DPH by chlorphentermine which binds in close proximity to the DPH binding sites. This quenching effect is much greater at a temperature above the transition temperature of the lipid (45°C) than at lower temperature (25°C), suggesting that the amphiphilic drug exhibits only a limited penetration of the interior of the lipid vesicles, i.e., the binding of chlorphentermine affects only those DPH binding sites near the vesicel surface. Nevertheless, these results show a second binding mode for the drug-lipid interaction which involves the hydrophobic interaction between the nonpolar moieties of the two species.

Hydrophobic interactions are important to the overall binding of drugs to phospholipids. **Table 1** shows the effect of several amine drugs of varying amphiphilic properties (as indicated by their structures) on the fluorescence of DPPC-bound ANS. The value A_t/A_b represents the fluorescence enhancement of the ternary complex over the binary complex. It can be seen that dopamine, which is not amphiphilic in nature due to the presence of the catechol group in their hydrophobic moiety, shows little or no interaction with DPPC. On the other hand, relatively strong amphiphiles, such as phentermine and chlorphentermine, exhibit relatively strong interactions with phospholipid.

The above results show a binding nature between the drug and lipid that is consistent with the amphiphilic characteristics of both species, i.e., the binding involves both hydrophobic and ionic interactions. The results indicate that, while the ionic interaction is most likely a cause for drug effect on surface activity of phospholipids, the hydrophobic interaction may serve as a stabilizing force for the binding of drug to lipid vesicles. One may consider that the hydrophobic interaction provides an orientation of the two species such that the ionic interaction is possible.

Quantitative aspects of the drug-lipid interaction

A Scatchard plot (equation *1*) for the binding of DPH to DPPC at 25°C is shown in **Fig. 4.** From the straight line plot, the values of n and K were calculated to be 0.0167 and 2.85×10^6 M^{-1}, respectively. These results suggest a strong hydrophobic binding between DPH and DPPC with a binding ratio of 1 DPH molecule per 60 lipid molecules. Since the number of lipid molecules per vesicle in unknown, the number of binding sites per lipid vesicle was not calculated. A plot that illustrates the competitive binding between chlorphentermine and DPH to DPPC (equation *2*) is shown in **Fig. 5.** The plot shows

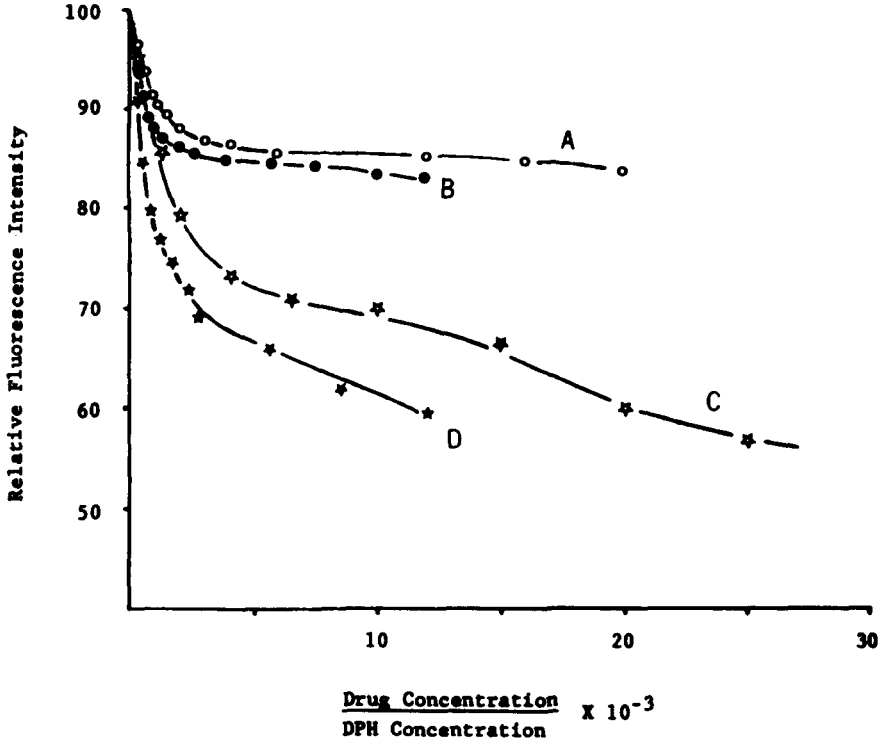
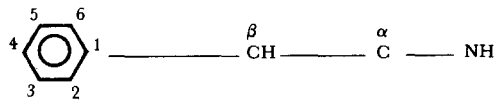


Fig. 3. Effect of chlorphentermine on the binding of DPH to DPPC at 25°C (circles) and at 45°C (stars) as indicated by fluorescence quenching. All solutions contain 5×10^{-5} M DPPC, a constant concentration of DPH, and varying concentrations of chlorphentermine (A: 5×10^{-7} M; B: 8×10^{-7} M; C: 5×10^{-7} M; D: 1.0×10^{-6} M). Fluorescence measurements were made at 440 nm with excitation at 368 nm.

TABLE 1. Chemical structure of selected amines and their effect on the fluorescence of DPPC-bound ANS



	Ring	β	α	Amino	A_t/A_b[a]
Dopamine	3-OH,4-OH	H	H,H	H	1.0
Phenylpropanolamine		OH	H,CH_3	H	1.50
Ephedrine		OH	H,CH_3	CH_3	1.45
Phentermine		H	CH_3,CH_3	H	3.70
Chlorphentermine	4-Cl	H	CH_3,CH_3	H	7.09

[a] A_t and A_b are the emission coefficients of ANS in the ternary (drug-lipid-ANS) and in the binary (lipid-ANS) complexes, respectively.

a straight line from data obtained in three separate experiments, and gives a value of 0.004 for n_1 and 8.04 $\times 10^3$ M^{-1} for K_1. These results suggest that about one-fourth of the bound DPH may be replaced by the binding of chlorphentermine. The relatively weak hydrophobic binding strength as indicated by K_1 suggests that these binding sites are near the surface of the lipid vesicles. Other amine drugs used in this study, which are less amphiphilic than chlorphentermine, did not quench the fluorescence of lipid-bound DPH.

The Scatchard plot for the binding of ANS to DPPC is shown in **Fig. 6.** From the slope and the intercept of the plot, the values of n and K were calculated to be 0.034 and 1.4 $\times 10^5$ M^{-1}, respectively. These results indicate a strong binding between the probe and the lipid with a molar binding ratio of about 1:30 (ANS:DPPC). The binding of the anionic probe to either PS or DPPG was not indicated by the fluorometric method, probably because these lipids exhibit an overall negative charge on the phosphate moiety at neutral pH. In the ternary systems, however, the anionic probe was found to interact with all three phospholipids. The results are shown in **Fig. 7,** where the binding data for solutions containing 10^{-2} M chlorophentermine, 2 $\times 10^{-5}$ M lipid, and varying concentrations of ANS are treated with equation *1*. The data for the ANS-

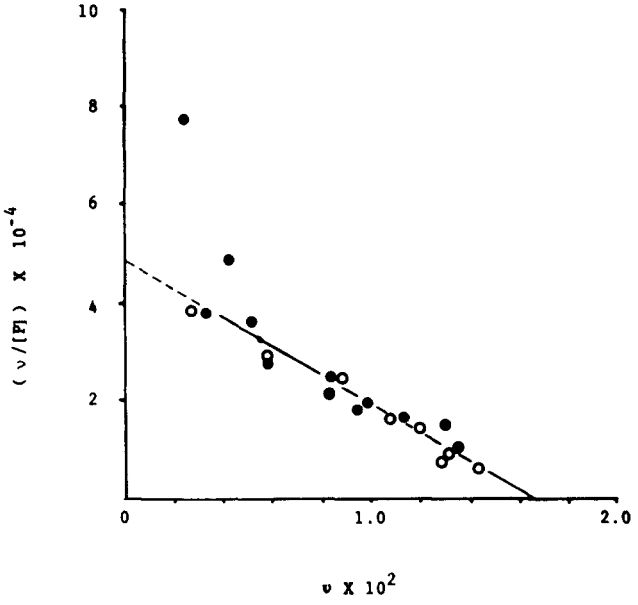


Fig. 4. Scatchard plot (equation *1*) for the binding of DPH to DPPC at 25°C measured at two lipid concentrations (● 5 × 10^{-5} M; ○ 2 × 10^{-5} M).

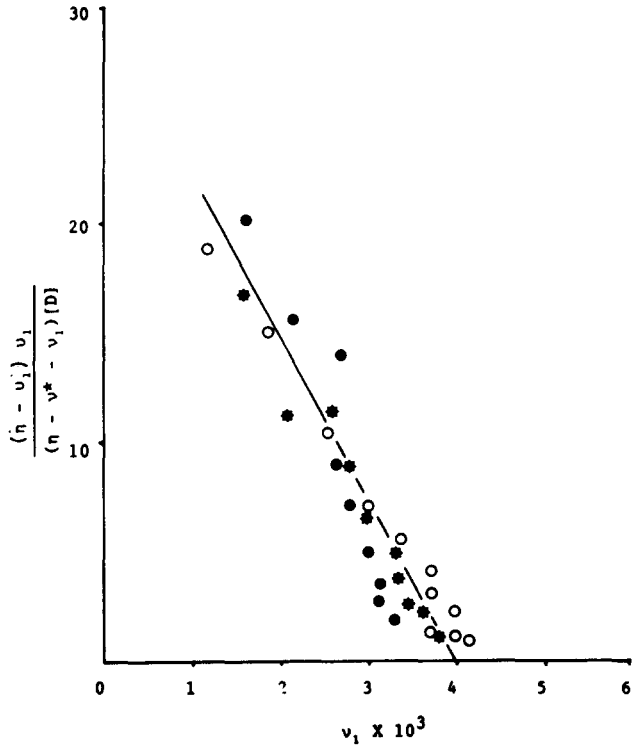


Fig. 5. Binding of chlorphentermine to DPPC measured by using DPH as a probe (equation *2*). Data were obtained from solutions containing 2 × 10^{-5} M DPPC, varying concentrations of chlorphentermine and ●) 5 × 10^{-7} M DPH; ✱) 8 × 10^{-7} M DPH; and ○) 2 × 10^{-6} M DPH.

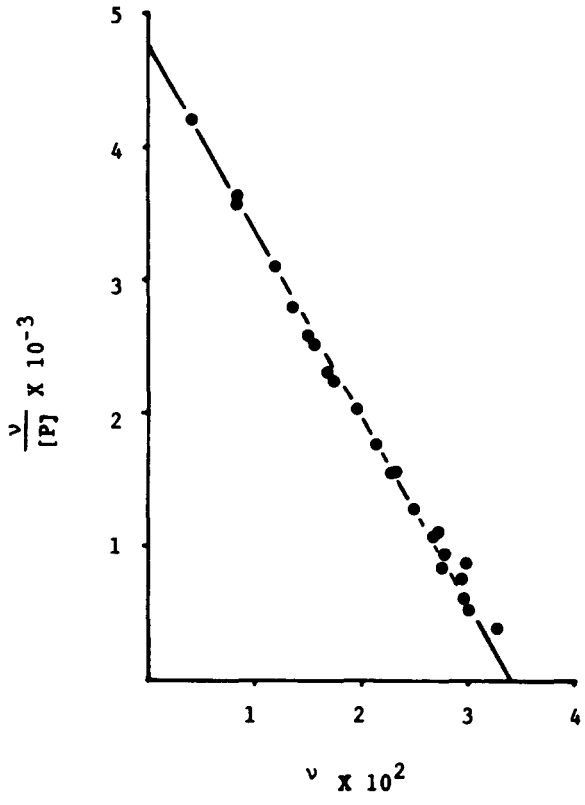


Fig. 6. Scatchard plot for the binding of ANS to DPPC for solutions containing 2×10^{-5} M DPPC and varying concentrations of ANS.

DPPC binding (curve A) shows a biphasic linear plot, indicating that in the presence of chlorphentermine the lipid has two classes of binding sites for the probe. At relatively low ANS concentrations, the plot is linear and gives a binding constant of 1.4×10^5 M^{-1}. This value is the same as that calculated for the binary ANS-DPPC binding, suggesting that the binding of the amphiphilic drug does not affect the primary binding of ANS to the lipid. However, the drug-lipid binding induces a secondary binding between the probe and the lipid as indicated by the linear plot in the region of high ANS concentrations. In the cases of PS and DPPG, the linear plots (curves B and C, respectively) show a single class of binding sites. Since the fluorometric method does not measure the primary binding of the probe to PS and DPPG, these data probably represent the drug-induced binding between the probe and the lipids. The overall binding capacity, n, calculated from Fig. 7 for all lipids is 0.067, which is twice the value calculated for the ANS-DPPC binary system. The chlorphentermine effect may be attributed to the ability of the cationic drug to neutralize the negative charges of the lipid and thus create a condition more conducive for the probe binding. The additional binding capacity for the probe also reflects the binding capacity of the lipid for the amphiphilic drug.

The effects of other amines on the binding of ANS to DPPC are shown in **Fig. 8.** For comparison, the solid line

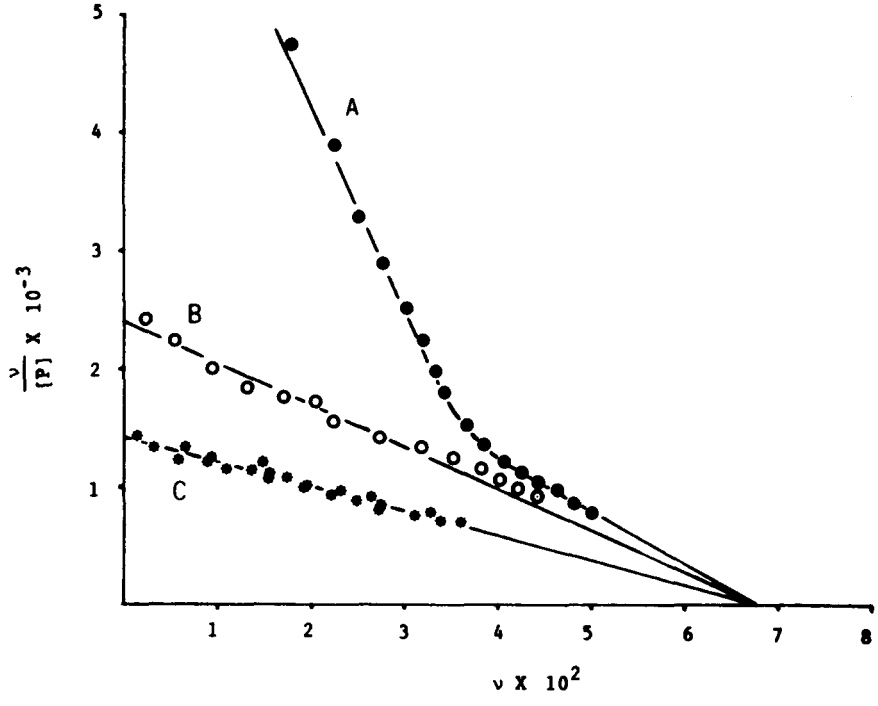


Fig. 7. Effect of chlorphentermine on the binding of ANS to A) DPPC, B) PS, and C) DPPG. Data were obtained from solutions containing 2×10^{-5} M lipid, 10^{-2} M chlorphentermine, and varying concentrations of ANS and treated using equation *1*.

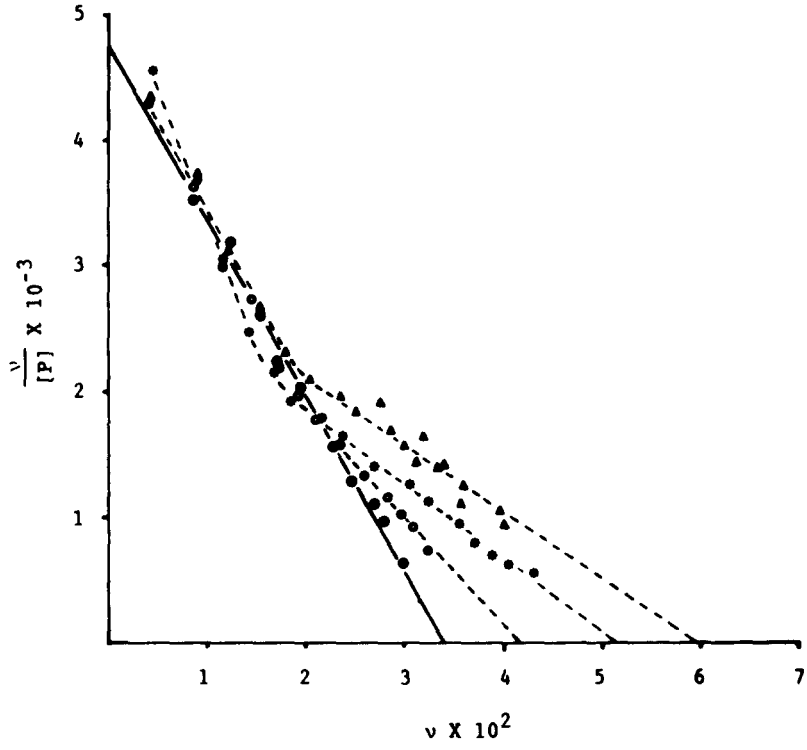


Fig. 8. Effect of dopamine (●), phenylpropanolamine (○), ephedrine (✱), and phentermine (△) on the binding of ANS to DPPC. Data were obtained from solutions containing 2×10^{-5} M DPPC, 10^{-2} M drug, and varying concentrations of ANS and treated using equation *1*.

represents the binding plot for the ANS-DPPC binary system. As can be seen, dopamine has no effect on the ANS binding, suggesting that it does not interact with the lipid. However, the binding of phenylpropanolamine, ephedrine, and phentermine to DPPC is clearly indicated by their ability to induce a secondary binding for ANS, which occurs at relatively high ANS concentrations. Based on their relative effects on the ANS-DPPC binding shown in Figs. 7 and 8, it may be concluded that these drugs interact with the lipid in the following decreasing order of affinity: chlorphentermine > phentermine > ephedrine > phenylpropanolamine > dopamine. Thus, although ANS is used to indicate the ionic interaction of the amines with phospholipid, it shows that the binding affinity of the amines to the lipid is closely related to the hydrophobicity of the drug's nonpolar moiety. The results on phentermine and chlorphentermine are consistent with those reported in the literature (9, 10, 19).

Drug inhibition of DPPC hydrolysis by phospholipase A_2

Phospholipase A_2 activity has been monitored using ANS as a fluorescence probe. **Fig. 9A** shows the change of ANS fluorescence as a function of time after the addition of 0.25 μg/ml of phospholipase A_2 to a solution containing 1×10^{-5} M DPPC, 5×10^{-5} M ANS, and 6×10^{-3} M Ca^{2+}. In the absence of the metal ion, the fluorescence did not change as a function of time upon addition of the enzyme. The enzymatic hydrolysis of DPPC into LPC is indicated by the increase in ANS fluorescence and results

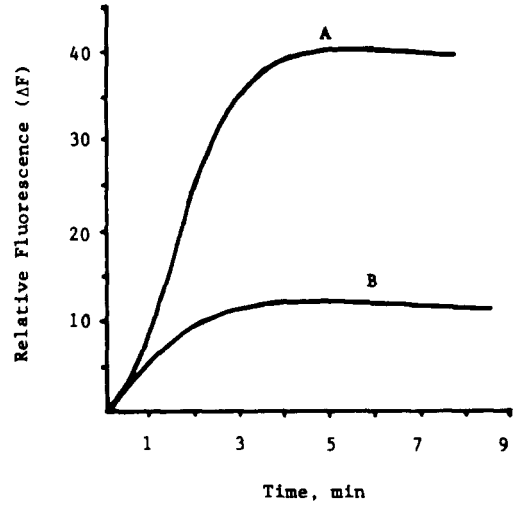


Fig. 9. Fluorescence change (ΔF) as a function of time upon addition of 0.25 μg/ml phospholipase A_2 to solutions containing 1×10^{-5} M DPPC, 5×10^{-5} M ANS, 6×10^{-3} M Ca^{2+}, and in the absence (A) and presence of 6×10^{-3} M chlorphentermine (B). Drug inhibition of enzyme activities is indicated by the reduction of fluorescence enhancement.

from the transformation of the Ca^{2+}-DPPC-ANS complex into the Ca^{2+}-LPC-ANS complex. The data shown in Fig. 9 are consistent with literature results (20, 21). It is also interesting to point out that the binding of ANS at the phosphocholine nitrogen group of DPPC does not appear to interfere with the enzymatic reaction, suggesting that the negative phosphate oxygen group of the lipid is an important group in regulating the lipids activity. Since the fluorescence studies indicate a binding of Ca^{2+} to phospholipid, it can be concluded that, in the presence of enzyme, the formation of a ternary complex in which Ca^{2+} is linked to both the lipid and the enzyme is essential for the enzymatic reaction.

Fig. 9B shows the effect of chlorphentermine on the hydrolysis of DPPC by phospholipase A_2, where the inhibitory effect of the drug is demonstrated by the reduction of fluorescence enhancement. The results also indicate that the presence of chlorphentermine reduces only the plateau level of the fluorescence intensity, but has no effect on the rate of fluorescence enhancement, i.e., both curves reach their plateau levels at approximately the same reaction time. This suggests that chlorphentermine affects mainly the binding equilibrium between Ca^{2+} and the lipid that precedes the enzymatic hydrolysis reaction. Further studies show that phentermine exhibits little or no effect on the hydrolysis of DPPC by phospholipase A_2, indicating that only strong amphiphiles whose binding with the lipid are significantly stabilized by hydrophobic interactions are able to inhibit the enzymatic degradation of phospholipids. Chlorphentermine possesses a stronger hydrophobic moiety than phentermine, thus, exhibits stronger binding. This may be the reason that, in chronically treated rats, large tissue accumulation was observed for chlorphentermine but not for phentermine, and that chlorphentermine, but not phentermine, induces lipidosis (5, 22).

CONCLUSION

This study demonstrates that phospholipids bind with cationic amphiphilic amines via both ionic and hydrophobic interactions. The ionic interaction occurs between the protonated amine group of the drug and the negative phosphate oxygen of the lipid, whereas the hydrophobic interaction invovles the nonpolar moieties of both species. The stronger hydrophobic interaction of the two species enhances the overall drug-lipid binding stability and thus may play an important role in the drug-induced lipidosis. The ionic interaction occurs only for amines possessing a nonpolar moiety that is capable of binding with the lipid. Our study shows a positive correlation between the amphiphilic characteristics of the drugs and their binding affinities. Drugs without a strong non-polar group, such as dopamine, do not interact with the lipid. On the other hand, highly amphiphilic drugs, such as chlorphentermine, and to a lesser extent, phentermine, exhibit marked ionic interaction with phospholipids.

Binding of amphiphilic amines has a direct effect on the biological activities of the lipids. The ionic interaction of strong amphiphiles can inhibit the metal ion-activated enzyme degradation of phospholipids by blocking the metal ion from binding to the lipid. Such inhibition may be an important cause for the drug-induced lipidosis. Furthermore, since the ionic interaction diminishes the amphiphilic characteristics of the lipids, it is likely that this interaction can also alter the physicochemical properties of the lipids and their surface activity.

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