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INTERACTIONS OF CHLORPROMAZINE AND IMIPRAMINE WITH ARTIFICIAL MEMBRANES INVESTIGATED BY EQUILIBRIUM DIALYSIS, DUAL-WAVELENGTH PHOTOMETRY, AND FLUORIMETRY

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Abstract—Binding of chlorpromazine and imipramine to liposomes of various synthetic lipids was investigated by equilibrium dialysis, dual-wavelength photometry, and fluorimetry. As proved by equilibrium dialysis, liposomes of all investigated lipids bound chlorpromazine to a greater extent than imipramine. At temperatures above the lipid phase transition the membranes bound more of both drugs than they did below. These results were confirmed by dual-wavelength photometry at low drug concentrations. Chlorpromazine and imipramine fluidized lipid bilayers below their transition temperatures. Less imipramine bound to positively charged liposomes as compared with membranes of zwitterionic phosphocholine. This emphasizes the importance of ionic interactions for the binding of imipramine to lipid membranes. Chlorpromazine binding, however, was little affected by ionic interactions. Both chlorpromazine and imipramine had the same effect on the fluorescence of 1-anilino-8-naphthalene-sulfonate (a probe for the polar part of the membrane), whereas the fluorescence of perylene (a probe for the inner hydrocarbon region of bilayers) was quenched by chlorpromazine but not by imipramine. From the results obtained with the three complementary methods it is concluded that chlorpromazine binds to the surface of membranes and also penetrates into the inner hydrocarbon phase of the bilayer, whereas imipramine only binds near the surface of the liposomes.

Chlorpromazine (CPZ) and imipramine (IP) are known to bind to biological membranes [1–7] which may be a site of pharmacological action [8, 9]. CPZ expands membranes [6, 9–11], and both CPZ and antidepressant drugs like IP and desipramine affect the phase-transition of lipid membranes [4, 8, 12]. Although the mechanism of binding and the localization of these drugs in the membrane is still controversial there is enough evidence that lipids are important binders [2, 4, 5, 7, 13–15]. It has been suggested that the pharmacological action of CPZ could be due to physiochemical changes in the lipid part of membranes [9].

The aim of the present study is to characterize the interaction of CPZ and IP with defined membrane systems composed of synthetic lipids. Lipids with different polar head groups (phosphocholines, *O*-methylphosphocholine, phosphatidic acid) and with fatty acid moieties of varying chain length or degree of unsaturation were used.

Four methods were used to characterize the binding of the drugs to liposomes:

Equilibrium dialysis yields the total amount of drug bound by the liposomal membranes. However, no conclusions as to the site and mechanism of the binding can be drawn.

The transfer of CPZ from a polar to a nonpolar environment leads to a red shift of the u.v. spectrum of CPZ. The consequence of this shift is a difference spectrum of CPZ bound to the membrane compared with CPZ free in aqueous solution [2, 16]. This spectral difference can be measured by dual-wavelength photometry [17]. The difference in absorbance ΔA increases with the amount of CPZ bound to the lipid membranes [2]. The advantage of dual-wavelength photometry as compared with equilibrium dialysis is the high sensitivity: CPZ concentrations as low as 10^{-6} M give measurable signals.

Perylene is a marker of the interior hydrocarbon phase of lipid bilayers [18]. If the drugs *quench the fluorescence of perylene* they must be located near the marker [18], i.e., in the interior of the membrane. The fluorescence of perylene increases sharply at the transition temperature of a membrane [19]. Thus, it would be possible to observe drug-induced phase-transition of the lipid.

ANS is a probe for the methylene groups near the polar surface of a membrane [18, 20]. CPZ and IP *enhance the fluorescence of ANS* in biological membranes [3, 14, 21] by increasing the quantum yield [14]. ANS fluorescence too shows marked anomaly in the temperature region of the lipid phase-transition [22].

MATERIALS AND METHODS

Reagents. The synthetic lipids (analytical grade) were purchased from Fluka, Buchs, Switzerland. Chlorpromazine was a gift from Bayer (GFR), imipramine from Ciba-Geigy, Basel, Switzerland. [^{14}C]-Imipramine and [^{35}S]-chlorpromazine were purchased from The Radiochemical Centre, Amersham, UK. Perylene (Fig. 1) was purchased from Aldrich Europe

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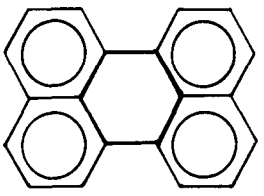

Fig. 1. Formula of the fluorescent probe, perylene.

(Belgium), 8-anilino-1-naphthalene-sulfonate (ANS) from Fluka. Phosphate buffer 0.05 M with 0.1 M KCl, pH 7.4 (unless otherwise stated) was used throughout. All reagents were of analytical grade and were used without further purification.

Preparation of liposomes. Liposomes were made of an appropriate amount of lipid or lipid mixture to obtain a final concentration of 150 μM lipid in buffer. The lipid solution was sonicated 20 min by an MSE 150 W sonicator (diameter of the tip: 19 mm) above the transition temperatures of the liposomes. If necessary, the solution was cooled during sonication and bubbled with nitrogen (unsaturated lipid). After sonication titanium particles were removed by centrifugation at 3000 rev./min. If necessary, liposomes were stored above their transition temperature. ANS-labelled liposomes were made by adding ANS to a final concentration of 50 μM before introducing the lipid in buffer. In order to label the liposomes with perylene, the dry lipid was dissolved in a chloroform solution of perylene and the solvent removed by a stream of nitrogen. Buffer was then added and the solution sonicated as described.

Equilibrium dialysis. Dialysis experiments were carried out according to Weder *et al.* [23, 24] with a Dianorm apparatus. Visking cellulose membranes of 25 μm thickness and a pore diameter of 1–2 nm were used to separate the 1 ml dialysis chambers. Time to reach equilibrium was $2\frac{1}{2}$ to 4 hr. No corrections were made with respect to loss of drug due to adsorption at the membrane or walls of the chambers. The concentration of IP was measured by counting the radioactivity of [^{14}C]-IP in both chambers with a Packard Tri-Carb liquid scintillation counter. CPZ was measured colorimetrically [25, 26] or by counting the radioactivity of [^{35}S]-CPZ.

The amount of bound ligand was calculated as follows:

$$c_{L\,b} = c_{L\,0} - c_{L\,f},$$

where $c_{L\,b}$, $c_{L\,f}$: concentration of bound and free ligand and $c_{L\,0}$: total ligand concentration.

$$a_l = 2\,a_{L\,b} + a_{L\,f},\ a_r = a_{L\,f},$$

where a_l, a_r: measured signal (radioactivity, absorbance) in the left (liposome) and right (ligand) chamber; $a_{L\,b}$, $a_{L\,f}$: signal of bound and free ligand; and $a_{L\,0}$: signal of the total ligand concentration.

$$a_l - a_r = 2\,a_{L\,b},\ a_l + a_r = 2\,a_{L\,0},$$

$$(a_l - a_r/a_l + a_r)\,c_{L\,0} = c_{L\,b}.$$

Dual-wavelength photometry. Absorbance differences were measured with a Perkin-Elmer 156 Dual-wavelength photometer with a thermostated cell holder. The wavelengths were set at 246 and 263 nm and the absorbance differences

$$\Delta A = A_{263} - A_{246}$$

measured. This difference is the maximum in the difference spectrum of CPZ in the presence of lipids [2]. One compartment of a quartz tandem mixing cuvette (total path length 0.87 cm) was filled with liposomes in buffer, the other with a solution of CPZ in buffer. ΔA was measured and set as $\Delta A_0 = 0$. After mixing the solutions the difference in absorbance to ΔA_0 was measured as ΔA. The suitable concentration range of CPZ was 1 to about 100 μM. Totally independent duplicate experimental series were carried out.

Fluorimetry. Fluorescence measurements were performed with a Hitachi Perkin-Elmer MPF 3 Spectrofluorimeter. 1 ml Polystyrene cells were used. 0.5 ml of liposome suspension were mixed with 0.5 ml of a drug solution of appropriate concentration. The excitation wavelength for the experiments with ANS (50 μM) was 381 nm, the emission wavelength 470 nm. The experiments with perylene (0.25 μM) were performed at 413 and 473 nm respectively. Fluorescence of CPZ, IP and liposomes alone in solution were negligible at the above conditions. The results were expressed in arbitrary units relative to the maximum fluorescence of the marker in each series of experiments. Totally independent duplicate experimental series were carried out.

RESULTS

Equilibrium dialysis. Figures 2–4 illustrate the importance of the physical state of the lipid bilayer for the binding of CPZ and IP. Liposomes of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine with a transition temperature of 37° [27] bound more CPZ or IP above their transition temperature than below (Figs. 2 and 3). Results with liposomes of other lipids are summarized in Table 1. More CPZ than IP was bound per mole of lipid at temperatures both below and above the phase transition of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Figs. 2 and 3). This was also the case with positively and negatively charged liposomes (Figs. 5–7).

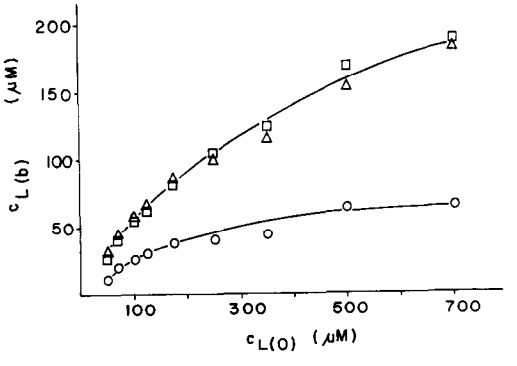

Fig. 2. Binding curves of the chlorpromazine liposome interaction above and below the transition temperature (equilibrium dialysis). Liposomes: 340 μM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, transition temperature 37° [27]. pH = 7.4. ○ 21°, △ 37°, □ 50°. Each point is the median of four experiments.

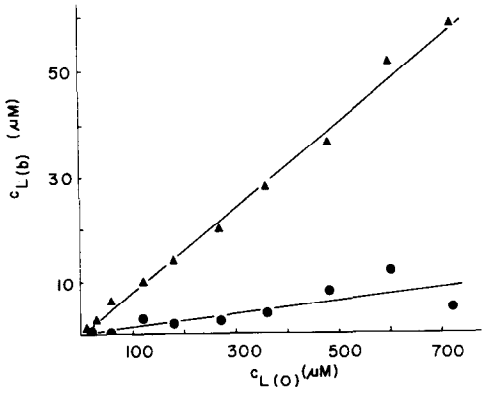

Fig. 3. Binding curves of the imipramine liposome interaction above and below the transition temperature (equilibrium dialysis). Liposomes: 150 μM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, transition temperature 37° [27]. pH 7.4. ● 22°, ▲ 50°. Each point is the median of three experiments.

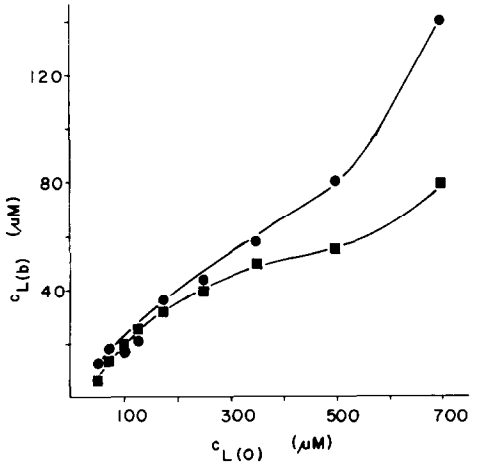

Fig. 4. Binding curves of the chlorpromazine liposome interaction at different temperatures below the transition temperature (equilibrium dialysis). Liposomes: 340 μM 1,2-distearyl-sn-glycero-3- phosphocholine, transition temperature 51° [27]. pH 7.4. ■ 37°, ● 42°. Each point is the median of four experiments. The curves at 22°, 27°, 32° are identical to the 37° curve.

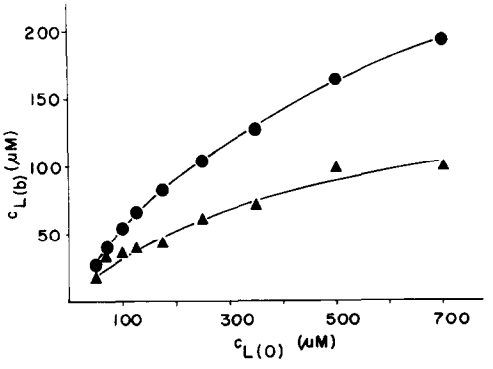

Fig. 5. Binding curves of the interaction of chlorpromazine and positively charged liposomes at different pH-values (equilibrium dialysis). Liposomes: 340 μM 1,2-dipalmitoyl-sn-glycero-3-*O*-methylphosphocholine. 37°. ● pH 7.4, ▲ pH 6.0. Each point is the median of four experiments.

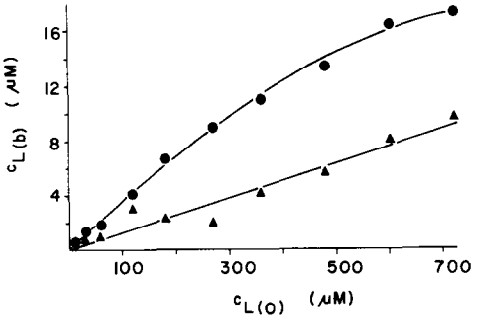

Fig. 6. Binding curves of the interaction of imipramine and positively charged liposomes at different pH-values (equilibrium dialysis). Liposomes: 150 μM 1,2-diplamitoyl-sn-glycero-3-*O*-methylphosphocholine. 37°. ● pH 7.4, ▲ pH 6.0. Each point is the median of three experiments.

Binding of CPZ to liposomes of some other phosphocholines was investigated. The amount of bound drug was found to be dependent only on the physical state of the lipid, but not on the chain length or double bonds of the hydrocarbon chains (Table 1).

The charge of the lipid membrane had a pronounced effect on the binding of CPZ and IP. At pH 7.4 positively charged liposomes of 1,2-dipalmitoyl-sn-glycero-3-*O*-methylphosphocholine bound a much smaller amount of IP than did liposomes of the corresponding zwitterionic phosphocholine at 37° (Figs. 3 and 6). In contrast, CPZ was bound by the positively charged liposomes to the same extent as by the zwitterionic liposomes above the transition temperature (Figs. 2 and 5). Binding of both CPZ and IP to positively charged liposomes increased with pH elevation from 6.0 to 7.4 (Figs. 5 and 6).

Binding of CPZ and IP to negatively charged liposomes (80%-1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 20%-3-phosphatidic acid) is shown on Fig. 7. CPZ seemed to reach a range of binding saturation (below 500 μM), whereas this was not the case with IP up to a concentration of 720 μM (Fig. 7).

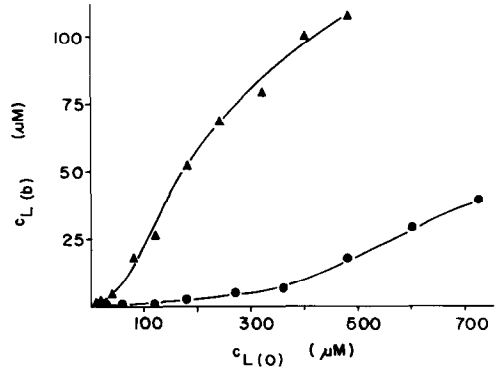

Fig. 7. Binding curves of the interaction of chlorpromazine and imipramine with negatively charged liposomes (equilibrium dialysis). Liposomes: 120 μM 1,2-dipalmitoyl-sn-glycero-phosphocholine + 30 μM 1,2-dipalmitoyl-sn-glycero-phosphatidic acid. 37°, pH 7.4. ▲ chlorpromazine, ● imipramine. Each point is the median of three experiments.

Table 1. Interaction of CPZ with various phospholipids above and below phase transition temperature

Method	Binder	*Transition temperature	Experimental temperature	Binding curve
Equilibrium dialysis	dioleyl-PC	−14	37	Fig. 2 upper curve
	dilauroyl-PC	0	37	
	dimyristoyl-PC	21	24, 30, 37, 45, 55, 60	
	dipalmitoyl-PC	37	37, 50	
Dual wavelength photometry	dioleyl-PC	−14	22	Fig. 8 upper curve
	dimyristoyl-PC	21	37	
	dipalmitoyl-PC	37	50	
	dipalmitoyl-PC	37	22	Fig. 8 lower curve
	distearyl-PC	51	32	
	dipalmitoyl-PA		22	

*[27]. All temperature in °C. Liposome composition: PC; 1,2-dialkyl-sn-glycero-3-phosphocholine; PA; 33%-phosphatidic acid +67%-phosphocholine.

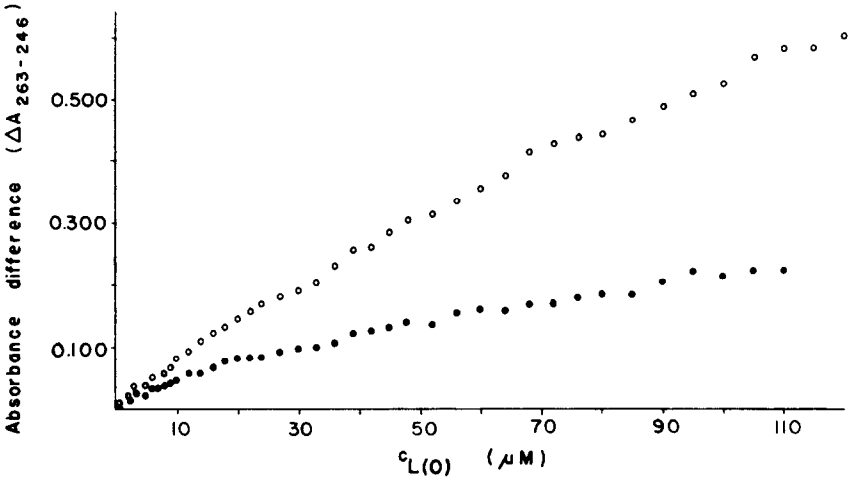

Fig. 8. Spectral change of chlorpromazine in the presence of liposomes below and above the transition temperature (dual-wavelength photometry). Liposomes: 150 μM 1,2-dipalmitoyl-sn-glycero-phophocholine, transition temperature 37° [27]. pH = 7.4. ● 22°, ○ 50°. Each point is one measurement.

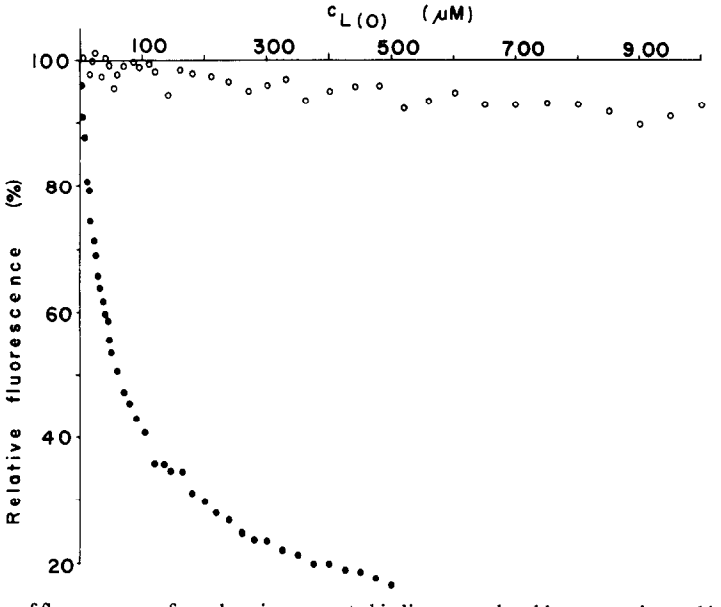

Fig. 9. Quenching of fluorescence of perylene incorporated in liposomes by chlorpromazine and imipramine. Liposomes: 150 μM 1,2-dilauroyl-sn-glycero-3-phosphocholine. 37°. pH 7.4. ● chlorpromazine, ○ imipramine. Each point is one measurement.

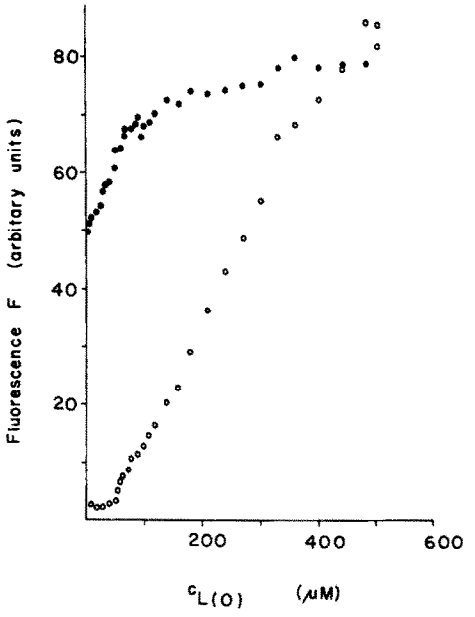

Fig. 10. Fluorescence of ANS incorporated in liposomes in the presence of chlorpromazine, 37°, pH 7.4. ● 150 μM 1,2-dioleyl-sn-glycero-3-phosphocholine. ○ 100 μM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine +50 μM 1,2-dipalmitoyl-sn-glycero-phosphatidic acid. Each point is one measurement.

Dual-wavelength photometry. Figure 8 shows the dependence of the spectral change of CPZ on the fluidity of the liposomal membrane. In accordance with the binding experiments performed by equilibrium dialysis a higher absorbance difference was observed when the lipid was in the liquid-crystalline state as compared with the gel state. Results with various lipids are summarized in Table 1.

Fluorimetry. Changes of the fluorescence of the probe rather than absolute fluorescence values were measured in the experiments with fluorescent probes.

With the fluorescent probe, perylene, the same results were obtained with liposomes of any lipid or lipid mixture investigated. The fluorescence of perylene incorporated in the saturated 1,2-dilauroyl- 1,2-dipalmitoyl-, the unsaturated 1,2-dioleyl-sn-glycero-3-phosphocholine, the positively and negatively charged liposomes mixed of 67%-1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 33% -3-*O*-methyl-phosphocholine or 33% -3-phosphatidic acid was markedly quenched by CPZ, but only weakly by IP. With all lipids mentioned above the same quenching curves were obtained. Typical experiments are shown in Fig. 9.

The influence of CPZ and IP on the fluorescence of ANS is shown in Figs. 10 and 11. Identical curves as with 1,2-dioleyl-sn-glycero-3-phosphocholine shown in the figure were obtained with the other lipids mentioned in the above paragraph, the only exception being the negatively charged liposomes of 67%-1,2-dipalmitoyle-sn-glycero-3-phosphocholine and 33%-3-phosphatidic acid. With this lipid mixture in the presence of CPZ no fluorescence increase could be detected up to a CPZ concentration of about 50 μM (Fig. 10). In the presence of IP, fluorescence increase began only at a concentration of about 200 μM IP (Fig. 10).

Control experiments showed that no fluorescence of CPZ or IP with liposomes occured in the absence of fluorescence probes. Fluorescence of CPZ with ANS in the absence of liposomes occured only at high CPZ concentrations upon formation of a precipitate. However, concentrations of free ANS and CPZ in the presence of liposomes did not reach this critical range, and no precipitate was observed within the time needed for performing fluorescence measurements.

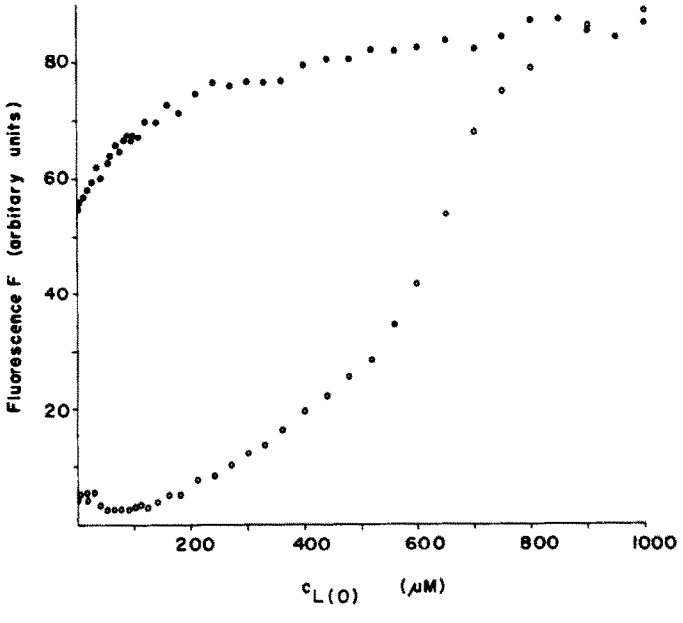

Fig. 11. Fluorescence of ANS incorporated in liposomes in the presence of imipramine. 37°, pH 7.4. ● 150 μM 1,2-dioleyl-sn-glycero-3-phosphocholine. ○ 100 μM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine +50 μM 1,2-dipalmitoyl-sn-glycero-phosphatidic acid. Each point is one measurement.

DISCUSSION

The results indicate that binding of both CPZ and IP to liposomes strongly depends on the physical state of the lipid bilayer. Liposomes in the gel state bound less of these two drugs than did liposomes of the same lipid composition in the liquid–crystalline state (Figs. 2, 3 and 8). The full binding capacity was reached when the phase transition of the membrane was reached (Fig. 2). When the lipid is in the gel state, CPZ is able to enhance isothermically the fluidity of a pure lipid membrane [8]. The expansion of erythrocyte membranes by CPZ [9–11] is also likely to result from a drug-induced disorder of the membrane structure. The fluidization of a lipid membrane below the transition temperature by increasing concentrations of CPZ resulted in an apparent cooperative effect in the binding of CPZ to the membrane (Fig. 4). However, no abrupt change of ANS or perylene fluorescence as a signal for a true phase transition [19, 22] could be observed (Figs. 9 and 10). Similar effects were observed with dual-wavelength photometry (Fig. 8). The absorbance difference signal is a measure for the transfer of CPZ from a polar to a hydrophobic environment [2, 16]. This signal was much larger when the lipid was in the liquid–crystalline rather than in the gel state. Apparently, CPZ can better penetrate into a fluid than into a rigid membrane. The experiments with dual-wavelength photometry complemented the results obtained by equilibrium dialysis. The photometric measurements (Fig. 8) were performed at a lower concentration range (1–100 μM CPZ) than the equilibrium dialysis experiments (50–700 μM CPZ). As in the high concentration range, sharp discontinuities in the binding of CPZ to liposomes did not occur.

Differences were observed in the binding of CPZ and IP to zwitterionic phosphocholines. More CPZ than IP was bound per mole of lipid (Figs. 2 and 3). When the results of the zwitterionic (Figs. 2 and 3) and the positively charged lipids (Figs. 5 and 6) at pH 7.4 above the transition temperature are compared, a marked difference between CPZ and IP emerges. CPZ was bound by both zwitterionic and positively charged liposomes to the same extent (Figs. 2 and 5). The amount of bound IP, however, was much smaller in the presence of a positively charged membrane as compared with a neutral one (Figs. 3 and 6). This fact suggests that the positive charge on the bilayer surface is a more potent repulsive force for IP than for CPZ at pH 7.4 or that binding sites for CPZ exist which are not affected by ionic forces. This second suggestion would be compatible with the fact that binding of IP was weaker than that of CPZ with any membrane investigated in this study. These findings agree with the results of the fluorescence experiments discussed below. At pH 6.0, where the ionization of IP and CPZ is higher than at pH 7.4, the binding of CPZ to positively charged liposomes is also reduced (Fig. 5).

In the case of negatively charged liposomes the results are less easily explained. The amount of CPZ bound per mole of negatively charged lipid was higher than per mole of zwitterionic or positively charged lipids (Figs. 2, 5 and 7). The CPZ cation seems to bind easily to negatively charged membranes. In contrast to CPZ, the binding curve of IP and negatively charged liposomes rose only at a concentration of about 200 μM (Fig. 7) The concave shape of the curve suggests a positive cooperative effect.

These results are in agreement with the results obtained by the experiments with the fluorescence probe, ANS. When ANS was incorporated into negatively charged liposomes a no-effect concentration range could be observed (Fig. 10) In this range practically no fluorescence was measurable, i.e., no ANS seems to be incorporated in the membranes. At concentrations of about 50 μM CPZ or 200 μM IP, ANS began to emit fluorescent light. At these threshold concentrations, ANS is likely to penetrate into the lipid bilayer after the cationic drugs have neutralized the negative surface charge on the bilayer. With all zwitterionic and positively charged lipids investigated, the enhancement of ANS fluorescence by CPZ and IP was identical (Figs. 10 and 11). Since ANS is a probe located near the surface of a membrane [18, 20] it is concluded that CPZ and IP bind in the same way near the polar surface of a lipid bilayer.

However, a difference in the binding of the two drugs becomes evident when the inner hydrocarbon region of the bilayer is observed by means of quenching of perylene fluorescence [18]. CPZ strongly quenched the fluorescence of perylene, whereas IP did so very weakly (Fig. 9). The strong quenching of perylene fluorescence by CPZ suggests that bound CPZ is located near the probe molecules, i.e., in the hydrocarbon core of the bilayer. These binding sites were found to be available for CPZ but not for IP, because IP did not quench the perylene fluorescence. As seen by the equilibrium dialysis experiments (Figs. 2–7), IP bound to a smaller extent to lipid membranes than did CPZ. There is much evidence from the fluorescence experiments that IP binds only to or near the surface of a lipid bilayer, whereas CPZ has additional binding sites in the interior of the membrane. The more planar structure of CPZ as compared with IP may be responsible for the fact that the former penetrates better into the membranes.

The conclusions drawn in this paper are compatible with results on the binding of desipramine to multilamellar [12] and single-lamellar [29] structures of egg phosphocholine by nuclear magnetic resonance, and on the binding of CPZ to liposomes as obtained by electron spin resonance [15], and with results on the interaction of CPZ with lipids [2] and fatty acids [16] obtained by difference spectrophotometry. However, the results reported here contradict the suggestion, that CPZ binds to lipids at high concentrations only [13]. The findings of this study agree with conclusions drawn from investigations made by scanning calorimetry and nuclear magnetic resonance [30]. These authors found CPZ-induced fluidization of multilamellar membranes, and suggest that CPZ pentrates into the lipid bilayer.

The three methods described in this paper (equilibrium dialysis, dual-wavelength photometry, fluorimetry) complemented one another. By equilibrium dialysis at high drug concentrations, and by dual-wavelength photometry at low drug concentrations, the overall membrane binding process could be observed. In the fluorimetric experiments the influence of bound drugs to a probe in the polar (ANS) and the hydrophobic (perylene) part of the membrane could be determined, and information was obtained about the localization of CPZ and IP in the membrane. It is concluded

that both CPZ and IP bind to about the same extent near the polar surface of pure lipid membranes and that CPZ has additional binding sites in the inner part of the bilayer. This additional pool is suggested to be responsible for the higher overall binding capacity of the liposomes for CPZ as compared with IP.

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