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THE BINDING OF CHLORPROMAZINE TO BILAYER LIPOSOMES. EVALUATION OF STOICHIOMETRIC CONSTANTS FROM EQUILIBRIUM AND STEADY STATE STUDIES

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Abstract—The binding of chlorpromazine to egg yolk lecithin bilayer liposomes has been studied with equilibrium dialysis as well as with a new steady state method.

The influence of the cholesterol amount previously incorporated into the liposomes to the binding of chlorpromazine was examined. The binding of chlorpromazine was evaluated in terms of stoichiometric constants and shows a strong positive cooperative binding step, followed by an equally strong negative cooperative step.

The first step, which is only detectable with the steady state method may be related to the partition of chlorpromazine into the lipophilic bilayer core, whereas the second and the following steps describe the overall saturation of the liposome membrane.

The binding of chlorpromazine is attenuated by increasing amounts of cholesterol present in the bilayer liposomes.

Investigating the binding to liposomes without cholesterol a saturation up to 75 per cent can be reached experimentally.

It is known that neuroleptic drugs like chlorpromazine (CPZ) are extremely fat soluble and surface active. Due to their very high partition coefficients, these drugs are very soluble in biological membranes. At rather high concentrations (μM range) the neuroleptics interact with the membranes in a nonspecific way and fluidize all pre-synaptic and vesicle membranes. This leads to an enhanced spontaneous release of neurotransmitters [1].

In contrast to the well known specific pre- and post-synaptic receptor binding sites for neuroleptics in the nanomolar drug concentration range, the drug-membrane interactions occurring in a much higher concentration range are nonspecific and can be attributed to the high partition ratios found between the drugs and the lipid membrane components. Because these nonspecific interactions can not yet be correlated to a part of the therapeutic effects or to untoward side effects of neuroleptics—for one reason because the free concentration of these drugs in the plasma water is extremely low [1]—we intended to study the binding mechanisms of CPZ in the concentration range of 5 μM to 5 mM to well defined model phospholipid bilayer membranes.

As the presence of cholesterol changes the dynamics of biological membranes, we further studied its influence on the binding of CPZ to our model membranes.

From the results of ESR spin label studies Leterrier *et al.* [2] suggested that CPZ interacts preferentially with the polar head-groups of the phospholipids arranged in multilamellar lecithin vesicles, whereas oxidized derivatives of CPZ are mainly found in the hydrophobic regions of the lecithin molecules. Similar results were found by the same authors [3, 4] with rat synaptic plasma membranes, pointing out that the CPZ-membrane interactions occur predominantly at the interface between the phospholipids and the proteins of the membranes.

Having examined the binding of CPZ and chlorimipramine to erythrocyte membrane ghosts and to liposomes of ghost lipids as well as to isolated ghost proteins Elferink [5] suggests that both the lipid and the protein phase are binding domains.

Seydel and Wassermann [6] found strong interactions of amphiphilic drugs with phospholipids like phosphatidylcholine and phosphatidylethanolamine from NMR-T_2 relaxation experiments. The presence of cholesterol antagonizes the drug actions, possibly due to permeability alterations.

Di Francesco and Bickel [7] showed with binding data evaluated from equilibrium dialysis studies that rat liver microsomes, mitochondria, mitochrondrial membranes, brain synaptosomes, myelin vesicles and red blood cells bind CPZ with relatively high affinity and large capacity. There was no detectable binding of CPZ to cytosol or mitochondrial matrix. Similar binding values were obtained with protein free liposomes made from mitochondria membrane lipids, red cell membrane lipids, microsomes and pure egg yolk lecithin.

From these results it could not be concluded whether CPZ and related drugs interact with the hydrophobic or polar region of the membrane lipids. Furthermore the antagonizing effect of cholesterol has not been taken into account.

MATERIALS AND METHODS

Lecithin was extracted from fresh egg yolks according to the procedure of Singleton [8]. It showed to be chromatographically pure and the value of the oxidation index described by Klein [9] was 0.11, indicating a slight oxidation of 0.6 per cent.

Cholesterol was purchased from Fluka, Buchs, Switzerland, and recrystallized twice from methanol.

Chlorpromazine hydrochloride (CPZ) was obtained from Rhodia S.A., Geneva, and used without further purification.

[^{35}S]-Chlorpromazine hydrochloride ([^{35}S]-CPZ) (original specific activity 8.3 mCi/mole) and L-α-dipalmitoyl-(2-palmitoyl-9,10-[^{3}H]) phosphatidyl choline ([^{3}H]-PC) (original specific activity 13 Ci/mM) were supplied by the Radio-chemical Centre, Amersham UK and Applied Science Lab. Inc., State College, USA. All other chemicals used in this work were of analytical grade.

1. *Preparation of single bilayer liposomes.* Different ethanol solutions containing about 8.10^{-5} M egg yolk lecithin (EYL) labelled with a known amount of [^{3}H]-PC and variable amounts of cholesterol (0, 25, 33 and 50% (w/w)) were evaporated to dryness under reduced pressure. The dried lipids and the lipid–cholesterol mixtures were then suspended in phosphate buffer (1 mM KH_2PO_4 with 0.16 M NaCl and adjusted to pH 6.0) and sonicated in a glass tube with a Branson Sonifier model B 12 at 40 W power level, protecting the mixture from oxidation with a nitrogen flow.

Sonication time varied from 30 to 60 min and the ambient temperature from 4° to 40° according to the cholesterol amounts added to the EYL. The sonicated vesicle dispersions were centrifuged at 100,000 *g* for 60 min at 4° to remove multibilayer vesicles and titanium particles lost from the sonifier tip. The vesicle preparations consisted after that treatment of more than 95 per cent of homogenous single shelled bilayers, which was examined according to the methods described by Huang [10].

To obtain a final concentration of $7–8.10^{-4}$ mole/l EYL (average molecular weight: 768) for the binding experiments, the vesicle preparations were diluted with buffer.

EYL concentrations were calculated by measuring the [^{3}H]-PC activity with a Searle Mark III liquid scintillation counter, equipped with a d.p.m. accessory and using PCS (Amersham/Searle) scintillation cocktail. The tritium activity was measured using a variable quench program with a ^{3}H efficiency of 48 per cent and a 0.25 per cent standard deviation of the net count rate at the 95 per cent confidence level.

2. *Steady state dialysis.* The flow-dialysis method described by Weder *et al.* [11] was modified by combining one stirred half cell as a closed compartment with a flow-through compartment separated by a semipermeable membrane. Figure 1 shows the setup of the dialysis system which was used. The method is based on the proportionality between the concentrations of a diffusing ligand in the reaction compartment RC and the diffusate, whereby a large concentration gradient across the membrane is established.

Since the diffusion kinetics of the ligand are highly dependent by means of the dialysis apparatus, i.e. membrane properties, volume and stirring speed in the RC and the flowrate in the flow compartment, the optimal dialysis conditions had to be determined experimentally.

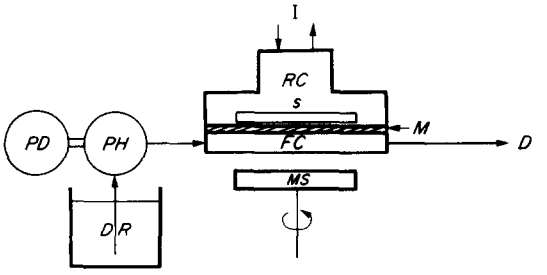

Fig. 1. Experimental setup.
FC, flow through compartment; RC, reaction compartment; M, membrane; S, stirring bar; MS, magnetic stirrer; PD, pump drive; PH, pump head; DR, dialysis fluid (buffer) reservoir; D, diffusate; I, injection holes.

Figure 2 demonstrates the linearity between the permeation rate (i.e. ligand concentration in the diffusate times flowrate) and the free ligand concentration in the reaction compartment during the experimental period [12]. The CPZ concentration in the diffusate was continuously monitored with an Uvicon LCD 725 flow cell spectrophotometer at a wavelength of 254 nm. The amounts of free, respectively bound ligand were calculated by extrapolation to the moment of binder injection as shown in Fig. 3. (labeled I).

The injection of a macromolecular binder into the reaction compartment causes a sudden concentration change of the free ligand due to binding, perturbating the original steady state conditions. Correspondingly the ligand concentration in the diffusate changes abruptly until the new steady state is established.

3. *Equilibrium dialysis.* The equilibrium dialysis experiments were performed with a DIANORM^{R} apparatus. All experiments were made at 22° during 150 min. Both half cells had a capacity of 1 ml and were separated by a cellulose membrane with a molecular weight cut off of about 10,000. The amount of free and bound CPZ in the liposome-containing compartment, as well as the free CPZ in the diffusate were analyzed by counting the [^{35}S]-CPZ activity simultaneously with the [^{3}H]-PC activity of the liposomes. For this purpose we used a ^{3}H/^{14}C dual label, variable quench program with 38 per cent ^{3}H, respectively 69 per cent ^{14}C efficiencies and equal statistical parameters as described above. As the ^{35}S beta energy range falls within that of ^{14}C, the ^{35}S activity could be measured with the ^{14}C program without use of an ^{35}S calibration curve. Proceeding that way, no correction due to losses of CPZ caused by adsorption to the cell halves and the membrane was necessary.

RESULTS AND DISCUSSION

The binding of CPZ to EYL liposomes has been studied in the absence as well as in the presence of

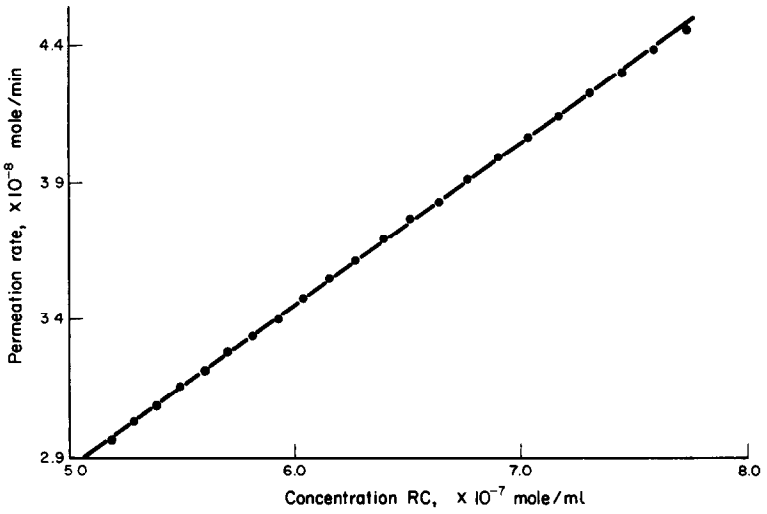

Fig. 2. Linear correlation between the permeation rate of CPZ and the CPZ concentration in the reaction compartment of an experiment during 30 min.
Initial CPZ concentration $8.3 \cdot 10^{-4}$ mole/l; flowrate 0.85 ml/min; volume RC 3.0 ml; stirrer speed 100 rev/min; cellulose membrane molecular weight cutoff 10,000; membrane area 7.85 cm^2; diffusion constant $K_D = 7.55 \cdot 10^{-3}$ cm/min.

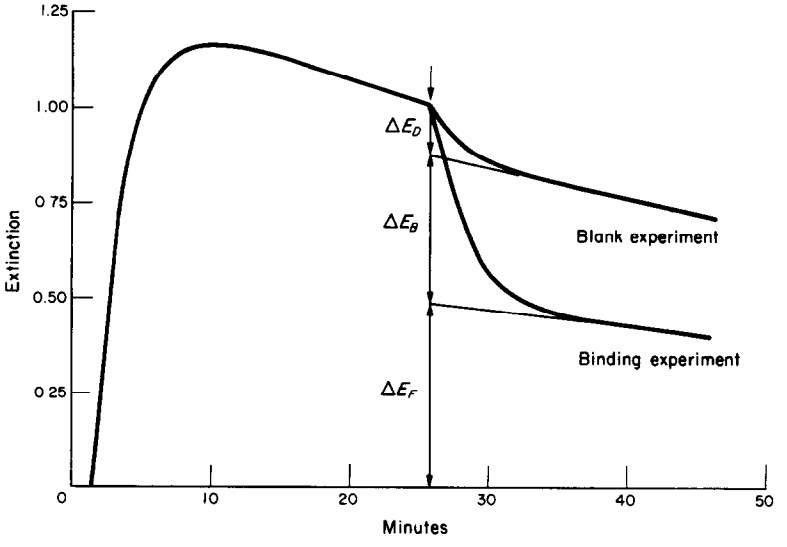

Fig. 3. Time dependence of CPZ concentration in the diffusate for a blank and a binding experiment. ΔE_D, extinction change due to dilution, caused by injection of 0.5 ml buffer or liposomes; ΔE_B, extinction change caused by a sudden decrease of free CPZ due to binding to liposomes: ΔE_F, corresponds to the remaining free CPZ concentration. (⇓) Injection of 0.5 ml buffer respectively liposome dispersion into the RC.

different amounts of cholesterol. The cholesterol was incorporated into the bilayers at amounts of 25, 33 and 50 per cent (w/w) during sonication. The two Scatchard plots (Figs. 4 and 5) show remarkable differences, especially in the high affinity range.

We could prove with the known binding of phenylbutazone to bovine serum albumin that both methods, the equilibrium dialysis and the steady state dialysis, yielded exactly the same results*. In the latter method the total CPZ concentration available for binding to the liposomes corresponds to the CPZ concentration in the RC at time of injection. From Fig. 3 it can be seen that steady state conditions are established after about 6 min in the blank experiment and 10–15 min in the liposome binding experiment. In contrast to these findings the steady state for phenylbutazone in absence and presence of bovine serum albumin is established within a shorter and equal period of time due to the very fast binding process. In our case the overall binding process is obviously much slower and in the order of magnitude of the dialysis process. It

* H. Hämmig and H. G. Weder, unpublished results.

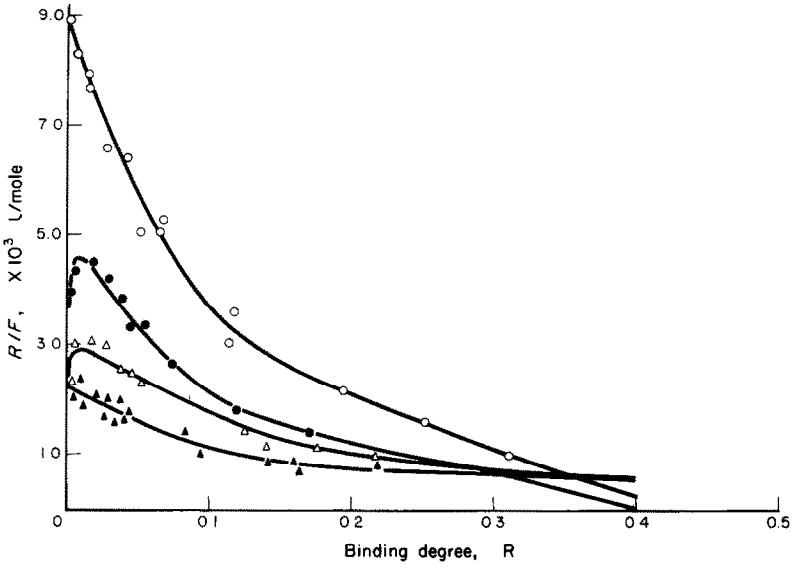

Fig. 4. Equilibrium dialysis.
Binding curves of the interaction of CPZ with EYL-liposomes in the CPZ concentration range of $5 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ mole/l. EYL mol. wt.: 768. (○) CPZ-EYL; (●) CPZ-EYL/25% (w/w) cholesterol; (△) CPZ-EYL/33% (w/w) cholesterol; (▲) CPZ-EYL/50% (w/w) cholesterol.

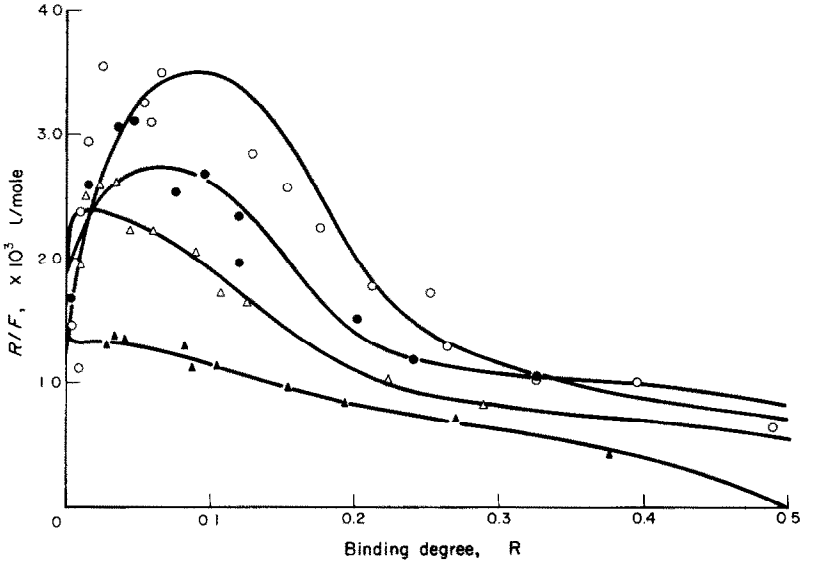

Fig. 5. Steady state dialysis.
Binding curves of the interaction of CPZ with EYL-liposomes in the CPZ concentration range of $5 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ mole/l. EYL mol. wt.: 768. (○) CPZ-EYL; (●)CPZ/EYL/25% (w/w) cholesterol; (△) CPZ-EYL/33% (w/w) cholesterol; (▲) CPZ-EYL/50% (w/w) cholesterol.

can be assumed that the primary interaction of CPZ with the liposome surface is a fast process—not detectable with the steady state technique—followed by a slow process attributed to the hydrophobic partition of the CPZ molecules into the lipophilic phase of the liposome bilayer. This partition of CPZ into the lipophilic bilayer core is possibly indicated by means of the positive cooperative effect observed with the kinetic method. On the other hand the investigation of the results of the equilibrium dialysis experiments that characterize the CPZ—liposome overall binding after equilibrium is reached, allow us not to distinguish between polar and hydrophobic interactions. (cf. Fig. 4).

Thus we analyzed the binding in terms of stoichiometric constants as described by Klotz [13], obtaining the values K_i of the constants from computer best fits [14]. These results are summarized in Tables 1 and 2. Then we correlated the stoichiometric binding constants in the affinity profile [13], representing a

Table 1. Stoichiometric constants of the binding of CPZ to EYL bilayer liposomes containing various amounts of incorporated cholesterol obtained from equilibrium dialysis. The experimental conditions are described in the Methods section and in the legends of Figs. 4 and 6.

Cholesterol content % (w/w)	$K_i \times 10^{-3}$ (M^{-1})				
	K_1	K_2	K_3	K_4	K_5
0	—	89.0	10.0	9.3	2.3
25	—	52.0	6.0	4.5	2.0
33	—	31.0	8.0	1.7	2.2
50	—	22.0	4.1	1.5	2.4

Table 2. Stoichiometric constants of the binding of CPZ to EYL bilayer liposomes containing various amounts of incorporated cholesterol obtained from steady state dialysis. The experimental conditions are described in the Methods section and in the legends of Figs. 5 and 7

Cholesterol content % (w/w)	$K_i \times 10^{-3}$ (M^{-1})				
	K_1	K_2	K_3	K_4	K_5
0	11.7	96.8	1.1	5.9	1.3
25	18.2	34.9	0.84	5.0	1.0
33	24.0	12.5	1.3	4.5	—
50	13.0	8.3	0.89	4.5	0.84

plot of $i \times K_i$ versus i, where i is the number of constants describing the ligand–binder interaction and K_i the individual stoichiometric constants. For the ideal case where the binding sites are completely independent and equivalent in intrinsic affinity, $i \times K_{i(\text{ideal})}$ is linear in i. Any deviation from linearity in this graph shows either accentuate or attenuate interactions between binding sites. Thus if $K_i > K_{i+1}$ and K_{i+1} lies below this ideal line, the interaction of the $(i + 1)$th step is attenuating, respectively negative cooperative. Conversely if K_{i+1} lies above the ideal line and $K_{i+1} > K_i$, the interaction is accentuating, respectively positive cooperative. Therefore the individual interactions of the binding process can be evaluated, provided that the binding sites of the system were originally equivalent and noninteracting.

Discussing the affinity profile of the equilibrium dialysis experiments, one can see that the binding of CPZ (i.e. K_2 in Table 1) is strongly attenuated, whereas by following the affinity profile, the second step (i.e. K_3 in Table 1) shows a positive cooperativity for 0 and 25 per cent (w/w) incorporated cholesterol. The constant K_2 results possibly from the overall saturation of the liposome membrane. The successive uptake of ligands occurs more or less without changing affinity. Increased amounts of cholesterol in the bilayer decrease the affinity constant K_2 significantly (cf. Table 1). At cholesterol contents of 33% (w/w) and higher percentages the positive cooperative effect of the second binding step disappears (see Fig. 6). All other following binding constants describe again noninteracting sites.

In contrast to the equilibrium dialysis results, the fitting procedures of the steady state data yield five stoichiometric constants. The additional constant K_1 represents a strong positive cooperative effect weakened by increasing amounts of cholesterol present in the bilayer.

A good correlation between K_1 and increasing cholesterol contents (cf. Table 2) up to 33% (w/w) can be shown, whereas the deviation from linearity at higher amounts can be explained in terms of the abrupt structural change of the lecithin bilayer which is consistent with the asymmetrical distribution of the lipid composition between the inner and the outer

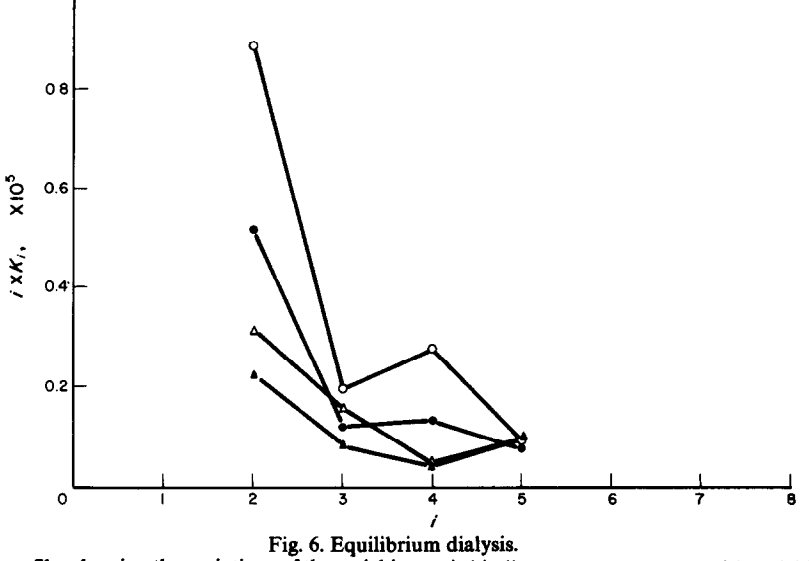

Fig. 6. Equilibrium dialysis.
Affinity profiles showing the variations of the stoichiometric binding constants, $i \times K_i$, with stoichiometric binding steps, i. The experimentally observed binding steps 1 to 4 have been shifted one unit to allow a better comparisong with the affinity profile of the steady state dialysis (cf. Fig. 7 and K values in Tables 1 and 2) which means that the values of $i \times K_i$ represent actually the $(i - 1) \times K_{i-1}$ values.
(○) CPZ-EYL; (●) CPZ-EYL/25% (w/w) cholesterol; (△) CPZ-EYL/33% (w/w) cholesterol; (▲) CPZ-EYL/50% (w/w) cholesterol.

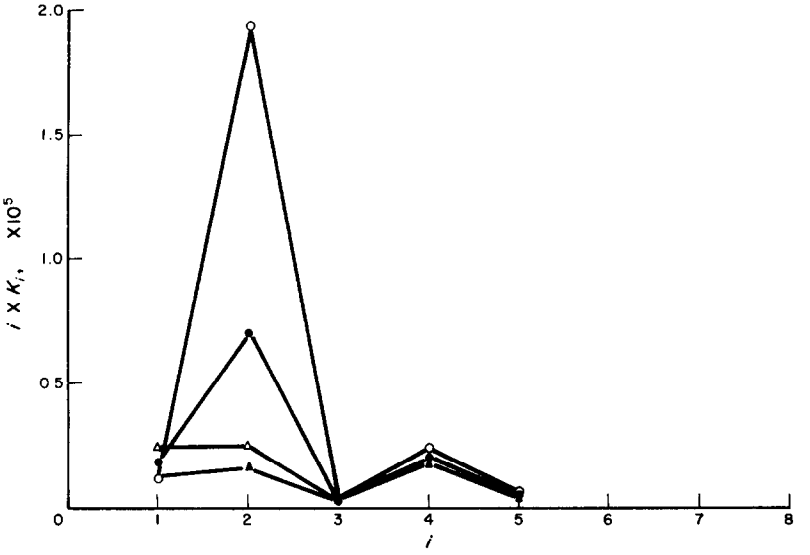

Fig. 7. Steady state dialysis.
Affinity profiles showing the variations of the stoichiometric binding constants, $i \times K_p$ with stoichiometric binding steps, i. (○) CPZ-EYL; (●) CPZ-EYL/25% (w/w) cholesterol; (△) CPZ-EYL/33% (w/w) cholesterol; (▲) CPZ-EYL/50% (w/w) cholesterol.

bilayer face [15, 16]. The additionally evaluated constant K_1 could be attributed to the slow partition of CPZ into the lipophilic moiety of the bilayer. The linear cholesterol dependent decrease of K_1 gives further evidence to the above described interaction mechanism.

The following strong negative cooperative effect can again be attributed to the overall saturation of the membrane, i.e. the polar surface and the lipophilic membrane core. The consecutive binding steps show more or less a similar course with both methods and are very difficult to interpret, as the binding affinities are too low and experimentally difficult to observe.

It is suggested by different authors [17, 18] that the molar ratio of the cholesterol–phospholipid complex is 1:1. At cholesterol concentrations below 33% (w/w) each sterol molecule is surrounded by fatty acid chains without cholesterol–cholesterol interactions, whereas at higher concentrations intermolecular interactions seem to be apparent. As our results show a weak binding to liposomes containing 50% (w/w) cholesterol and considerable binding when cholesterol is absent, we assume that CPZ could occupy a similar space around the fatty acid chains in the lecithin bilayer as cholesterol does. The interactions of CPZ with the lipophilic lecithin region are certainly different from

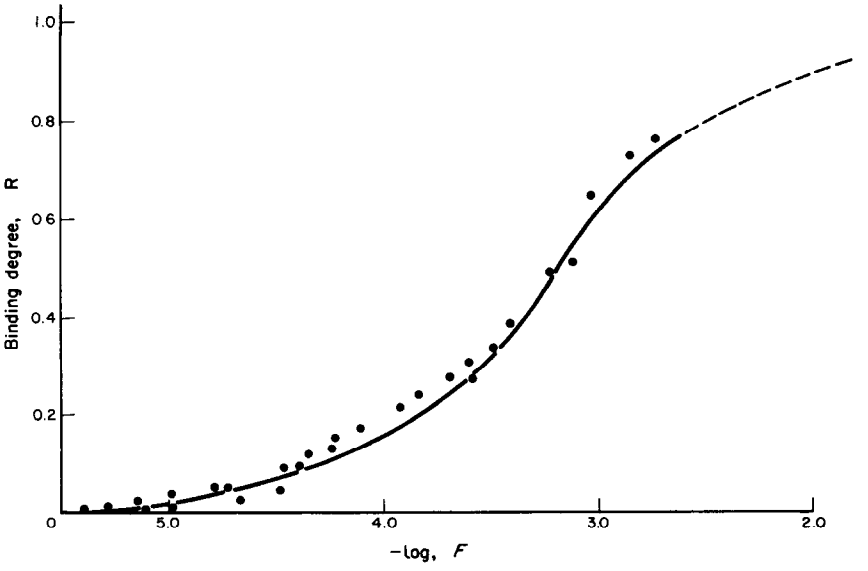

Fig. 8. Binding data of CPZ to EYL liposomes without cholesterol, represented in terms of binding degree R versus logarithm of free CPZ (log F). CPZ concentration range $5 \cdot 10^{-6}$ to $5 \cdot 10^{-3}$ mole/l.

the cholesterol–lecithin interactions due to the different molecular structures.

The fact that CPZ still binds to a certain degree to liposomes with 50% (w/w) finds an explanation in the asymmetrical lipid distribution found in such bilayers, leaving excess "free" lecithin molecules in the outer layer, allowing CPZ to bind [16].

By plotting the experimental R values against the logarithm of free ligand (log F) one obtains a sigmoidal saturation curve with half saturation at its inflection point [19]. Unfortunately it is in most of the cases impossible to cover the whole concentration range of the curve due to experimental difficulties.

In the case of CPZ the critical micelle concentration (cmc) is reached at about 5.10^{-3} mole/l [20]. Reaching the cmc the first order diffusion kinetics are therefore perturbed (see Fig. 8). From the found experimental saturation (up to 75 per cent) and the shape of the curve in Fig. 8, it can be assumed that the total molar binding ratio tends toward one and represents again the binding of CPZ to the polar surface of the bilayer and its partition into the lipophilic bilayer core.

Our results have shown that nonspecific interactions of amphiphilic drugs like chlorpromazine with lipid membranes are hard to describe by means of relatively simple binding models, and that the amount of cholesterol present in such membranes is of paramount importance.

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