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Partition of chlorpromazine into lipid bilayer membranes: the effect of membrane structure and composition

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Partition coefficients, k_p, of chlorpromazine between the aqueous phase and lipid bilayer vesicles were determined as function of drug concentration, lipid chain length, cholesterol content and temperature encompassing the range of the lipid phase transition. Radioactivity and absorption measurements were performed to determine the k_p values. Up to a concentration of $3 \cdot 10^{-5}$ M, the partition coefficient is independent of chlorpromazine concentration, whereas it decreases drastically at higher chlorpromazine concentrations, at which membrane lysis is observed. Membrane structure is not disturbed at less than $3 \cdot 10^{-5}$ M chlorpromazine, as was concluded from electron paramagnetic resonance studies measuring TEMPO partitioning and order degree. However, the lipid phase-transition temperature decreases and is broadened at higher chlorpromazine concentrations. From fluorescence measurements, we conclude the formation of chlorpromazine micelles at concentrations higher than $5 \cdot 10^{-5}$ M in chlorpromazine in the absence of lipids and the formation of mixed micelles in the presence of lipids. The effect of lipid chain length on k_p values was investigated. The partition coefficient decreases from 8100 in dilauroyl- to 3400 in dipalmitoylphosphatidylcholine vesicles, both at 50°C, that is, above their corresponding phase-transition temperature t_t. At $t < t_t$ the k_p values are strongly reduced, by at least a factor of 10, depending on lipid chain length and membrane composition. It is possible to establish a lipid phase-transition curve from the temperature-dependent measurements of the k_p values. Cholesterol within the lipid membrane strongly decreases k_p. At 20 mol% cholesterol in dipalmitoylphosphatidylcholine membranes, the partition coefficient is reduced from 3400 to 2300. This value is well comparable to the k_p value obtained in erythrocyte ghosts. In contradiction to earlier experiments by Conrad and Singer (Biochemistry 20 (1981) 808–818), this value in a biological membrane could be obtained by the hygroscopic desorption as well as the centrifugation method. From our experiments we are justified in further considering artificial bilayer membranes as models for biological membranes.

Introduction

Lipid membranes play a crucial role in the phenomenon of local anesthesia [1]. The Meyer-Overton rule [2] states that the anesthesizing concentration of a narcotic is always constant within a membrane. This is at least true for neutral or positively charged anesthetics. Conclusively, the anesthetic action of a substrate depends on its membrane solubility expressed by a partition coefficient which is the equilibrium concentration between the lipid and the aqueous phase. In a biological system one has to deal with a complex

Abbreviations: DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; SL5, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl.

equilibrium situation. Different types of lipid, phase separation phenomenon as well as lipid-protein-interaction leading to a domain structure have to be considered [3–5]. The question of whether the site of anesthetic action is in the membrane lipid, the membrane protein or in the lipid/protein interface is still unanswered. The membrane expansion theory [6] opposes the protein perturbation hypothesis [7].

Perturbation of the lipid-protein interaction or of lateral phase separation within a membrane may as well act as the primary site of the anesthetic action [8]. Charged lipids in the surrounding of a protein are especially sensitive to local anesthetics [9].

To obtain experimental evidence for such theories in natural membranes is difficult. Therefore artificial bilayer membranes are in widespread use as models for biological membranes to investigate the effect of drugs. Very recently, the use of artificial bilayer membranes as model membranes became doubtful. Singer and co-workers [10–12] reported in a series of papers that amphipaths, including many anesthetics and tranquillizers, are excluded from biological membranes but are readily soluble in artificial bilayer membranes. They postulated an 'internal pressure' of the lipids in the biological membranes that does not exist in lipid vesicles and serves to exclude the amphipaths from biological material. The hypothesis of Conrad and Singer [10] rested mainly on the use of a new filtration technique to separate cells or vesicles from the aqueous phase. They assume the formation of amphipath micelles that adsorb to the cell surface and are stripped off during the filtration. Using the conventional centrifugation technique, these micelles are thought to be pelleted together with the cells, leading to erroneously high partition coefficients. Pjura et al. [13] investigated the partition of fatty acids and could not find any differences in its solubility in lipid vesicles or plasma membranes using either method. These results are in agreement with experiments of Bondy and Remien [14]. Forrest et al. [15] could not detect any micelles in their NMR study. Luxnat et al. [16] reported comparable partition coefficients in biological membranes using the centrifugation and the filtration techniques. This controversy is not satisfactorily solved. Comicellation between lipids and amphipaths may occur and an even more important question is how the physical state of the membrane may control the uptake of an amphipath.

In this paper we studied the partition of chlorpromazine in phosphatidylcholine bilayer vesicles. Lipid chain length, cholesterol content and temperature critically determine the partition coefficients. Chlorpromazine partitions into erythrocyte membranes with a value comparable to cholesterol-rich artificial bilayer vesicles. Chlorpromazine concentrations higher than $2.8 \cdot 10^{-5}$ M lead to erroneously low partition coefficients due to comicellation with lipids extracted from the membranes.

Materials and Methods

Lipids and probes. Lipids (dimyristoyl-, dipalmitoyl-, dilauroylphosphatidylcholine) were purchased from Fluka (Neu-Ulm, F.R.G.), checked by TLC and used without further purification. Chlorpromazine hydrochloride was obtained from Serva (Heidelberg, F.R.G.). [^{3}H]Chlorpromazine hydrochloride (New England Nuclear) with a specific activity of 26.5 Ci/mmol was used for the radioactivity measurements. The ESR experiments were carried out by the spin-label technique using the fatty-acid nitroxide labels SL5 and TEMPO. Both were obtained from EGA-Chemicals (Steinheim, F.R.G.).

Large unilamellar vesicles. Large unilamellar vesicles were prepared by a modification of the method of Szoka and Papahadjopoulos [17]. A 25 mM solution of phosphatidylcholine or phosphatidylcholine/cholesterol in chloroform/buffer (2 : 1, v/v) was sonicated briefly (Branson sonifier) until the mixture became a homogeneous dispersion. In a typical experiment, 17 mg of lipid was dissolved in 1 ml chloroform/buffer. The buffer solution throughout the experiments was 140 mM NaCl in 10 mM Tris-HCl at pH 7.0. The organic solvent was then removed at $t > t_t$ under reduced pressure by a rotary evaporator. The final aqueous suspension was washed three times by centrifugation ($12\,000 \times g$, 15 min) in 20 vol. buffer. The final pellet was resuspended in 10–30 ml of buffer solution to make up a lipid concentration of 0.2–1.0 mg/ml.

Vesicles used in the ESR experiments were not resuspended. Spin-labeled fatty acid SL5 was added in a concentration of 10 mmol/mol lipid to the organic phase before evaporation.

Erythrocytes. Erythrocytes were prepared from human blood by centrifugation and washing three times with 0.9% NaCl at 4°C ($2000 \times g$, 10 min). Erythrocyte ghosts were prepared from these cells by lysis in 10 mM Tris-HCl (pH 7.0) at 4°C and by washing five times in 10 vol. of this buffer according to the method of Dodge et al. [18]. The final ghost sediment was dispersed in isotonic buffer and incubated 2 h at 37°C to reseal the cells.

Phosphate determination. Lipid concentration was calculated from phosphate analysis. First, ghost or vesicle suspensions were heated at 200°C for 1 h with a few drops of 70% perchloric acid. Phosphate was determined by a modification of the method of Chen et al. [19]. After cooling of the samples, 3.5 ml distilled water, 0.45 ml 70% perchloric acid, 0.5 ml 2.5% ammonium molybdate and 0.5 ml 10% ascorbic acid were added. After 5 min at 100°C the absorbance was measured at 800 nm.

Determination of partition coefficient. 0.4–1 ml of vesicles or cell suspensions were incubated with chlorpromazine at a given concentration. Ghosts were incubated at 37°C and vesicles at a temperature above the lipid phase-transition temperature. After 30 min the equilibrated membranes were centrifuged for 15 min at $12\,500 \times g$.

Absorbance measurements. After centrifugation, the extinction of chlorpromazine in the supernatant was determined as a measure of concentration. The partition coefficient, k_p, was calculated from [20]:

$$k_p = \frac{E_{tot} - E_{free}}{E_{free}} \cdot \frac{V_{(b)}}{V_{(m)}}$$

where E_{tot} is the measured extinction of the chlorpromazine solution before and E_{free} is the extinction of the supernatant after incubation and centrifugation of the vesicles; V_m is the volume of membranes and V_b the volume of the buffer.

Membrane volume, V_m, was taken to be that of the lipid compartment of the membrane. It was calculated from phosphate analysis and known cholesterol content and a density of 1 g/ml for lipids. The partition coefficient was corrected for lipid remaining in the supernatant. In a typical experiment a 1 ml suspension of membranes contained $2.8 \cdot 10^{-5}$ M chlorpromazine.

Radioactivity measurements. The radioactivity measurements were performed by addition of [^{3}H]chlorpromazine at an activity of 1 μCi/ml ($3.7 \cdot 10^{-8}$ M) to unlabeled chlorpromazine to obtain the given concentration. 50 μl aliquots of supernatant were dissolved in 50 ml Ready Solv MP (Beckmann) and the radioactivity was measured with a Tri-Carb scintillation counter. The partition coefficient was directly determined from radioactivity in the supernatant:

$$k_p = \frac{cpm_{(m)}}{cpm_{(b)}} \cdot \frac{V_{(b)}}{V_{(m)}}$$

where cpm represents the counts per min in membrane (m) or buffer (b). $Cpm_{(m)}$ was calculated from total cpm minus $cpm_{(b)}$.

Hygroscopic desorption. Hygroscopic desorption was performed according to Conrad and Singer [10] with the limitations mentioned in Ref. 16. The pore diameter of the filter was 0.2 μm.

Spectroscopic methods. Absorbance measurements were carried out with a Varian Cary 118 spectrometer. Fluorescence spectra were taken with a fluorescence spectrometer Perkin Elmer MPF3. Emission spectra were measured after irradiation at the maximum of excitation. Excitation spectra were measured at a fixed emission at $\lambda = 452$ nm. ESR measurements were performed with a Varian ESR spectrometer E4. Samples were thermostatically controlled by a heated nitrogen flow. Lipid phase-transition temperature was determined by the TEMPO partitioning method [21]. The order degree of the fatty acid spin label SL5 was calculated by

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - A_{xx}}$$

where $A_{\parallel}$ is the hyperfine splitting constant parallel and $A_{\perp}$ the corresponding value perpendicular to the external field. $A_{zz} - A_{xx}$ are constants and yield 25 G [20].

Results

Partition coefficients at variable chlorpromazine concentration

Partition coefficients, k_p, of chlorpromazine between phosphatidylcholine bilayer vesicles and the aqueous phase were determined by either measuring the radioactivity of ^{3}H-labelled chlorpromazine or by measuring the absorbance of chlorpromazine at $\lambda = 254$ nm in the supernatant after centrifugation or hygroscopic desorption. Either combination yielded comparable results. Absorption measurements could be performed only at chlorpromazine concentrations greater than $2.8 \cdot 10^{-5}$ M. The results for DMPC membranes are given in Fig. 1. The k_p value is constant at 5400 ± 500 up to $2.8 \cdot 10^{-5}$ M chlorpromazine and decreases abruptly at higher chlorpromazine concentrations. The loss of lipid in the supernatant or the filtrate is less than 1% at less than $2.8 \cdot 10^{-5}$ chlorpromazine. Considerably higher amounts of lipid were found at higher chlorpromazine concentrations. Obviously lipid is extracted from the bilayer membrane to form small aggregates which may not be centrifuged at low speed or which do not remain on top of the filter. This result may be construed as a comicellation of lipid and chlorpromazine starting at $2.8 \cdot 10^{-5}$ M chlorpromazine and finally leading to membrane lysis at higher chlorpromazine-concentrations.

Although strongly dependent on chlorpromazine-concentration, the k_p value does not change with lipid concentration. A constant value of 5400 was found at $2.8 \cdot 10^{-5}$ chlorpromazine with varying lipid concentrations between 1.1 and 0.2 mg/ml.

Micelle formation of chlorpromazine

Amphipathic molecules tend to form micellar aggregates in aqueous solution, as is well known for detergents. For chlorpromazine, a critical micellar concentration of $4 \cdot 10^{-3}$ M was reported from surface tension measurements [22]. Structural changes in membranes, however, are induced far below this concentration. Our results given in Fig. 1 clearly show a lysis of lipid bilayers at about $3 \cdot 10^{-5}$ M. We therefore investigated the aggregation behavior by a fluorescence study. Chlorpromazine is a major tranquillizer or a local

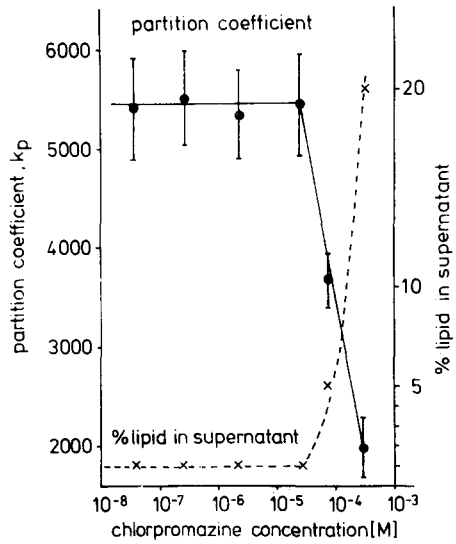

Fig. 1. Partition coefficients of chlorpromazine in DMPC-bilayer vesicles at $t = 37$°C are given as function of chlorpromazine concentration. The amount of lipid remaining in the aqueous phase as determined from phosphate analysis is also given. The lipid concentration is 1 mg/ml.

anesthetic in the sense of Seeman [23]. From a physical point of view, chlorpromazine is a fluorescent dye. Very often fluorescent dyes exhibit a specific shift in their absorption or fluorescence spectra if the polarity of the surrounding medium is changed [24]. Dye aggregates may also be spectroscopically different from monomeric dyes in solution [25]. The concentration dependences of the fluorescence and the excitation spectra of chlorpromazine in 0.14 M NaCl/10 mM Tris-HCl buffer at pH 7.0 are shown together with the absorption spectrum in Fig. 2.

The absorption spectrum (Fig. 2a) is characterized by a band at $\lambda = 254$ nm and a broad band with a maximum at $\lambda = 305$ nm. No change in the absorption maximum was observed with increasing chlorpromazine concentration. The fluorescence spectra (Fig. 2b) are shifted to longer wavelength at chlorpromazine concentrations above $5 \cdot 10^{-5}$ M. At less than $5 \cdot 10^{-5}$ M chlorpromazine, the fluorescence maximum is at 452 nm, whereas at $2.8 \cdot 10^{-3}$ M, for example, it is found at 460 nm. The red shift is even more pronounced in the excitation spectra (Fig. 2c). Fluorescence was recorded at 450 nm; the excitation wavelength was varied. The excitation maxi-

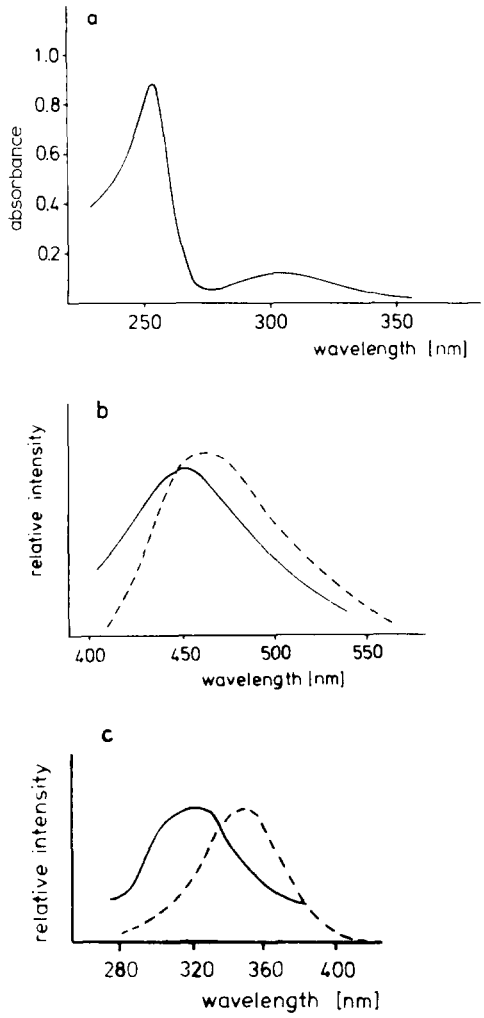

Fig. 2. (a) Absorption spectra of $2.8 \cdot 10^{-5}$ M chlorpromazine in solution at pH 7.0 and in 0.14 M NaCl in 10 mM Tris-HCl. (b) Fluorescence spectra of chlorpromazine at $2.8 \cdot 10^{-5}$ M (————) and $2.8 \cdot 10^{-3}$ (— — —). The ordinate gives only relative intensities, different from each spectrum. The excitation was at $\lambda = 325$ and 354 nm, respectively, which are the excitation maxima obtained from the excitation spectra shown in (c). (c) Excitation spectra of chlorpromazine in solution as in (b). The fluorescence was measured at 450 nm; the irradiation wavelength was varied and is given on the abscissa. The ordinate gives only relative intensities. The recorder was not corrected for absorbance changes with increasing concentration. The intention here is to demonstrate the red shift of the excitation maximum with concentration.

mum at $2.8 \cdot 10^{-5}$ M chlorpromazine was found at 325 nm and is red-shifted with increasing concentration, e.g., 354 nm at $2.8 \cdot 10^{-3}$ M. The wavelength shift of the excitation maximum, λ_{max}, is given in Fig. 3 as function of chlorpromazine concentration. The excitation maximum is constant up to $3 \cdot 10^{-5}$ M. A step-like increase to $\lambda_{max} = 340$ nm occurs at about $5 \cdot 10^{-5}$ M, which is followed by a shallow increase at higher concentrations. These fluorescence data clearly evidence the formation of chlorpromazine aggregates at about $5 \cdot 10^{-5}$ M, which is considerably lower than the value assumed by Conrad and Singer [11]. Hence, we should reckon with the possibility that chlorpromazine concentrations higher than $3 \cdot 10^{-5}$ M may induce membrane lysis by the formation of mixed micelles containing membrane components.

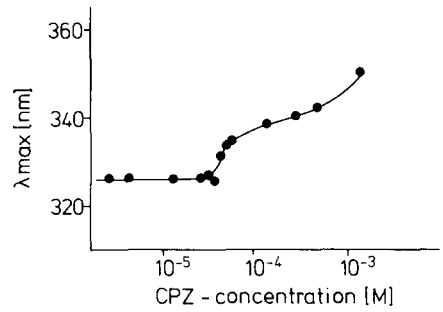

Fig. 3. Excitation maximum of spectra shown in Fig. 2c as function of chlorpromazine (CPZ) concentration. A step-like increase in λ_{max} occurs at $5 \cdot 10^{-5}$ M, which may be construed as the formation of chlorpromazine-aggregates.

The effect of chlorpromazine on the phase transition of lipid bilayers

From the partition coefficients we learn that at a chlorpromazine-concentration of $2.8 \cdot 10^{-5}$ M and a lipid concentration of 2.5 mg/ml, 5 mmol chlorpromazine are dissolved per mol lipid. This value increases to about 40 mmol per mole lipid at $2.8 \cdot 10^{-4}$ M chlorpromazine. The question arises of whether chlorpromazine disturbs the lipid bilayer structure at the concentrations used in this study. ESR spectroscopy using the spin-label technique is well suited to answering this question. We performed TEMPO partitioning as well as order degree measurements on the spin-labeled fatty acid, SL5. The results are shown in Fig. 4. Up to $2.8 \cdot 10^{-5}$ M chlorpromazine almost no effect could be observed; $2.8 \cdot 10^{-4}$ M considerably broadens the phase transition and reduces the

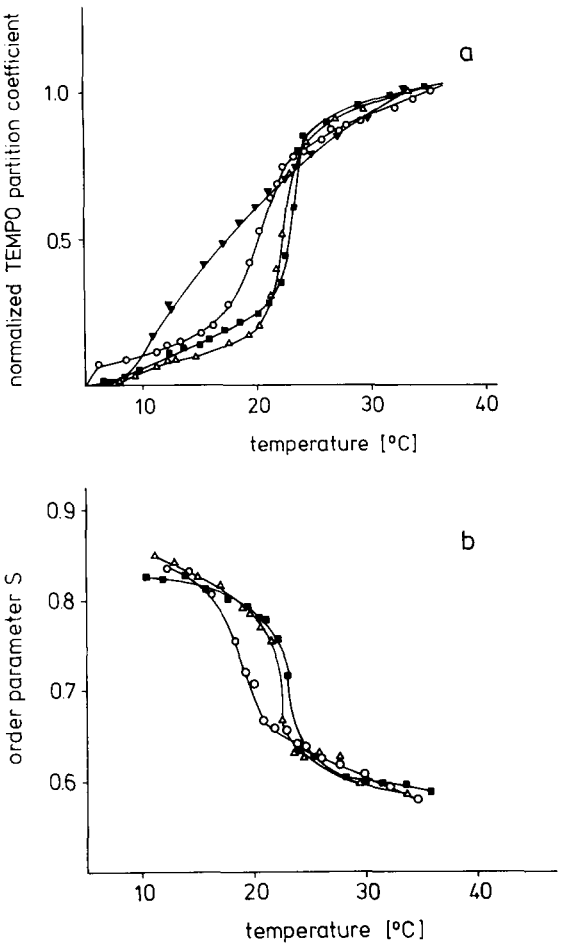

Fig. 4. (a) TEMPO partitioning was used to measure thermotropic phase-transition curves of DMPC in the presence of chlorpromazine. Chlorpromazine concentrations: ■, pure lipid; △, $2.8 \cdot 10^{-5}$ M chlorpromazine; ○, $2.8 \cdot 10^{-4}$ M chlorpromazine; ▼, $c = 2.2 \cdot 10^{-3}$ M chlorpromazine. (b) Order degree of SL5 in DMPC bilayer vesicles in the absence and in the presence of chlorpromazine as function of temperature. Symbols as in (a).

midpoint temperature in both sets of experiments. The lipid order is strongly reduced at $t = 20°C$. Above and below the phase-transition temperature of DMPC ($t_t = 23°C$), chlorpromazine does not change the lipid order. At even higher concentrations the phase transition completely disappears (e.g., $2.2 \cdot 10^{-3}$ M chlorpromazine in Fig. 4a).

It is important to note that lysis occurs above $2.8 \cdot 10^{-5}$ M chlorpromazine (e.g., Fig. 1). The measurements exhibited in Fig. 4 were performed with the centrifuged pellet, whereas the supernatant containing both chlorpromazine and lipid was withdrawn. Thus the measured disturbance of membrane structure occurs in the intact lipid bilayer vesicles.

The effect of cholesterol and lipid chain length on the k_p value

Cholesterol strongly reduces the k_p value. The partition coefficients given in Table I were obtained by absorption measurements after centrifugation at $2.8 \cdot 10^{-5}$ M chlorpromazine. Radioactivity measurements yield the same values. k_p values are identical with centrifuged or filtered vesicles. The uncertainty of the measurements should not vitiate the conclusion that cholesterol considerably reduces the partition coefficient. Obviously, membrane structure determines the drug incorporation.

These results prompted us to investigate the effect of lipid chainlength. The values presented in Table I were obtained at temperatures above the corresponding lipid phase-transition temperature. Short-chain lipids show a drastically increased drug uptake. The k_p value changes from 3400 in DPPC to 8100 in DLPC-bilayer vesicles at 50°C.

TABLE I

PARTITION COEFFICIENTS, k_p, IN CHOLESTEROL-CONTAINING DMPC MEMBRANES AND IN PURE DLPC, DMPC AND DPPC MEMBRANES

The chlorpromazine concentration was $2.8 \cdot 10^{-5}$ M; absorption measurements were performed to determine the chlorpromazine concentration in the supernatant of centrifuged vesicles. Samples were incubated above the lipid phase-transition temperature for 30 min. A value for erythrocyte membranes is included.

Membrane composition	k_p	Incubation temperature (°C)
DMPC	5400 ± 500	37
DMPC + 5% cholesterol	3400 ± 300	37
DMPC + 10% cholesterol	2700 ± 300	37
DMPC + 20% cholesterol	2300 ± 200	37
DLPC	8100 ± 100	50
DMPC	5000 ± 200	50
DPPC	3400 ± 300	50
Erythrocyte ghosts	2700 ± 300	37

Temperature dependence

The partition of chlorpromazine in pure DMPC, in pure DPPC and in cholesterol-containing membranes was investigated in a temperature range that covers the lipid pre- and main transition temperatures. Results are given in Fig. 5.

The partition coefficient in DMPC vesicles (Fig. 5a) increases from 550 ± 100 at 1°C to about 5400 at $t > 25$°C, which is above the lipid phase-transition temperature of DMPC ($t_t = 23$°C). A gradual increase was observed between the pre- and main transition temperature (14–23°C). Cholesterol reduces the k_p value both below and above the phase-transition temperature. The transition region characterized by the increased partition coefficient is drastically broadened.

The observed effects in DMPC vesicles are even more pronounced in DPPC bilayer vesicles (Fig. 5b). The partition coefficient increases to about 1000 at the pretransition temperature of the lipid ($t_p = 33$°C). A further increase to 3500 was observed in a narrow temperature range ($\Delta t = 1$ K) at $t = 41$°C which is the phase-transition temperature. Cholesterol reduces the partition coefficient to zero at $t < t_p$ (within the limits our determination). With increasing temperature, k_p increases smoothly between 30 and 45°C to 1200 at $t > t_t$. The steplike increase in k_p at the pretransition temperature observed in pure DPPC vesicles has diminished in the presence of cholesterol.

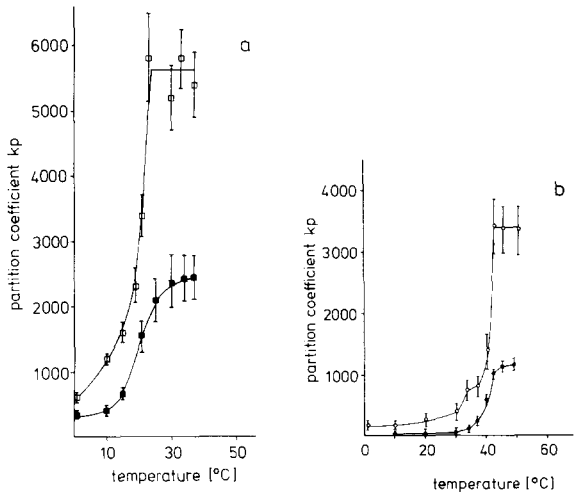

Fig. 5. (a) Temperature-dependence of the partition coefficients of chlorpromazine in DMPC vesicles (□) and DMPC vesicles containing 20 mol% cholesterol (■). (b) DPPC vesicles (○) and DPPC vesicles containing 20 mol% cholesterol (●). Vesicles were incubated and centrifuged at the corresponding temperature.

Biological membranes

We have determined the partition coefficient in erythrocyte ghosts to check the hypothesis of Singer et al. [10] who reported the complete extrusion of chlorpromazine from biological membranes. We found the value $k_p = 2700 \pm 300$, which is an average of five determinations. This value was calculated from radioactivity as well as absorption measurements. Both centrifugation and the hygroscopic desorption filtration technique were used to separate the cells. It should be pointed out that there is in our hand no difference in the partition coefficients determined by each method. The uncertainty of the determination should not vitiate the conclusion of the present study. A complete extrusion of amphipaths does not occur in biological membranes and the similarities of the partition coefficients in cholesterol-containing artificial bilayer vesicles and erythrocyte ghosts should result in a common mode of interaction.

Discussion

We investigated the membrane/buffer partition coefficients of chlorpromazine in artificial bilayer and in erythrocyte membranes. The hygroscopic desorption filtration technique and the conventional centrifugation method were applied to separate the vesicles or cells. Chlorpromazine in the pellet or supernatant was quantified by measuring the radioactivity of ^{3}H-labeled drug or by absorption measurements. Our primary result is that no essential difference exists in the partition of chlorpromazine in artificial and biological membranes. The partition coefficient, k_p, in erythrocyte membranes is 2700 ± 300, independent of the separation method. This value is very close to the k_p value observed in DMPC vesicles containing 10 mol% cholesterol. To conclude: in agreement with Roth and Seeman [2], Pjura et al. [13] and Bondy and Remien [14] and in contrast to Conrad and Singer [11], we present strong evidence that there is no difference in the partition of chlorpromazine in lipid vesicles and red-cell ghosts.

The similarities should result in a common mode of anesthetic action.

The main intention of the present study is to illuminate the biophysical properties of a membrane that controls the amphipath partition and to illuminate the physical behavior of the amphipath itself in aqueous solution.

The chlorpromazine concentration is an important aspect to which attention has to be paid. Our fluorescence study indicates the formation of chlorpromazine aggregates at concentrations above $5 \cdot 10^{-5}$ M. This concentration might be called a critical micelle concentration. The use of higher chlorpromazine concentrations leads to erroneously low partition coefficients (e.g., Fig. 1) and to considerable loss of lipid in the pelleted or filtered vesicles. This result must be construed as a comicellation of lipid and amphipath which finally leads to a complete membrane lysis at high chlorpromazine concentrations. Conrad and Singer [11] used $6 \cdot 10^{-5}$ M solutions of chlorpromazine, which is too high in the light of our results; Pjura et al. [13] used $1.3 \cdot 10^{-6}$ M solutions and yielded results in satisfactory agreement with ours. These results may explain some of the discrepancies between partition coefficients in comparable systems ranging from some hundreds to some thousands. However, further difficulties have to be taken into account. Recently we reported values of $k_p \approx 2000$ in DMPC membranes [16]. During the continuation of our earlier work we used a new batch of ^{3}H-labelled chlorpromazine and obtained higher partition coefficients. We investigated this effect and noticed that radioactive chlorpromazine solutions exposed to light or after storage even in the dark are subject to considerable degradation, which results in lower partition coefficient due to a low solubility of the degradation product. Consequently, we now used freshly prepared chlorpromazine solutions for each measurement and avoided excessive light exposure. We have to state that the main conclusion of our earlier paper, namely the reduction of k_p in cholesterol-containing membranes, is correct. The given interpretation holds, although the absolute values were too small. The relative change is confirmed now and is independent of the method of vesicle separation and of the method of concentration determination.

Amphipaths are known to perturb the structure of artificial and natural membranes [9,26,27]. The effect of various cationic amphiphilic drugs on the phase transition temperature of phosphatidylcholine vesicles has been reported before in various papers [28–31]. Drug-binding constants were determined by Rooney and Lee [32] in order to gain insight into the electrostatic interaction between membranes and charged drugs. Here we were interested in the concentration range over which chlorpromazine affects lipid order and the lipid phase transition. $2.8 \cdot 10^{-5}$ M was found to be a critical value. No effect was observed at $2.8 \cdot 10^{-5}$ M or less, whereas the lipid phase transition of artificial bilayer vesicles is drastically broadened at higher concentrations. It is important to note that only pelleted vesicles were used for ESR measurements. The lipid order is strongly reduced in the lipid phase transition region but not at low ($t < 15$°C) or high ($t > 25$°C) temperatures, even in the presence of $2.8 \cdot 10^{-4}$ M chlorpromazine in solution, which corresponds to 40 mmol/mol lipid in the fluid bilayer membrane.

These experiments show the high capacity of fluid membrane for the uptake of amphipaths and, moreover, the high sensitivity of transitional states or phase boundaries to derangements.

We now come to the physical parameters of lipid bilayers that determine the partition coefficients. Lipid chain length strongly affects amphipath solubility. The k_p values are reduced in long-chain lipids, which is easy to accept in the light of an increased chain-chain interaction that prevents amphipath incorporation. This also holds for the temperature dependence of k_p in the range of the lipid phase-transition temperature. Low partition coefficients were found in the quasicrystalline state below the phase transition temperature. Interestingly enough, we observed a step-like change in k_p at the pretransition temperature, t_p, in DPPC membranes. The temperature range between t_p and the main transition temperature, t_t, is known as the ripple structure or $P_{\beta'}$ phase [33]. This lipid phase is characterized by defect structures that can be regarded as fluid channels between rigidified domains of crystalline lipid [34]. Obviously, these defect structures already allow considerable uptake of drugs.

Cholesterol reduces the k_p values both below

t_p and above t_t. The pretransition diminishes. Cholesterol is known to increase the order of fluid membranes and to decrease the order to crystalline lipid phases [35]. Therefore, the decrease in k_p at $t > t_t$ is as expected. The somewhat unexpected decrease in k_p at $t < t_p$ may be explained by the assumption that possible vacancies which might incorporate chlorpromazine are already filled with cholesterol, thus inhibiting further substrate incorporation. In cholesterol DPPC membranes and below t_p the k_p values approach zero.

We conclude that partition coefficients are strongly influenced by the physical state of a membrane, which may be triggered by external parameters like temperature or by membrane composition. The phase-transition region is especially sensitive to changes in drug uptake. Under extreme conditions such as low temperatures and in the presence of cholesterol it is possible to inhibit drug incorporation. Such an exclusion of amphipaths has not been observed and can probably not be anticipated in biological membranes that exist in a fluid state preserved by a heterogeneous membrane composition.

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