The pH-Dependence in the Partitioning Behaviour of (*RS*)-[^{3}H]Propranolol Between MDCK Cell Lipid Vesicles and Buffer

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Purpose. The pH-dependent partitioning of (*RS*)-[^{3}H]propranolol between unilamellar vesicles of MDCK cell lipids and buffer was determined.
Methods. Partitioning studies were performed by means of equilibrium dialysis at 37°C between pH 7 and 11 at a molar propranolol/lipid ratio in the membrane of 10^{-6}.
Results. The partition-pH diagram was bell-shaped. The highest apparent partition coefficient was 1797 at pH 9.7, the lowest was 805 at pH 6.9. Curve fitting with a combination of Henderson-Hasselbalch equations revealed an inflection point at the apparent pK_a of propranolol, i.e. 9.7, and two additional pK_a values at pH 7.7 and 10.0. The first one corresponds to the pK_a of free fatty acids (FFA) within lipid bilayers and the other one to the pK_a of phosphatidylethanolamine (PhE). The true partition coefficients (P) of the neutral as well as the ionised solute were fitted for each ionisation status of the membrane. The highest P, i.e. 2123, was calculated for neutral propranolol in the membrane with deprotonated FFA and protonated PhE.
Conclusions. The partitioning behaviour of (*RS*)-[^{3}H]propranolol in a complex membrane/buffer system can be described when considering ionisation changes of drug and lipids.

KEY WORDS: partition coefficient; liposome; MDCK cell lipids; propranolol; lipophilicity; drug-lipid membrane interactions.

INTRODUCTION

Among the studies on drug partitioning in liposome/buffer systems only few concentrate on the pH-dependence (1–3). Recently, Pauletti and Wunderli-Allenspach (4) established a standardised phosphatidylcholine (PhC) liposome/buffer system to quantitatively study the pH-dependent partitioning behaviour of drugs. It permits to determine the apparent partition coefficient (D) as a function of the pH over a wide pH range. (*RS*)-[^{3}H]propranolol was used as a model compound. These partitioning studies are based on equilibrium dialysis which allows the determination of the concentration in both phases without disturbance of the equilibrium. With radioactively labelled propranolol concentrations as low as 10^{-8} M could be achieved, resulting in concentration-independent D. As aqueous phase a universal buffer solution with physiological osmolality and constant ionic strength was used. While pH values much higher or lower (≥ 2 pH units) than the pK_a of propranolol reveal the true partition coefficient (P) of the neutral or protonated drug, both molecular species, i.e. the neutral as well as the protonated, contribute to D at pH values around the pK_a. Beside the drug also the lipid membrane undergoes pH-dependent changes. Indeed, two inflections in the D-pH diagram were found with the PhC liposome/buffer system, one at the pK_a of propranolol, i.e. 9.25, and the other one at the pK_a of PhC, i.e. 1.97.

This ideal partitioning behaviour encouraged us to examine the influence of a more complex lipid mixture on the partitioning behaviour of (*RS*)-[^{3}H]propranolol. Lipids from kidney epithelial (MDCK) cells were used to produce liposomes, called MDCKsomes. For the analysis of partitioning data membranes containing ionisable lipids are handled as consisting of several membrane species, depending on the lipid charges. Each membrane species is regarded as a separate partitioning system for the neutral as well as for the ionised drug. D at one particular pH represents the overall concentration ratio of the solute species in all membrane species. It is calculated according to the ionisation states of the solute and the lipids. This approach is successfully used to analyse the partitioning data obtained for (*RS*)-[^{3}H]propranolol in the MDCKsome/buffer system.

MATERIALS AND METHODS

Chemicals

(*RS*)-Propranolol HCl #P-0884 was from Sigma. From Merck: sodium cholate #12448 and methanol (HPLC grade) for lipid extraction and HPLC. All other solvents were from Romil (Shepshed, UK), HPLC quality. Radioactively labelled compounds were either from NEN/Du Pont as [Methyl-^{14}C]-choline, 2 GBq/mmol #NEC 141 and [2,4-^{3}H(N)]-cholic acid, 0.48 TBq/mmol #NET 382, or from Amersham Int.: (S)-3-phosphatidyl[N-methyl-^{14}C]choline-1,2-dipalmitoyl (^{14}C-DPPhC), 2.15 GBq/mmol #CFA 630; (S)-3-phosphatidyl[N-*methyl*-^{3}H]choline-1,2-dipalmitoyl (^{3}H-DPPhC), 3.00 TBq/mmol #TRK 673; [Carboxyl-^{14}C]cholic acid sodium salt, 1.99 GBq/mmol #CFA 482 and (*RS*)-[4-^{3}H]propranolol hydrochloride, 533 GBq/mmol #TRK 495. Reference lipids were from Lipid products (Nutfield, UK), Siegfried (Switzerland), or Sigma. All other chemicals were of analytical grade.

MDCK Cells

MDCK cells, a gift from M. Paccaud, Institut d'Hygiène, Geneva, Switzerland, were cultured as monolayers in MEM #12-104-54 containing 5 % foetal calf serum (Flow Laboratories). Cells grown for 3–4 days were scraped. Lipids were extracted with chloroform/methanol, washed (5) and dried in a nitrogen stream.

Separation and Quantification of Lipids

Lipids were analysed by HPLC (Varian). For lipid separation (6) we used a Spherisorb S3W Si-60 3μm, 4.6 × 150 mm column from PhaseSep (Norwalk, USA). Lipids were detected with a mass detector 950/14 (ACS, UK) with a sensitivity of 1 μg. Free fatty acids (FFA) were determined by thin layer chromatography (TLC), the total lipid concentration in partitioning experiments by phospholipid quantification according to Stewart (7). Standard solutions were measured gravidimetrically.

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Liquid Scintillation Counting (LSC)

Dual label LSC of ^3H and ^{14}C was performed with a Beckman LS6800.

Liposomes

MDCKsomes (10 mg lipid/ml) were prepared by the detergent dialysis method (4) with a lipid/sodium cholate ratio of 1 (w/w). Dialysis was against 10 mM phosphate buffered saline (PBS), pH 7.4. The residual detergent, determined with ^{14}C-labelled cholate and ^3H-DPPhC, corresponded to a molar lipid/detergent ratio of 360. Liposome size and zetapotential ζ were analysed by dynamic light scattering (DLS) and microelectrophoresis using a ZetaSizer 3 (Malvern Instruments, UK). The size intensity distribution was calculated according to the cumulants (monomodal) method, which results in the z average mean value and the polydispersity factor. ζ was determined at 37°C after incubation for 5 h.

Equilibrium Dialysis for Partitioning

Partitioning experiments were performed by means of equilibrium dialysis (Macro 1 cells, 1.0 ml; cellulose membrane, M_r cut-off 10,000, #10.16, Dianorm, Germany) at 37°C during 5 h at which time equilibrium is reached at both pH 7 and 11. We used a universal buffer solution containing phosphate, citrate and borate, adjusted to 230 mmol/kg ionic strength with NaCl. The osmolality was about 300 mmol/kg (4). Change of buffers in the liposome suspension was achieved by dialysis. Equilibration of protons through the lipid membrane takes less than 5 min (4). At the start the liposome suspension contained 500 Bq/ml ^3H-labelled (10^{-9} M) and non labelled (*RS*)-propranolol, as indicated. The lipid concentration was 2 mg/ml (100 Bq ^{14}C-DPPhC per mg lipid). ^3H- and ^{14}C-activities from both chambers were determined by LSC (95–100% recoveries).

Calculation of D

D is defined as the ratio of the solute concentrations between the lipophilic phase, e.g. the lipid membranes, and the hydrophilic phase at equilibrium:

$$D = \frac{C_L}{C_B} \qquad (1)$$

C_L, molar solute concentration in the lipid membrane; C_B, molar solute concentration in the buffer solution.

As C_L cannot be measured directly, D is calculated (4):

$$D = \frac{C_{LB} - C_B}{C_B} \cdot \frac{V_{LB}}{V_L} + 1 \qquad (2)$$

C_{LB}, drug concentration in the liposomes containing chamber; V_{LB}, sample volume of the liposome suspension; V_L, volume of the lipophilic phase (calculated with a density of 1 g/ml) within V_{LB}.

The molar ratio r of propranolol to lipid in the lipid membrane was calculated as follows:

$$r = \frac{C_{LB} - C_B}{C_l} \qquad (3)$$

C_l, molar lipid concentration within the sample

Data Analysis

Curve fitting of D as a function of pH was according to eqs. 5 and 6 (see Results) with proFit 4.1 (QuantumSoft, CH) using the Levenberg-Marquardt algorithm. The parameters with the lowest chi^2 values were chosen at a confidence interval of 90%.

Apparent pK_a of Propranolol Within the MDCKsome Surface Buffer Layer

The pH of the stationary buffer layer above a charged membrane differs from the bulk pH. The apparent pK_a is calculated as follows:

$$pK_a(\text{apparent}) = pK_a(\text{intrinsic}) - \frac{F \cdot \zeta}{2.303 \cdot R \cdot T} \qquad (4)$$

F, Faraday constant; R, gas constant; ζ, zetapotential, i.e. electrical potential at the shear plane of the vesicle. We assume that the potential stays constant over the whole stationary buffer layer, based on the assumption that the surface potential is similar to the ζ under these conditions (8). The apparent pK_a of propranolol (37°C) within the MDCKsome surface buffer layer equals 9.7, as calculated from the intrinsic pK_a, i.e. 9.24 (4), and from ζ at pH 9.5, i.e. −30 mV.

RESULTS

Characterisation of MDCKsomes

The compositions of MDCK cell lipid extracts and MDCKsomes were studied by HPLC. No significant difference was found between the two. A representative chromatogram of a MDCK cell lipid extract is shown in Fig. 1. TLC analysis of the MDCKsome lipids showed about 10% of total lipids as FFA.

The size distribution of the MDCKsomes was characterised 24 h after preparation (Material and Methods): the z average mean value was 187 ± 6 nm (n = 5), the polydispersity factor

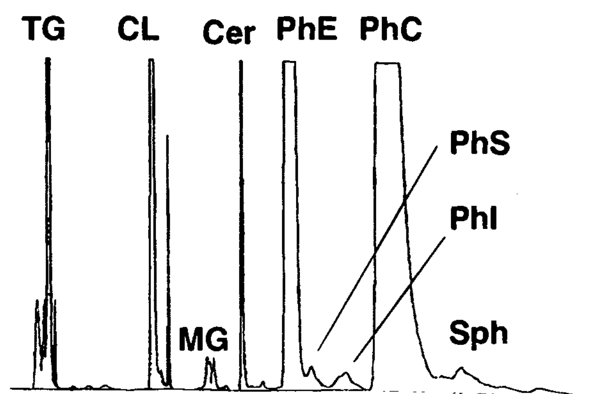

Fig. 1. HPLC chromatogram of a MDCK cell lipid extract. The chloroform/methanol 2/1 (v/v) extract from MDCK cells (n = 3; 0.1 mg lipids per extract) was separated by HPLC and detected with a mass (light scattering) detector (Material and Methods). TG, triglycerides (5.5 ± 1.2% w/w); CL, cholesterol (14.9 ± 2.2% w/w); MG, monoglycerides; Cer, cerebrosides (1.3 ± 1.5% w/w); PhE, phosphatidylethanolamine (23.6 ± 1.9% w/w); PhS, phosphatidylserine; PhI, phosphatidylinositol; PhC, phosphatidylcholine (35.0 ± 3.1% w/w); Sph, sphingomyeline.

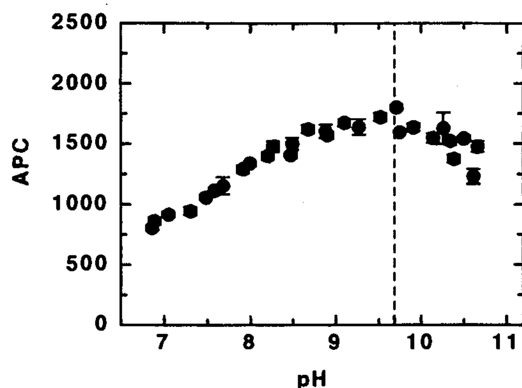

Fig. 2. Partitioning behaviour of (*RS*)-[^{3}H]propranolol in the MDCKsome/SUBS system. The apparent partition coefficients (D) were studied by means of equilibrium dialysis at 37°C at pH values between 7 and 11. Data are from 3 sets of independent experiments. The apparent pK_a of propranolol at 37°C equals 9.7 (----).

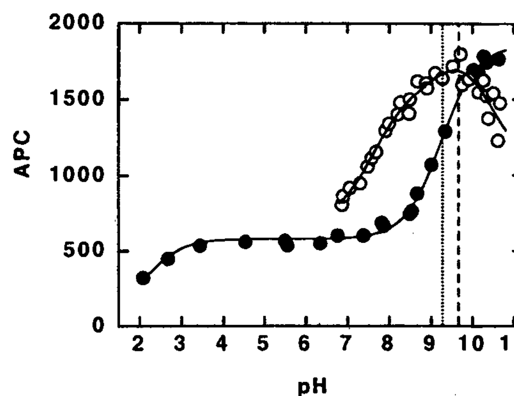

Fig. 3. Data analysis of partitioning behaviour of (*RS*)-[^{3}H]propranolol in liposome/SUBS systems. Lipophilic phase: (○) MDCKsomes; (●) PhC-liposomes (data are from Pauletti and Wunderli-Allenspach (4)). Curve fitting was performed using the equations described in the text. (——) best fit, i.e. lowest chi^2; (----) apparent pK_a of propranolol, i.e. 9.7; (····) intrinsic pK_a of propranolol, i.e. 9.24 (4). The resulting fit parameters are listed in Tab. I.

0.077 ± 0.018. Electron micro graphs of freeze fractured liposome suspensions in SUBS of pH 7.0, 7.6, 9.6 and 11.2 revealed unilamellar vesicles (not shown). The stability of liposomes was tested by DLS after 5 h of equilibrium dialysis at 37°C. As indicated by constant z average values and polydispersity factors liposomes were stable at pH ≥ 7. Below pH 7 the z average mean and polydispersity factor increased during equilibrium dialysis, and below pH 4 the MDCKsomes aggregated visibly. Above pH 11 ^{14}C-activity, i.e. a ^{14}C-PhC hydrolysis product, was found in the buffer phase after partitioning experiments.

pH-Dependent Partitioning

The partitioning behaviour of (*RS*)-[^{3}H]propranolol (10^{-9} M) in the MDCKsome/SUBS system was examined between pH 7 and 11 (Material and Methods). pH values were determined at 37°C immediately after dialysis. D was calculated according to eq. 2. The resulting D-pH diagram is bell-shaped (Fig. 2). D is highest, i.e. about 1800, at pH 9.7. From there it decreases to about 800 at pH 7 and 1400 at pH 11.

In order to describe the D-pH diagram quantitatively we used a combination of Henderson-Hasselbalch equations. For each membrane species a P for the neutral (P_n) and the ionised propranolol (P_i) is defined. The resulting D corresponds to the sum of all P after their correction for the ionisation state of the drug and the membrane lipids. Best fits were obtained with the following equation:

$$D = \frac{1}{1 + 10^{pK_a - pH}} \left[\frac{P_n[pH_{m1}/pH_{m2}]}{(1 + 10^{pH_{m1} - pH})(1 + 10^{pH - pH_{m2}})} + \frac{P_n[<pH_{m1}]}{1 + 10^{pH - pH_{m1}}} + \frac{P_n[>pH_{m2}]}{1 + 10^{pH_{m2} - pH}} \right] + \frac{1}{1 + 10^{pH - pK_a}} \left[\frac{P_i[pH_{m1}/pH_{m2}]}{(1 + 10^{pH_{m1} - pH})(1 + 10^{pH - pH_{m2}})} + \frac{P_i[<pH_{m1}]}{1 + 10^{pH - pH_{m1}}} + \frac{P_i[>pH_{m2}]}{1 + 10^{pH_{m2} - pH}} \right] \quad (5)$$

pH_{m1} and pH_{m2}, fitted interfacial pK_a values of membrane lipids. The term in brackets behind P_n or P_i defines the respective membrane species. To fit the data the pK_a, i.e. the calculated apparent pK_a of (*RS*)-propranolol (37°C) in the MDCKsome surface buffer layer, was kept fixed at 9.7 (Materials and Methods). The fitted curve is illustrated in Fig. 3. As indicated in Table I, two additional points of inflection can be determined, one around pH 7.7, the other one around pH 10.0, which is indicative for two distinct changes in the ionisation status of the membrane. Due to the stability of the MDCKsomes only a limited pH range can be covered with the partitioning experiments. Furthermore the pK_a values of solute and lipids are within a narrow range of 2.3 pH units. Therefore the fitted P_n and P_i values show large uncertainties at a confidence interval of 90%.

Table I. Fit Parameters of the pH-Dependent Partitioning of (RS)-[^{3}H]Propranolol in the MDCKsome/SUBS and the PhC-Liposome/SUBS Systems

	Membrane derived inflection points		P at Various Membrane States				
			Above pH_{m1} below pH_{m2}		Below pH_{m1}	Above pH_{m2}	
Liposomes	pH_{m1}	pH_{m2}	P_n	P_i	P_i	P_n	P_i
MDCK	7.66 +/− 0.31	9.97 +/− 0.91	2123 [a]	1568 [a]	710 [a]	1193 [a]	1609 [a]
PhC	2.46 +/− 0.54	—	1858 +/− 24	580 +/− 17	216 +/− 181	—	—

Note: The experimental data from partitioning studies were fitted as described in Fig. 3. The apparent pK_a of propranolol was kept fixed at 9.7 (MDCKsomes) or 9.24 (PhC-liposomes) during the fit procedure (see Results). $P_n[<pH_{m1}]$ is not relevant for the curve description.

[a] high uncertainties due to a high degree of superposition in a limited pH range (confidence limits 90%).

For the PhC-liposome/buffer system (4) data analysis can be performed with the same approach. In this case eq. 5 can be reduced to eq. 6, which takes into account that PhC liposomes have only one membrane pK_a and omits P_n below pH_m, where the contribution of the neutral propranolol can be neglected:

$$D = \frac{1}{1 + 10^{pka-pH}} \cdot \frac{P_n[>pH_m]}{1 + 10^{pH_m-pH}} + \frac{1}{1 + 10^{pH-pK_a}} \cdot \left[\frac{P_i[>pH_m]}{1 + 10^{pH_m-pH}} + \frac{P_i[<pH_m]}{1 + 10^{pH-pH_m}}\right] \quad (6)$$

In this case, based on $\zeta = 0$ mV, the apparent pK_a of propranolol is set equal to the intrinsic pK_a, i.e. 9.24 (37°). The fitted curve for the D-pH diagram of (*RS*)-[^{3}H]propranolol in the PhC-liposome/buffer system is also shown in Fig. 3. Fit parameters are summarised in Tab. I. The apparent pK_a of PhC in the PhC-liposome/buffer system (4) was fitted as 2.5. As the ionisation curves of solute and lipid do not overlap, $P_n[>pH_m]$ and $P_i[>pH_m]$ values can be determined with low uncertainties at 90% confidence limit.

Concentration-Dependent Partitioning

To study the pH-dependent partitioning behaviour as a function of the solute concentration, partitioning experiments were performed over a large solute concentration range. (*RS*)-[^{3}H]propranolol was kept constant at 10^{-9} M, additional unlabelled (*RS*)-propranolol was added up to 10^{-4} M. D at pH 7 as well as at pH 11 is constant, i.e. concentration-independent, up to a molar propranolol/lipid ratio of 0.01 in the membrane (Fig. 4).

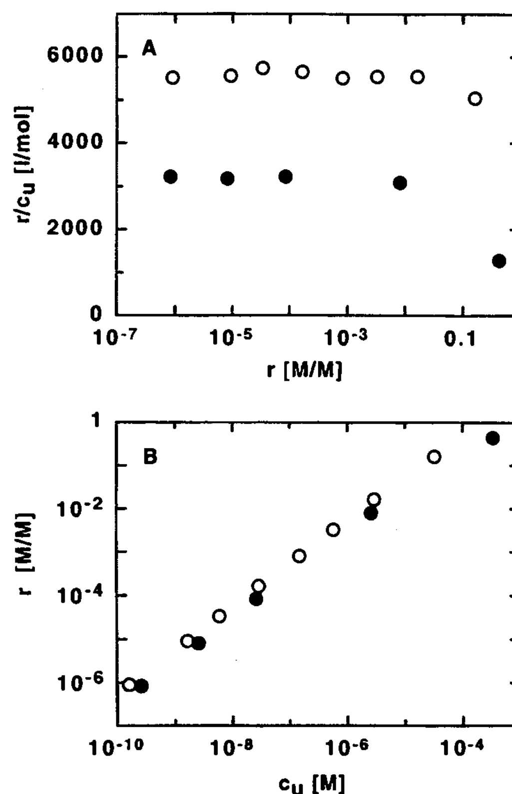

Fig. 4. Concentration-dependent association of (*RS*)-propranolol to MDCKsomes. The concentration ratios of (*RS*)-propranolol between MDCKsomes and SUBS were determined by means of equilibrium dialysis at 37°C. (A) Semilogarithmic Scatchard plot of the propranolol-lipid association. (B) Langmuir adsorption isotherm. r, molar ratio in the membrane of propranolol to lipids; c_u, molar propranolol concentration in buffer. (●) pH 7.2; (○) pH 11.2.

DISCUSSION

With the use of membranes as partitioning systems it has been recognised that also charged molecules can associate with the lipophilic phase (1–4). The concept which has been developed and used with octanol has to be revised accordingly. This paper demonstrates that complex D-pH diagrams can be analysed on the assumption that neutral and ionised molecules each have their distinct partitioning pattern in every single ionisation status of the membrane. The D-pH diagrams of (*RS*)-[^{3}H]propranolol in the MDCKsome and the PhC-liposome/SUBS systems can be described quantitatively according to the Henderson-Hasselbalch equation using the apparent pK_a of propranolol and one or two additional membrane-derived pK_a. The ionisation change of PhC leads to the point of inflection around pH 2 in the PhC-liposome/SUBS system (4). The primary amine group of PhE appears as the inflection point at pH 10.0 in the MDCKsome system (manuscript in preparation). PhE accounts for 24% (w/w) of the total lipid in the MDCKsomes. Tsui et al. (9) reported an apparent pK_a (50 mmol/l ionic strength) of 10.6 in PhC/PhE-liposomes (70/30 mol/mol). The intrinsic interfacial pK_a was calculated as 9.7. In the MDCKsomes it is 9.5 assuming that the surface potential equals ζ (37°C, −32 mV at pH 10.0; eq. 4). This correspondence supports our hypothesis that pH-dependent changes in D are caused by lipid protonation/deprotonation in the membrane together with the ionisation changes of the solute. The change in D around pH 7.7 in the MDCKsome/SUBS system is explained by the presence of about 10% FFA in the lipid membrane. The respective intrinsic interfacial pK_a is 7.4, calculated with the ζ of −20 mV at pH 7.7. This inflection is also found with PhC/FFA-liposomes (95/5, w/w) (manuscript in preparation). The interfacial pK_a of medium- and long-chain (C≥10) fatty acids within lipid membranes indeed lies between pH 6 and 8 (10).

Since MDCKsomes contain less than 0.1% phosphatidic acid (pK_a 8.5) it is unlikely, to be responsible for the inflection at pH 7.7. Cholic acid (pK_a 6.4) can be excluded as well, since freeze-thaw-filter liposomes, which contain no residual cholate, showed the same results (data not shown).

Another explanation for D changes could be a pH-dependent asymmetry in the lipid membrane as shown by NMR studies on mixed phospholipid bilayers (11). pH-Dependent changes in lipid distribution could possibly mimic a lipid pK_a in the D-pH diagram.

The fitted P_n of 2123 in the MDCKsome system between pH 7.7 and 10.0 is in the range of the fitted P_n for the PhC-liposome system, i.e. 1858 (4). In PhC liposomes D decreases as soon as the propranolol is partly protonated, i.e. pH < 10. In the MDCKsomes a membrane ionisation state exists between pH 7.7 and 10.0, where the protonated propranolol has a P_i which is not much lower than the P_n, i.e. 1568. Negatively charged lipids, i.e. FFA, seem to attract the protonated propranolol more than the PhC does. With pH values lower than 7.7, i.e. neutralised negative

charges of the FFA, the protonated propranolol has a P_i of 710, very similar to the P_i in PhC liposomes, i.e. 580. At pH 7.4 D in the MDCKsome/buffer system is 1036 whereas it is 598 in the PhC liposome/buffer system. Above the pK_a of 10.0 P_n decreases to 1193 in the MDCKsomes. This decrease can also be seen in other systems containing lipids with a primary amine group (manuscript in preparation).

As we used the enantiomer for experiments, we cannot distinguish between *R*- and *S*-propranolol. Partitioning studies with *R*-oxprenolol and *S*-oxprenolol in a liposome/buffer system, though at higher solute concentration, revealed no difference between the two enantiomers (12).

Our experiments showed, that the propranolol to lipid ratio in the membrane has to be lower than or equal to 10^{-2}. This was also postulated by Flewelling and Hubbell (13) and can be derived from the comparison of the drug-lipid association mass balance and the partition coefficient (14).

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