Free Fatty Acids Cause pH-Dependent Changes in Drug-Lipid Membrane Interactions Around Physiological pH

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Received December 20, 1996; accepted March 6, 1997

Purpose. The influence of oleic acid (OA) and phosphatidylethanolamine (PhE) as membrane constituents on the partition behavior of (*RS*)-[^{3}H]propranolol between unilamellar liposomes and buffer was studied as a function of pH.

Methods. Partition studies were performed by means of equilibrium dialysis at 37°C over a broad pH range at a molar propranolol to lipid ratio in the membrane of 10^{-6}.

Results. As compared to the standard phosphatidylcholine (PhC)-liposome/buffer partition system PhE and OA have an enhancing effect on the apparent partition coefficient (D) of (*RS*)-[^{3}H]propranolol between pH 6 and 11. Data analysis with Henderson-Hasselbalch equations revealed that the neutral propranolol has a higher affinity to membranes containing net neutral charged PhE than to pure PhC-liposomes. Net negatively charged PhE seems to have no significant influence on the partitioning. Deprotonated OA caused an increase in the true partition coefficient (P) of the protonated propranolol. Neutral OA showed no influence on the partitioning. From the fit D vs pH curves and from zeta potential measurements of the liposomes the intrinsic pK_a values of the membranous lipids were calculated as 7.5 to 7.8 for OA and 9.7 to 9.8 for PhE.

Conclusions. Since the pK_a of membranous OA is close to the physiological pH and D depends on the ionisation state of OA, small pH changes around the physiological pH may cause large differences in drug-lipid membrane interactions.

KEY WORDS: partitioning; liposome; propranolol; drug-lipid membrane interactions; free fatty acid; phosphatidylethanolamine.

INTRODUCTION

The affinity of a drug to the lipid membranes in the body determines its pharmacokinetic behavior, particularly its distribution in the organism. To quantify this affinity, a parameter to express the lipophilicity of a compound has been introduced, the partition coefficient. As early as 1891 Nernst formulated the conditions to define a true partition coefficient (P), which is the concentration ratio of one molecular species between a lipophilic phase and a hydrophilic phase at equilibrium (1). If Nernst conditions are not fulfilled, e.g. if a solute exists in more than one ionisation status, only an apparent partition coefficient (D) can be determined.

In the past large data bases have been established with lipophilic solvent/buffer systems (2). To provide a better model for the partitioning in vivo, membranes have been chosen as lipophilic phases (3–5). A standardised liposomal partition system using phosphatidylcholine (PhC) liposomes has been introduced (6), which permits to study partition diagrams (D = f(pH)) over the whole pH range under Nernst conditions. Partition studies with (*RS*)-[^{3}H]propranolol revealed that the D-pH diagram follows the ionization curves of the model drug propranolol and of PhC. These findings were confirmed with other drugs (7). In liposomal partition systems—contrary to the situation in conventional solvent partition systems, where only the partitioning of the neutral species plays a role,—the partitioning of the ionised compound is also important. P can thus be determined for the neutral (P_n) and the ionised (P_i) species (6,8).

In an effort to come closer to the in vivo situation, membranes consisting of a complex lipid mixture were used as lipophilic phase (8). Liposomes from lipids of Madin Darby canine kidney (MDCK) cells were produced, the so called MDCKsomes. With (*RS*)-[^{3}H]propranolol as a model solute an ideal partition behavior was found also in this case. The bell-shaped D-pH diagram could be described with a combination of Henderson-Hasselbalch equations. Inflections were found at the apparent pK_a of propranolol, i.e. pH 9.7, and at pH 7.7 and a 10.0. The latter two were tentatively assigned to the apparent pK_a of free fatty acids (FFA) and of phosphatidylethanolamine (PhE).

To analyse the contribution of the single lipids to the partition patterns liposomes were produced consisting of two or three lipid components. Based on the data from MDCKsomes, combinations of PhC together with PhE and/or oleic acid (OA) were chosen to possibly mimic the partition characteristics of the complex membrane. The surface charge of the liposomes at various pH values was determined as zetapotentials (ζ) and therefrom the apparent pK_a values of propranolol in the various liposomal systems were calculated as previously described (8). With all liposomes tested the D-pH diagrams for (*RS*)-[^{3}H]propranolol can be described with a combination of Henderson-Hasselbalch equations using the apparent pK_a of propranolol and the apparent pK_a values of the lipids.

MATERIALS AND METHODS

Chemicals

(*RS*)-Proparanolol HCl #P-0884 was from Sigma. From Merck: sodium cholate #12448 and methanol (HPLC grade). All other solvents were from Romil (Shepshed, UK), HPLC quality. Radioactively labelled compounds were either from NEN/Du Pont as [2,4-^{3}H(N)]-cholic acid, 0.48 TBq/mmol #NET 382 and [1-^{14}C]-oleic acid (^{14}C-OA), 1.9 GBq/mmol # NEC 317 or from Amersham Int.: (S)-3-phosphatidyl[N-methyl-^{14}C]choline-1,2-dipalmitoyl (^{14}C-DPPhC), 2.15 GBq/mmol #CFA 630 and (*RS*)-[4-^{3}H]propranolol hydrochloride, 533 GBq/mmol #TRK 495. Egg phosphatidylcholine (PhC)

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ABBREVIATIONS: OA, oleic acid; PhE, phosphatidylethanolamine; PhC, phosphatidylcholine; D, apparent partition coefficient; P, true partition coefficient; Pn, true partition coefficient of neutral solute; Pi, true partition coefficient of ionised solute; apparent pK_a, bulk pH, at which the pH in the stationary buffer layer above the membrane equals the intrinsic pK_a; intrinsic pK_a, pK_a as determined by titration or as estimated from apparent pK_a; pHm, membrane derived inflection point in D-pH diagrams, i.e. apparent pK_a of membranous lipids; ζ, zetapotential; FFA, free fatty acids; SUBS, standardised universal buffer solution; pd, polydispersity factor in dynamic light scattering.

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and phosphatidylethanolamine (PhE), grade I, were from Lipid products (Nutfield, UK); oleic acid # 75090 was from Fluka (Switzerland). All other chemicals were of analytical grade.

Liposomes

Liposomes (10 mg lipid/ml) with various lipid compositions were prepared by either of the following three methods: detergent dialysis (DD-liposomes) (6), freeze-thaw-filter technique (FTF-liposomes) (9), or extrusion through 0.1 μm polycarbonate membranes after 5 freeze-thaw cycles (Ex-liposomes) (10). MDCKsomes contain 35% (w/w) PhC, 24% PhE, 15% cholesterol, 5.5% triglycerides, 1.3% cerebrosides and traces of monoglycerides, phosphatidylserine, -inositol, and sphingomyeline (8). In addition we found 3% FFA (unpublished results). Residual detergent in DD-liposomes was determined with ^{3}H-labelled cholate and ^{14}C-DPPhC. Dual label LSC of ^{3}H and ^{14}C was performed with a Beckman LS6800. Liposome size distribution and ζ were analysed by dynamic light scattering (DLS) and micro electrophoresis using a ZetaSizer 3 (Malvern Instruments, UK) as described previously (8). pH Values were adjusted by dilution of the liposome preparations in standardized universal buffer solution (SUBS) containing phosphate, citrate, borate, chloride and sodium with an osmolality of around 300 mmol/kg and an ionic strength of 230 mmol/kg (6, 11). Equilibration of protons through the lipid membrane takes less than 5 min (6).

Partition Experiments

Partition experiments were performed by means of equilibrium dialysis at 37° C during 5 h in SUBS as described previously (6). At the start the liposome suspension (2 mg/ml) contained 500 Bq/ml ^{3}H-labelled (10^{-9} M) (*RS*)-propranolol or ^{14}C-OA ($3 \cdot 10^{-7}$ M), which was added 24 h prior to equilibrium dialysis. Radioactivity from both chambers was determined by LSC (95–100% recoveries). The exact lipid concentration in partition experiments was determined by enzymatic choline quantification (12).

Calculations and Data Analysis

Calculations were performed as previously described (6, 8). In brief the following equations were used:

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

C_{LB}, molar solute concentration in the liposomes containing chamber; C_B, molar solute concentration in the buffer solution; 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}.

Due to membrane charges the pH in the stationary buffer layer above the membrane differs from the bulk pH. If a particular pH is assigned to any process at the membrane surface, the bulk pH has to be corrected in order to estimate the pH within the stationary buffer layer above the membrane, i.e. at the site of action (eq. 2). ΔpH is therefore used to calculate the apparent pK_a, i.e. the bulk pH at which the pH in the stationary buffer layer (pH(above membrane)) equals the intrinsic pK_a of the solute and vice versa for the lipids:

$$\Delta pH = pH(bulk) - pH(above\ membrane) = -\frac{F \cdot \zeta}{2.303 \cdot R \cdot T} \qquad (2)$$

F, Faraday constant; R, gas constant; ζ, zetapotential, i.e. electrical potential at the shear plane of the vesicle. ζ of the various liposomes at pH 7.8, 9.5 and 10.0, which are used for the calculation of the apparent pK_a of propranolol and the intrinsic pK_a of membranous lipids are listed in Tab. 1.

Curve fitting of D as a function of pH was done 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%. The equations were adapted to the various liposome/buffer systems as follows:

PhC-liposomes (pH 2 to 11) and PhC/OA-liposomes (pH 4 to 11):

$$D = \frac{1}{1 + 10^{pK_a - pH}} \cdot \frac{P_n[>pH_{m1}]}{1 + 10^{pH_m - pH}} + \frac{1}{1 + 10^{pH - pK_a}} \cdot \left[\frac{P_i[>pH_{m1}]}{1 + 10^{pH_m - pH}} + \frac{P_i[<pH_{m1}]}{1 + 10^{pH - pH_m}} \right] \qquad (3)$$

MDCKsomes and PhC/PhE/OA-liposomes (pH 5 to 11):

$$D = \frac{1}{1 + 10^{pK_a - pH}} \cdot \left[\frac{P_n[pH_{m1}/pH_{m2}]}{(1 + 10^{pH_{m1} - pH})(1 + 10^{pH - pH_{m2}})} + \frac{P_n[<pH_{m1}]}{1 + 10^{pH - pH_{m1}}} + \frac{P_n[>pH_{m2}]}{1 + 10^{pH_{m2} - pH}} \right] + \frac{1}{1 + 10^{pH - pK_a}} \cdot \left[\frac{P_i[pH_{m1}/pH_{m2}]}{(1 + 10^{pH_{m1} - pH})(1 + 10^{pH - pH_{m2}})} + \frac{P_i[<pH_{m1}]}{1 + 10^{pH - pH_{m1}}} + \frac{P_i[>pH_{m2}]}{1 + 10^{pH_{m2} - pH}} \right] \qquad (4)$$

PhC/PhE-liposomes (pH 6 to 11):

$$D = \frac{1}{1 + 10^{pK_a - pH}} \cdot \left[\frac{P_n[>pH_m]}{1 + 10^{pH_m - pH}} + \frac{P_n[<pH_m]}{1 + 10^{pH - pH_m}} \right] + \frac{1}{1 + 10^{pH - pK_a}} \cdot \frac{P_i[<pH_m]}{1 + 10^{pH - pH_m}} \qquad (5)$$

pK_a, pK_a of propranolol, in eq. 3 to 5 the apparent pK_a (see above) is used, as calculated from the intrinsic pK_a (37°C) 9.24 (6) and ΔpH from Table I. $P_n[>pH_m]$, P_n above pH_m; $P_n[<pH_m]$, P_n below pH_m; $P_n[pH_{m1}/pH_{m2}]$, P_n above pH_{m1}/below pH_{m2}·pH_m, fit membrane derived inflection point in partition curve, i.e. apparent pK_a of membranous lipids, pH_{m1} and pH_{m2}, inflection points around pH 2 or 8, respectively, and 10.

For the fit procedure pK_a was kept fixed while the P_n, P_i and pH_m values were calculated from the partition data.

RESULTS

Characterisation of Liposomes

Liposomes were prepared and characterised with regard to their size distribution and, in the case of DD-liposomes,

Table I. Zetapotentials and Δ pH Values of Various Liposomes

Liposomes	pH 7.8[a]		pH 9.5[a]		pH 10[a]	
	ζ[mV]	Δ pH	ζ[mV]	Δ pH	ζ[mV]	Δ pH
FTF-PhC/PhE (67/33 mol/mol)	−3	0.05	−10	0.16	−12	0.19
Ex-PhC/OA (88/12) and (76/24)	−12	0.19	−17	0.27	−18	0.29
Ex-PhC/PhE/OA (55/22/23)	−18	0.29	−21	0.34	−22	0.35
DD-MDCKsomes[b]	−13	0.21	−30	0.48	−33	0.53
DD-PhC	0	0	0	0	0	0

[a] pH values chosen according to the respective pK_a values of membranous OA, propranolol and PhE.
[b] Composition of MDCKsomes: 35% (w/w) PhC, 24% PhE, 15% cholesterol, 5.5% triglycerides, 3% FFA, 1.3% cerebrosides and traces of monoglycerides, phosphatidylserine, -inositol, and sphingomyeline (Material and Methods). ζ of various liposomes in SUBS were measured on a ZetaSizer 3 at 37° C. From these ΔpH, i.e. the difference between the bulk pH and the pH of the stationary buffer layer above the membrane were calculated according to eq. 2 and used for pK_a correction (see Tables II and III).

residual detergent contents. Ex-liposomes containing PhC, PhE and/or OA were all between 70 and 90 nm with polydispersity factors (pd) lower than 0.1. Detergent dialysis- (DD-) PhC/PhE-liposomes were around 50 nm, DD-PhC liposomes around 70 nm (both pd < 0.1). DD-MDCKsomes were larger (~200 nm, pd < 0.1) and FTF-liposomes ~300 nm (pd ≈ 0.3). As judged by the polydispersity factors homogeneous populations were obtained by the extrusion as well as the detergent dialysis method. All methods produced unilamellar vesicles as revealed by electron microscopy (not shown). Residual detergent of DD-PhC liposomes was 1 cholate per 700 PhC molecules (6), in DD-MDCKsomes 1 per 360 (8). To control the stability of the liposomes during partition experiments, size distribution was examined before and after equilibrium dialysis. No significant changes were found within the pH ranges used.

OA-containing liposomes were checked for a pH-dependent loss of OA by partition experiments at pH values between 4 and 11 using ^{14}C-OA in Ex-PhC/OA-liposomes (76/24 mol/mol) (Material and Methods). D of deprotonated OA (pH 8 to 11) was determined as 10^5. From this we conclude that the composition of the membrane doesn't significantly change when the FFA are ionised. The highest concentration of OA ever appearing in the buffer phase in the partition experiments is $4 \cdot 10^{-5}$ M, as calculated from D.

Liposomes were also characterized by ζ over the examined pH range (Material and Methods). The ζ-pH diagrams are shown in Fig. 1. PhC-liposomes show neutral surface charge between pH 2 and 10.5. Above pH 10.5 ζ becomes negative. In this pH range hydrolysis of PhC was shown (8), resulting in FFA anions and other negatively charged hydrolysis products. MDCKsomes seem to have a relatively high density of negatively charged groups on their surface. ζ reaches from −12 mV at pH 6 to −40 mV at pH 11. No clear inflection can be seen which reflects the presence of several ionisable groups with different pK_a values. Below pH 8 the PhC/PhE-liposomes show a neutral surface as the PhC-liposomes do. At pH values above 8 the deprotonation of PhE (pK_a around 10 in lipid membranes, (13)) causes negative ζ values. Liposomes containing OA as PhC/OA- and PhC/PhE/OA-liposomes reveal a sigmoidal ζ-pH curve due to the pK_a of OA between 6 and 8 in lipid membranes (14). At pH values where OA is neutral, the membrane is neutral as well. From the ζ values the pH differences between the stationary buffer layer around the liposomes and the bulk buffer phase were calculated (Table I).

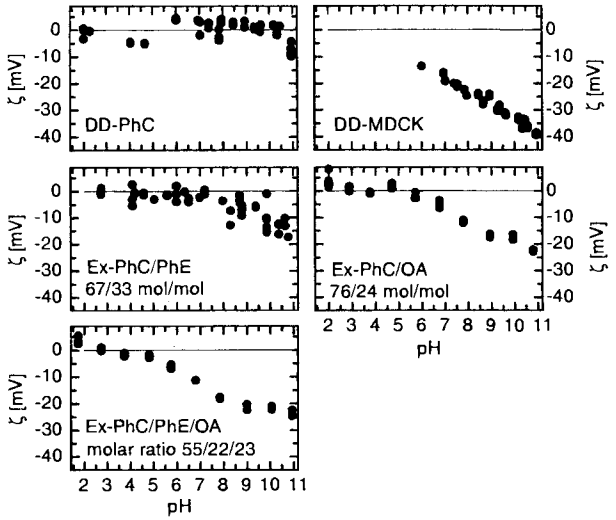

Fig. 1. Zetapotentials of various liposomes. Liposomes were prepared and characterized as described in Material and Methods. The zetapotential ζ was analysed in SUBS by micro electrophoresis and dynamic light scattering using a ZetaSizer 3 (Malvern Instruments, UK).

Influence of Cholate on the pH-Dependent Partition Behavior of (RS)-[^{3}H]propranolol

When D-pH diagrams of MDCKsomes were analysed, two points of inflection were found which were tentatively attributed to the influence of PhE and OA (8). Therefore, the influence of PhE and OA on the partitioning of *(RS)*-[^{3}H]propranolol was explored. To exclude a superposing effect of residual cholate, we first compared partitioning in DD-PhC/PhE-liposomes to FTF-PhC/PhE-liposomes and both of them to FTF-liposomes which contained exogeneously added cholate. Partition experiments were performed under standard conditions (Material and Methods). With the three types of liposomes the resulting D-pH diagrams for *(RS)*-[^{3}H]propranolol are bell-shaped (Fig. 2). Most strikingly, in the absence of cholate (FTF-PhC/PhE-liposomes, 67/33 mol/mol) maximal D values are significantly higher than in the presence of cholate. For DD-PhC/PhE-liposomes (molar ratio of PhC/PhE/cholate = 67/33/1.4) the difference is about a factor of two around pH 9.5 to 10. Exogeneously added cholate (FTF-PhC/PhE/cholate-liposomes 67/33/1.8) shows the same effect as the residual cholate in the DD-lipo-

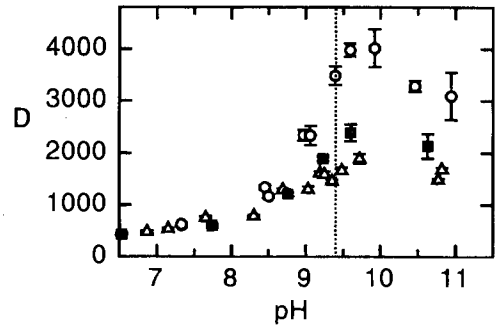

Fig. 2. Influence of cholate on the partition behavior of (*RS*)-[^{3}H]propranolol in the PhC/PhE-liposome/SUBS system. The apparent partition coefficients (D) were determined by means of equilibrium dialysis at 37°C at pH values between 7 and 11. Δ DD-PhC/PhE liposomes (molar ratio PhC/PhE/cholate = 67/33/1.4); ○ FTF-PhC/PhE-liposomes (67/33, no cholate); ■ FTF-PhC/PhE/cholate liposomes (67/33/1.8). The apparent pK_a of propranolol at 37°C equals 9.4 (----).

somes. No significant difference is found between the inflection points of the curves (not shown). To exclude the influence of cholate as an additional component on the calculated P values, further studies were performed with FTF- and Ex-liposomes exclusively.

Influence of PhE and OA on the pH-Dependent Partition Behavior of (RS)-[^{3}H]propranolol

The results of the partition studies with FTF-PhC/PhE-liposomes (67/33 mol/mol) and Ex-PhC/OA-liposomes (88/12) and (76/24) are presented in Fig. 3. The fit inflection points (pH_m) are around 10 for the PhE-containing liposomes and between 7.6 and 7.9 for the FFA-containing liposomes. Fit pH_m, P_n and P_i are listed in Tab. II. Comparison of the partition profiles with the one from the standard PhC-liposome/buffer system shows, that the partitioning of the neutral propranolol (P_n) is only influenced by the presence of net neutral charged PhE in the membrane but not significantly by the presence of FFA. However, the presence of deprotonated OA in the lipid membrane causes an increase of P_i of propranolol ($P_i[pH_{m1}/pH_{m2}]$). Neutral OA has no significant influence on the partitioning of the protonated propranolol, ($P_i[<pH_{m1}]$ is similar to $P_i[pH_{m1}/pH_{m2}]$ in the PhC-liposome system). Since the amount of neutral propranolol at pH values where OA is neutral is very low, data analysis gives no information about the influence of neutral OA on Pn ($P_n[<pH_{m1}]$). The pH_m values, which correspond to the apparent pK_a values of the lipids within the membrane, were corrected for ΔpH (Material and Methods). The resulting intrinsic pK_a values of the membranous OA or free fatty acids mixture (MDCKsomes), respectively, and of membranous PhE are listed in Tab. III. These values are in perfect agreement with published values (13,14). The pH_{m1} in the PhC-liposome system corresponds to the pK_a of membranous PhC (6).

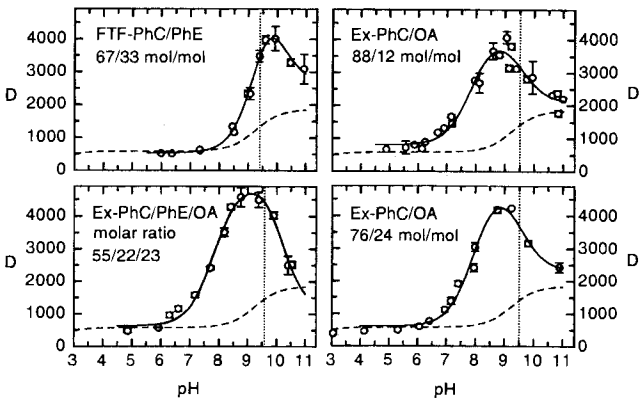

Fig. 3. Data analysis of the partition behaviour of (*RS*)-[^{3}H]propranolol in the PhC/PhE-, PhC/OA- and PhC/PhE/OA-liposome/SUBS systems. Curve fitting was performed using the equations described in the text. (——) Best fit, i.e. lowest chi^2; (.....) apparent pK_a of propranolol; (----) partitioning in the DD-PhC-liposome standard system (6,8).

For direct comparison with MDCKsomes PhC/PhE/OA-liposomes (molar ratio 55/22/23) were prepared. The lipid ratio was based on the recently determined composition of the MDCKsomes (8). Fig. 4 shows the lipophilicity profiles of (*RS*)-[^{3}H] propranolol in the liposome systems studied in this work compared to that from the MDCKsome system. Though Ex-PhC/PhE/OA liposomes and MDCKsomes show similar shapes in the D vs pH diagrams and reveal similar inflection points (Table II), the curves do not overlap. MDCKsomes show much smaller D values between pH_{m1} and pH_{m2}, resulting in lower P_n and P_i within this pH range.

DISCUSSION

The composition of lipid membranes is of high relevance for drug-lipid membrane interactions. The pH-partition diagram for a certain solute follows the ionisation curves of the solute and the lipids. The present work deals with two lipids, namely FFA and PhE, which seem to have a significant influence on the partition behavior of the model solute (*RS*)-[^{3}H]propranolol in mammalian cell membranes (8). In liposomes containing PhC and 12% (mol/mol) or more OA and/or 23% or more PhE, (*RS*)-[^{3}H]propranolol showed a strikingly different partition behavior than in pure PhC-membranes (6). Both, OA and PhE, led to an increase in D between pH 6 and 11. Data analysis allowed us to explore the influence of these lipids in more detail. The affinity of a drug to a particular lipid membrane strongly depends on the ionisation states of the drug and of the lipid components. As shown previously (14) and as confirmed in this work, membranous free fatty acids have a pK_a value around the physiological pH. This means, that in membranes containing FFA drug-lipid membrane interactions around physiological pH values are very sensitive to small pH changes, an aspect which is of relevance for in vivo processes like absorption, distribution, and elimination.

FFA show much higher pK_a values within lipid membranes, namely pK_a 6 to 8 (14), than in aqueous solution, where it equals ~4.8 (15). Since the difference between the pK_a of aqueous and membranous FFA is given by the difference between P_n and P_i (16), P_i for FFA must be ~10^3 times lower than P_n (based on a pK_a of 7.8, Table III). To check redistribution of OA to the hydrophilic phase after deprotonation, the pH-dependent partition behavior of OA was tested in the PhC/OA-liposome (76/24 mol/mol) system. At pH > 8 D values were in the range of 10^5 which means that the change in the partition profile of propranolol around the pK_a of membranous OA is

Table II. Fit Parameters of the pH-Dependent Partitioning of (*RS*)-[^{3}H]Propranolol in Various Liposome/SUBS Systems

Liposomes (molar ratio)	Apparent pK_a of propranolol[a]	Membrane derived inflection points		P at various membrane states: Above pH_{m1}/ below pH_{m2}		Below pH_{m1}[c]	Above pH_{m2}	
		pH_{m1}	pH_{m2}	P_n	P_i	P_i	P_n	P_i
FTF-PhC/PhE (67/33)	9.40	—	9.89 ± 0.19	7820 ± 752	519 ± 97	—	2655 ± 304	—
Ex-PhC/OA (76/24)	9.51	7.97 ± .15	—	2313 ± 357	3717 [d]	610 ± 179	—	—
Ex-PhC/OA (88/12)	9.51	7.87 ± 0.19	—	2085 ± 205	3439 [d]	805 ± 169	—	—
Ex-PhC/PhE/OA (55/22/23)	9.58	7.81 ± 0.26	10.11 ± 159	6013 [d]	4879 [d]	631 ± 198	1067 [d]	852 [d]
DD-MDCKsomes[b]	9.70	7.66 ± 0.31	9.97 ± 0.91	2123 [d]	1568 [d]	710 ± 140	1193 ± 292	1609 [d]
DD-PhC[b]	9.24	2.46 ± 0.54	—	1858 ± 24	580 ± 17	216 ± 181	—	—

[a] The apparent pK_a of propranolol was kept fixed during the fit procedure (see Material and Methods).
[b] FTF-liposomes revealed similar results. Composition of MDCKsomes see Table I.
[c] $P_n[<pH_{m1}]$ is not relevant for the curve description.
[d] High uncertainties due to a high degree of superposition in a limited pH range (confidence limits 90%). The experimental data from partition studies were fit with a combination of Henderson-Hasselbalch equations (Material and Methods).

directly caused by the ionisation change of OA and not by a change in the membrane composition, i.e. the loss of OA anions from the membrane. However, at high pH values the concentration of $4 \cdot 10^{-5}$ M ionised OA (calculated for PhC/OA-liposomes, 76/24 mol/mol) in the aqueous phase could have an influence on the partitioning, since total drug concentration is 10^{-9} M.

PhE seems to influence D only at its net neutral stage, where the amine is protonated (pK_a 10). Most likely the positive charge of its primary amine interacts with the secondary amine group of the neutral propranolol. This is supported by the fact, that there is no effect on the protonated propranolol.

The fit pK_a values of the membrane lipids are in good agreement with the results of zetapotential measurements. Membranes containing OA are negatively charged above the pK_a of OA but neutral at pH values where OA is protonated. PhE also causes negative surface charge when deprotonated in the PhC/PhE-liposomes. This effect is superimposed by the effect of OA in the PhC/PhE/OA-liposomes.

We tried to mimic the partition behavior of propranolol in the MDCKsome/SUBS system by using PhC/PhE/OA-liposomes. Though the inflection points of the D-pH curves are in best agreement, the curves do not overlap. This is not unexpected since MDCKsomes contain less FFA and also other additional lipids, which may influence the partitioning.

Regarding the D-pH diagram of (*RS*)-[^{3}H]-propranolol in the PhC/PhE/OA-liposome system it is not obvious, that there are three inflection points, namely the one at the pK_a of FFA, the one at the pK_a of propranolol and the third one at the pK_a of PhE. Therefore we tried to fit the curve with two inflection points only. As Fig. 5 shows, three inflections give a superior fit. The same is true for the MDCKsome system (not shown).

These studies reveal that the partition coefficient of the ionised solute P_i can be higher than the one of the neutral solute P_n. This must be due to electrostatic interactions. From this point of view partition coefficients alone are not sufficient for the prediction of membrane permeation. The neutral solute with less electrostatic affinity to the lipids headgroups can probably move much easier across the bilayer than the ionised molecule,

Table III. Intrinsic pK_a of Membranous PhE and OA/FFA

Liposomes	pK_a of FFA	pK_a of PhE
FTF-PhC/PhE (67/33 mol/mol)		9.70
Ex-PhC/OA (76/24)	7.78	
Ex-PhC/OA (88/12)	7.68	
Ex-PhC/PhE/OA (55/22/23)	7.52	9.76
DD-MDCKsomes	7.45	9.44

Note: Intrinsic pK_a values of the membranous lipids were calculated from the fit inflection points (pH_m) of the partitioning curves (Table II) and from ζ (Table I) as described (Material and Methods, eq. 2).

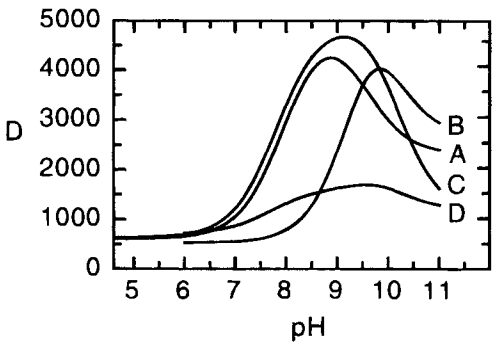

Fig. 4. Comparison of the partition profiles of (*RS*)-[^{3}H]propranolol in various liposome/SUBS systems. A, Ex-PhC/OA-liposomes (76/24 mol/mol); B, FTF-PhC/PhE-liposomes (67/33); C, Ex-PhC/PhE/OA-liposomes (55/22/23); D, MDCKsomes (8). Drawn are the fit curves as derived from partition data (Fig. 3).

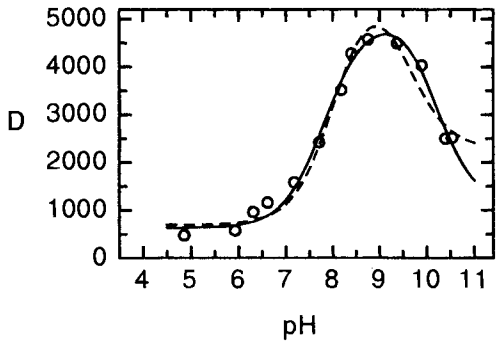

Fig. 5. Partitioning of (*RS*)-[^{3}H]propranolol between Ex-PhC/PhE/OA-liposomes (molar ratio 55/22/23) and SUBS. (——) Data are fit based on 3 inflections (pK_a of OA, propranolol and PhE). (- - - -) Data are fit based on 2 inflections (at pH ~8 and pH ~10).

which sticks to the headgroups. An additional component for the prediction of permeation behavior would therefore be the "flip-flop" of the solute, i.e. the movement between the two lipid layers.

ACKNOWLEDGMENTS

We thank Dr. Alex Avdeef for all the helpful discussions and Dr. Ernst Wehrli for the competent electron microscopic work.

REFERENCES

1. W. Nernst. *Z. Phys. Chem.* **8**:110–139 (1891).
2. C. Hansch, A. Leo, and D. Hoekman. *Exploring QSAR. Hydrophobic, Electronic, and Steric Constants.* ACS Professional Reference Book. American Chemical Society, Washington, DC, 1995.
3. H. Miyoshi, H. Maeda, N. Tokutake, and T. Fujita. *Bull. Chem. Soc. Jpn.* **60**:4357–4362 (1987).
4. C. J. Alcorn, R. J. Simpson, D. E. Leahy, and T. J. Peters. *Biochem. Pharmacol.* **45**:1775–1782 (1993).
5. R. P. Austin, A. M. Davis, and C. N. Manners. *J. Pharm. Sci.* **84**:1180–1183 (1995).
6. G. M. Pauletti and H. Wunderli-Allenspach. *Eur. J. Pharm. Sci.* **1**:273–282 (1994).
7. C. Ottiger and H. Wunderli-Allenspach. *Eur. J. Pharm. Sci.,* in press.
8. S. D. Krämer and H. Wunderli-Allenspach. *Pharm. Res.* **13**:1851–1855 (1996).
9. H. Wunderli-Allenspach, M. Günthert, and S. Ott. *Biochemistry* **32**:900–907 (1993).
10. M. J. Hope, M. B. Bally, G. Webb, and P. R. Cullis. *Biochim Biophys. Acta.* **812**:55–65 (1985).
11. T. Teorell and E. Stenhagen. *Biochem. Z.* **299**:416–419 (1938).
12. M. Takayama, S. Itoh, T. Nagasaki, and I. Tanimizu. *Clin. Chim. Acta* **79**:93–98 (1977).
13. F. C. Tsui, D. M., Ojcius, and W. L. Hubbell. *Biophys. J.* **49**:459–468 (1986).
14. R. Lieckfeldt, J. Villalaín, J. Gómez-Fernández, and G. Lee. *Pharm. Res.* **12**:1614–1617 (1995).
15. A. Spector. *J. Lipid Res.* **16**:165–179 (1975).
16. J. Miyazaki, K. Hideg, and D. Marsh. *Biochim. Biophys. Acta* **1103**:62–68 (1992).