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Modelling of interaction of basic lipophilic ligands with cytochrome *P*-450 reconstituted in liposomes. Determination of membrane partition coefficients of *S*-(−)-nicotine and *N*,*N*-diethylaniline from spectral binding studies and fluorescence quenching

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The spectral interaction of *N*,*N*-diethylaniline and *S*-(−)-nicotine with cytochrome P450IIB4 reconstituted into large unilamellar vesicles could properly be described by a model for interaction of basic lipid-soluble ligands with membrane-bound acceptor sites in which linear partitioning of non-ionized ligand in the membrane is postulated. Apparent spectral dissociation constants K_s^{app} for type I binding of *N*,*N*-diethylaniline and for type II binding of *S*-(−)-nicotine increased linearly with increasing lipid volume fraction α_L of the proteoliposomes. From plots of K_s^{app} vs. α_L, the membrane partition coefficient of each ligand was calculated. The apparent affinity of cytochrome P450IIB4 for the ligands increased as the pH was raised from 6.0 to 8.5. However, effective dissociation constants were virtually independent of the pH, indicating that only the uncharged form of the basic ligands interact with cytochrome P450IIB4. For each compound, the apparent quenching rate constants k_q^{app} derived from the Stern-Volmer plots for dynamic quenching of the fluorescence intensity of 8-(2-anthryl)octanoic acid in liposomes, decreased with increasing liposomal concentration. Plots of $(k_q^{app})^{-1}$ vs. α_L of the liposomes yielded the overall bimolecular quenching rate constant k_q of each quencher. The k_q value for *S*-(−)-nicotine was about three orders of magnitude less than that for *N*,*N*-diethylaniline. The values of the partition coefficient of *N*,*N*-diethylaniline, obtained from the binding studies and the fluorescence quenching measurements, were identical (on average, K_p amounted to 383). Analysis of the quenching data of *N*,*N*-diethylaniline with Scatchard plots likewise revealed that the association of the compound with liposomal membranes is a pure partition process.

Introduction

Numerous amphiphilic and lipophilic compounds are metabolized by the hepatic microsomal monooxygenase system or bind to its terminal hemoprotein component cytochrome *P*-450 [1–3]. Several kinetic and binding studies have demonstrated that these metabolic and equilibrium processes cannot adequately be described by mathematical expressions for homogeneous systems. For example, the rate of benzpyrene hydroxylation was non-linear with microsomal protein concentration [4]. Apparent Michaelis constants K_m^{app} of monooxygenase-catalyzed reactions increased with increasing enzyme concentration [5–7]. A similar dependence of the apparent spectral dissociation constant K_s^{app} on protein (or phospholipid) concentration was observed for the interaction of several lipophilic exogenous compounds [8–11] and steroids [12–15] with cytochrome *P*-450 in microsomes or proteoliposomes. The problem was quantitatively approached by Parry et al. [16] who pointed to the biphasic nature of membrane suspensions and assumed that lipid-soluble compounds partition between the aqueous phase and the lipid compartment of the membrane. Allowing for different orientation, these authors distinguished between lipid-faced and fully hydrophilic aqueous-faced binding sites. Heirwegh et al. [17] formulated a similar model

Abbreviations: PC, egg L-α-phosphatidylcholine; PE, egg phosphatidylethanolamine; DPPA, dipalmitoylphosphatidic acid.

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in which two additional types of binding site, namely aqueous-faced amphiphatic and mixed sites, were considered. The binding models presented in Refs. 16 and 17 were formulated for non-ionized compounds.

In the present paper, a quantitative approach for description of the interaction of ionizing ligands of type $BH^+ \rightleftharpoons B + H^+$ with membrane-bound acceptor sites is developed. The model was applied to analyze the binding of *S*-(−)-nicotine and *N,N*-diethylaniline to reconstituted cytochrome P450IIB4, and to determine their membrane partition coefficient. The ligand binding model was verified by measurement of the accumulation of each compound in liposomes, using steady-state fluorescence quenching of the membrane probe 8-(2-anthryl)octanoic acid. Partition coefficients were obtained by Stern-Volmer analysis of the quenching results. Furthermore, the quenching data of *N,N*-diethylaniline were analyzed by Scatchard plots which permits to discriminate between binding and partition of a quencher.

Materials and methods

Chemicals

Egg L-α-phosphatidylcholine and egg phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Dipalmitoylphosphatidic acid was from Sigma (St. Louis, MO, USA). The phospholipids migrated as a single spot on thin-layer chromatography on silica (solvent, chloroform/methanol/water, 65:25:4, v/v; I_2 staining). *n*-Octylamino-Sepharose 4B was prepared as described by Guengerich and Martin [18]. DEAE-Sepharose Fast Flow and Sepharose 4B-CL were obtained from Pharmacia (Uppsala, Sweden), and hydroxylapatite from Bio-Rad (Richmond, CA, USA). Renex 690 was kindly provided by the Atlas Chemical Division of ICI Belgium. *n*-Octyl β-D-glucopyranoside (Boehringer GmbH, Mannheim, Germany) and sodium cholate (Serva Feinbiochemica GmbH, Heidelberg, Germany) were used without further purification. *S*-(−)-Nicotine and *N,N*-diethylaniline, both supplied by Janssen Chimica (Beerse, Belgium) were purified by vacuum distillation prior to use. 8-(2-Anthryl)octanoic acid was synthetized by the method of Kaplun et al. [19]. Tris, gold label (Aldrich Chemie, Brussels, Belgium), methanol and chloroform, both of fluorimetric grade (Merck, Darmstadt, Germany) and all other reagents, of analytical grade, were used as received.

Isolation of cytochrome P450IIB4

Cytochrome P450IIB4 was isolated from liver microsomes of phenobarbital-pretreated rabbits, according to published procedures [20,21], with minor modifications. All purification steps were performed at 4°C. The preparation of the microsomes, the solubilization, poly(ethylene glycol) 6000 precipitation and DEAE-based ion exchange chromatography proceeded as described by Haugen and Coon [20], except that dialyzed 6–12% poly(ethylene glycol) fractions were chromatographed on DEAE-Sepharose Fast Flow instead of DEAE-cellulose, and that the cytochrome P450IIB4-containing fraction was eluted with 0.01 M Tris-HCl buffer (pH 7.7) containing 20% (v/v) glycerol, 0.5% Renex 690, 1 mM EDTA and 0.2 mM dithiothreitol. The following steps of the isolation were carried out essentially as described by Hansson and Wikvall [21]. All potassium phosphate buffers for the further procedure contained 20% (v/v) glycerol, 0.2 mM EDTA and 0.2 mM dithiothreitol. Nonionic detergent was removed by extensive washing of the DEAE-Sepharose Fast Flow eluate on hydroxylapatite with 10 mM potassium phosphate buffer (pH 7.4), after which cytochrome P450IIB4 was eluted with 300 mM potassium phosphate buffer (pH 7.4) containing 0.4% sodium cholate. The eluate was diluted 6-fold with 20% (v/v) glycerol containing 0.4% sodium cholate, 0.2 mM EDTA and 0.2 mM dithiothreitol, and applied to an *n*-octylamino-Sepharose 4B column (40 nmol cytochrome *P*-450 per ml gel; column height/diameter = 15) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.4% sodium cholate. After washing the column with two column volumes of the same buffer, the protein material was eluted with the equilibration buffer containing 0.1% Renex 690. The eluted cytochrome *P*-450 was diluted 5-fold with 20% (v/v) glycerol containing 0.2% Renex 690, 0.2 mM EDTA and 0.2 mM dithiothreitol, and applied to a hydroxylapatite column that had been equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.2% Renex 690. After washing the column with 2 column volumes of the equilibration buffer, cytochrome P450IIB4 was eluted with 70 mM potassium phosphate buffer (pH 7.4) containing 0.2% Renex 690. Removal of the nonionic detergent by chromatography on hydroxylapatite and elution of cytochrome P450IIB4 was performed as described above. The final preparation was dialyzed three times for 8 h against 25 volumes of 0.1 M Tris-HCl buffer (pH 7.5) containing 20% (v/v) glycerol and 0.2 mM EDTA (hereafter called 'standard buffer'), adjusted to a concentration of 30 nmol of cytochrome *P*-450 per ml and stored at −20°C.

Cytochrome *P*-450 was determined by the method of Omura and Sato [22]. Protein concentrations were estimated by the method of Lowry et al. [23] using bovine serum albumin as standard, with modifications for interfering substances [24]. The purified preparation had a specific content of 17.2 nmol of cytochrome *P*-450 per mg of protein and was homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

Preparation of liposomes and proteoliposomes

Large unilamellar vesicles were prepared by a detergent dialysis technique, based on published procedures [25,26]. For the preparation of proteoliposomes, a chloroform solution of either 38.25 mg (for the binding studies with *S*-(−)-nicotine) or 45.9 mg (for the binding studies with *N,N*-diethylaniline) of phospholipid (PC/PE/DPPA, 2:1:0.06, w/w) was evaporated as a thin lipid film in a small conical vessel under a stream of argon. The phospholipid composition was similar to that used for the preparation of vesicles with microsome-like properties [27,28]. Remaining solvent was removed under reduced pressure for 1 h. The dried phospholipids were solubilized with *n*-octyl β-D-glucopyranoside at a detergent/phospholipid ratio of two (w/w) in 3 ml of standard buffer, after which 3 ml (90 nmol) of cytochrome P450IIB4 stock-solution was added. Mixed detergent-phospholipid-protein micellar suspensions were dialyzed for 16 h at 4°C against 3 litres of standard buffer (preparations for the binding assays with *S*-(−)-nicotine and *N,N*-diethylaniline in Tris-HCl buffer) or against 50 mM potassium phosphate buffer (pH 7.0) containing 20% glycerol and 0.2 mM EDTA (preparations for the binding assays with *N,N*-diethylaniline in phosphate buffer), using a Lipoprep flow-through dialyzer (Diachema, Langnau a.A/Zürich, Switzerland). Buffer exchange of the proteoliposomal suspensions using the buffers for the binding assays, was performed by gel filtration on Sepharose 4B-CL. For determination of the membrane partition coefficients of the ligands, the entire proteoliposomal preparations were chromatographed on a 1.6 × 40 cm column of the gel filtration medium. Subsequently, the proteoliposomal preparations were diluted to different phospholipid concentrations. For assessment of the pH-dependence of the ligand binding, 1-ml samples of proteoliposomal suspension were run on a 1.6 × 10 cm column of Sepharose 4B-CL, and adjusted to a cytochrome *P*-450 concentration of 2 nmol/ml.

Liposomes were prepared by dissolving 38.25 mg of dried phospholipid (PC/PE/DPPA, 2:1:0.06, w/w) with 75 mg of *n*-octyl β-D-glucopyranoside in 6 ml of 0.1 M Tris-HCl buffer, pH 8.5 (for fluorescence quenching studies with *S*-(−)-nicotine) or pH 7.9 (for fluorescence quenching studies with *N,N*-diethylaniline) containing 20% (v/v) glycerol and 0.2 mM EDTA. Clarified lipid suspensions were dialyzed for 16 h at room temperature against 3 litres of the respective solubilization buffer, using the Lipoprep apparatus. The vesicle preparations were diluted to the desired phospholipid concentrations. Each suspension was labeled with fluorophore by addition of 8-(2-anthryl)octanoic acid, dissolved in a small volume (< 10 μl) of methanol. The probe to lipid molar ratio of the liposomal dilutions varied between 1:100 and 1:800. Uptake of the probe in the vesicles was achieved by vortex mixing for 15 s. Negative staining electron microscopy was performed with 1% uranyl acetate. A value of 1 g/ml was accepted for the density of the vesicles [29]. The diameter of the liposomes varied between 150 and 250 nm. On the assumption that the liposomes had a mean diameter of 176 nm [26], a bilayer thickness of 4 nm [30], and that the total liposomal bilayer volume was available for partition, it was calculated that there are $2.9 \cdot 10^5$ phospholipid molecules per vesicle and that a vesicle suspension of 1 mg/ml corresponds to a $4.5 \cdot 10^{-6}$ millimolar concentration of vesicles.

Phospholipid concentrations were assayed by quantitation of inorganic phosphate [31,32].

Binding studies with reconstituted cytochrome P450IIB4

Binding experiments were carried out at 30°C with 2 ml of proteoliposomal suspension in both the sample and reference cuvette, using an Aminco/SLM DW-2000 UV/VIS spectrophotometer (SLM Instruments, Urbana, IL, USA). The measurements for the determination of the membrane partition coefficient of *S*-(−)-nicotine were done in 0.1 M Tris-HCl buffer, pH 7.8 or 8.3, and for *N,N*-diethylaniline in 50 mM potassium phosphate buffer (pH 7.0) or 0.1 M Tris-HCl buffer (pH 7.6), each buffer containing 20% (v/v) glycerol and 0.2 mM EDTA. The effect of pH on the binding of the ligands was assayed in 0.1 M Tris-HCl buffers and in 50 mM potassium phosphate buffers of different pH between 6.0 and 8.5, all containing 20% (v/v) glycerol and 0.2 mM EDTA. Stock solutions of *S*-(−)-nicotine (15–60 mM) were made in water, and of *N,N*-diethylaniline (30–60 mM) in 66% aqueous methanol. Because of increased instability of cytochrome *P*-450 above pH 8.0 [33], binding measurements with *S*-(−)-nicotine at pH 8.3 and 8.5 were carried out as quickly as possible. A baseline of zero absorbance was recorded to compensate for differences between the two cuvettes. Microlitre aliquots (total volume < 20 μl) of the ligand solutions were added to the sample cuvette, with identical volumes of water (binding studies with *S*-(−)-nicotine) or 66% aqueous methanol (binding studies with *N,N*-diethylaniline) being added to the reference cuvette, using Agla micrometer syringes (Wellcome Research Laboratories, Beckenham, UK). Difference spectra for the binding of *S*-(−)-nicotine and *N,N*-diethylaniline to cytochrome P450IIB4 were recorded between 370 and 490 nm and between 350 and 450 nm, respectively. For the determination of the partition coefficients of the ligands, titration curves were established at various proteoliposomal P450IIB4 concentrations, varying between 1 and 5 nmol/ml for the binding measurements with *N,N*-diethylaniline, and between 0.95 and 4.7 (0.95 and 6.6) nmol/ml for the binding measurements with *S*-(−)-nicotine at pH 8.3 (at pH 7.8). Apparent spectral dissociation constants K_s^{app} were calculated,

using the computer program of Cleland [34]. The present proteoliposomal systems were prepared in such a way that in the binding experiments, protein-bound ligand could be neglected (Heirwegh, K.P.M. and Vermeir, M., unpublished data). Membrane partition coefficients were determined by fitting Eqn. 11 to (α_L, K_s^{app})-data pairs.

Fluorescence quenching measurements

All fluorescence measurements were performed at 30°C with 2 ml of liposomal solution in 1 cm × 1 cm quartz cuvettes. The probe's fluorescence spectra were registered using a Spex Fluorolog 212/Datamate at an excitation wavelength of 360 nm. The fluorescence quenching of 8-(2-anthryl)octanoic acid in PC/PE/DPPA (2:1:0.06, w/w) liposomes by *S*-(−)-nicotine or *N*,*N*-diethylaniline was measured at different liposomal concentrations. Fluorescence quenching titrations were performed, using Agla micrometer syringes. Microlitre (1–18 μl) quantities of *N*,*N*-diethylaniline were added to the vesicle dilutions from a 100 mM stock solution in 80% aqueous methanol, the final volume of methanol in the vesicle suspensions, after addition of probe and the highest quencher amount, never exceeding 1.25% of the total volume. At this concentration, no influence of the methanol on the fluorescence spectrum of 8-(2-anthryl)octanoic acid could be observed. Aliquots of *S*-(−)-nicotine (10–120 μl) were added undiluted to the liposomal suspensions

pK_a values of the ligands

The pK_a values of *S*-(−)-nicotine and *N*,*N*-diethylaniline were assumed to be 8.0 [35] and 6.5 [36], respectively.

Theory

Ligand binding to a membrane-bound acceptor site

The following model describes the interaction of ionizable, lipid-soluble compounds of type $BH^+ \rightleftharpoons B + H^+$ with membrane-bound acceptor sites. The membrane is considered as a single homogeneous bulk phase, and the lumenal compartment of the vesicles and the surrounding aqueous medium as a single aqueous phase with identical local concentrations of ligand. Furthermore, it is assumed that only the neutral form of the basic compounds accumulates into the membrane and that equilibrium binding to the acceptor site solely occurs with the uncharged form of the ligand. Besides, linear solute partition in the bilayer membrane will be postulated. A mathematical expression is derived which allows one to determine the membrane partition coefficient of the ligand and the 'effective dissociation constant' [17] for the ligand-acceptor binding which is independent of the effects of ligand ionization and of ligand partitioning into the membrane.

Consider an ionizable ligand undergoing an acid–base equilibrium in the aqueous phase $BH^+ \rightleftharpoons B + H^+$. Define $[B]_A$ and $[BH^+]_A$ as the aqueous concentrations of unionized and ionized ligand, respectively, and K_a as the acid-base dissociation constant, given by:

$$[BH^+]_A \cdot K_a = [B]_A \cdot [H^+] \quad (1)$$

Neglecting acceptor-bound ligand, the total mass of ligand can be expressed as:

$$V_T \cdot [B_0] = V_A \cdot [BH^+]_A + V_A \cdot [B]_A + V_L \cdot [B]_L \quad (2)$$

where $[B]_L$ is the concentration of neutral ligand in the lipid compartment (with volume V_L), $[B_0]$ the total concentration of ligand, ionized and unionized, in the total system (with volume V_T), and V_A the volume of the aqueous phase.

Defining the membrane partition coefficient K_p as:

$$K_p = [B]_L / [B]_A \quad (3)$$

and the lipid volume fraction of the membrane suspension, α_L, as:

$$\alpha_L = V_L / V_T \quad (4)$$

Eqn. 2 can be rearranged to:

$$[B]_A = [B_0] / \{(1 - \alpha_L)(10^{(pK_a - pH)} + 1) + K_p \cdot \alpha_L\} \quad (5)$$

and

$$[B]_L = K_p \cdot [B_0] / \{(1 - \alpha_L)(10^{(pK_a - pH)} + 1) + K_p \cdot \alpha_L\} \quad (6)$$

Eqns. 5 and 6 express the local ligand concentrations $[B]_A$ and $[B]_L$ in terms of the total ligand concentration $[B_0]$.

Consider the equilibrium binding of the ligand to a membrane-bound acceptor site $E_i + B_i \rightleftharpoons EB_i$ with

$$[EB]_i \cdot K_s = [E]_i \cdot [B]_i \quad (7)$$

K_s is the dissociation constant for equilibrium binding of unionized ligand, $[B]_i$ is the concentration of free ligand and $[E]_i$ and $[EB]_i$ are the concentrations of ligand-free and occupied binding sites in phase i, respectively. The total concentration of acceptor sites in phase i is given by:

$$[E_0]_i = [E]_i + [EB]_i \quad (8)$$

Substitution into Eqn. 7 gives

$$[EB]_i = [E_0]_i \cdot [B]_i / (K_s + [B]_i) \quad (9)$$

Allowing for different sidedness and nature of the binding site, one can distinguish between hydrophilic aqueous-faced sites and sites with some hydrophobic

character (aqueous-faced amphiphilic, lipid-faced and mixed sites) [17]. The ligand concentration relevant for the binding equation will be dependent on the type of binding site. Rearranging in Eqn. 9 the local concentration of free ligand $[B]_i$ by the expressions of Eqns. 5 and 6 gives:

$$[EB]_i = [E_0]\cdot[B_0]/(K_s^{app} + [B_0]) \quad (10)$$

where the apparent dissociation constant K_s^{app} is given by:

$$K_s^{app} = K_s'\cdot\{(1-\alpha_L)(10^{(pK_a-pH)}+1) + K_p\cdot\alpha_L\} \quad (11)$$

K_s' has been termed the 'effective dissociation constant' [17]. For aqueous-faced hydrophilic sites:

$$K_s' = K_s \quad (12)$$

where K_s is the true dissociation constant relating to a single phase system. For lipid-faced and mixed sites:

$$K_s' = K_s/K_p \quad (13)$$

For aqueous-faced amphiphilic sites K_p can be replaced by a related quantity [17]. By measuring the apparent dissociation constant K_s^{app} at various lipid volume fractions α_L, the membrane partition coefficient K_p and the effective dissociation constant K_s' can be calculated from Eqn. 11. K_s' pertains to the binding of neutral ligand to the acceptor site in one of both phases and should be independent of the pH of the aqueous phase. Thus, if the supposition that only the neutral form of the ligand interacts with the acceptor is correct, a plot of pK_s' vs. pH should give a horizontal line, irrespective of the type of binding site.

Fluorescence quenching

For a fluorophore that is solubilized in a lipid bilayer, the fluorescence quenching is dependent on the concentration of quencher molecules in the membrane, $[Q]_L$, given by:

$$[Q]_L = K_p\cdot[Q]_A \quad (14)$$

where $[Q]_A$ represents the quencher concentration in the aqueous phase. If the quencher dissociates in the aqueous phase, $QH_A^+ \rightleftharpoons Q_A + H_A^+$, the aqueous concentration of neutral quencher, $[Q]_A$, is a function of its dissociation constant K_a and of the pH of the aqueous phase:

$$[Q]_A = [QH^+]_A\cdot 10^{(pH-pK_a)} \quad (15)$$

where $[QH^+]_A$ is the aqueous concentration of ionized quencher. The total mass of quencher is:

$$[Q]_T V_T = ([QH^+]_A + [Q]_A)\cdot V_A + [Q]_L\cdot V_L \quad (16)$$

Taking into account Eqns. 14–16 and assuming that only the neutral form of the quencher partitions into the membrane, the Stern-Volmer relationship, written as a function of the total quencher concentration $[Q]_T$ can be expressed as:

$$I_0/I = 1 + k_q^{app}\cdot\tau_0\cdot[Q]_T \quad (17)$$

where I_0 and I denote the fluorescence intensities in the absence and presence of quencher, τ_0 represents the lifetime of the probe in the absence of quencher while the apparent quenching rate constant k_q^{app} is given by Eqn. 18:

$$1/k_q^{app} = \left[1/k_q - \frac{10^{(pK_a-pH)}+1}{k_q K_p}\right]\cdot\alpha_L + \frac{10^{(pK_a-pH)}+1}{k_q K_p} \quad (18)$$

where k_q is the overall bimolecular rate constant of quenching. Thus, the reciprocal of the apparent quenching rate constant k_q^{app} is linearly dependent on the lipid volume fraction α_L. k_q^{app} values are obtained as the slopes of the Stern-Volmer plots, divided by τ_0. By plotting $1/k_q^{app}$ vs. α_L, the partition coefficient K_p and the bimolecular quenching rate constant k_q can be calculated from Eqn. 18.

Partition and binding

In order to distinguish between binding and/or partition of a quencher, an alternative model can be applied to analyze fluorescence quenching data [37,38]. The main assumption is that I_0/I at a particular quencher concentration is only dependent on the average number of quenchers per vesicle, $\langle Q\rangle$, and independent of the quenching mechanism. For the interaction of an ionizing quencher with a vesicle v,

$$\begin{array}{ccc} Q_a + v & \rightleftharpoons & Q_l \\ K_a \Big\| & & \Big\| H^+ \\ QH_a^+ & & \end{array}$$

Scheme I.

the total concentration of quencher $[Q]_T$ can be expressed as [39]:

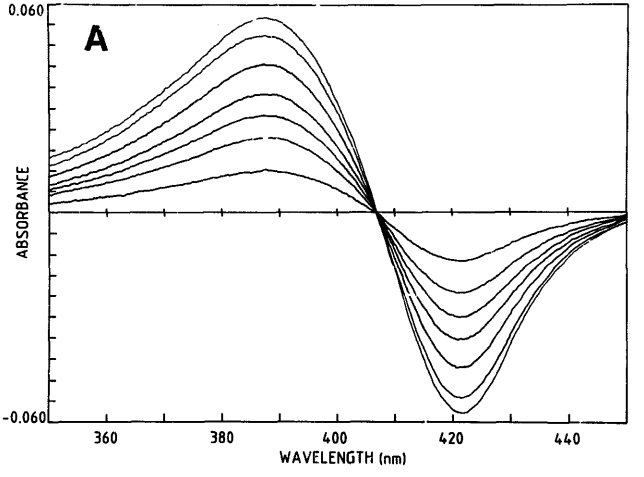
$$[Q]_T = \{\langle Q\rangle\cdot(1 + 10^{(pK_a-pH)})/K_{eq}\} + \langle Q\rangle\cdot[v] \quad (19)$$

where K_{eq} is an equilibrium distribution constant defined as:

$$K_{eq} = [Q_l]/([Q_a]\cdot[v]) \quad (20)$$

and $\langle Q\rangle$ is given by:

$$\langle Q\rangle = [Q_l]/[v] \quad (21)$$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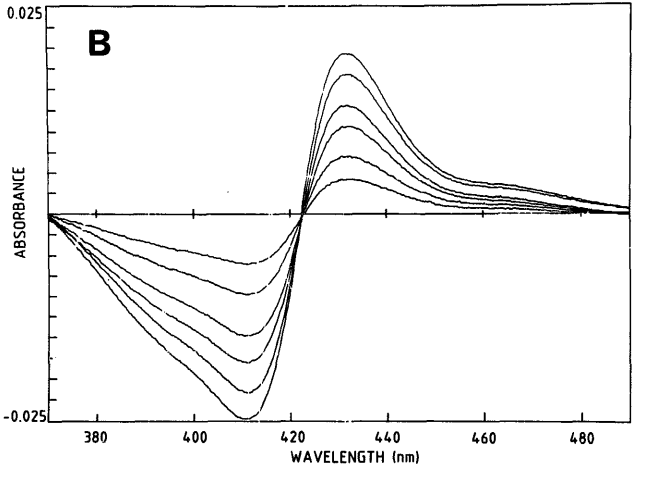
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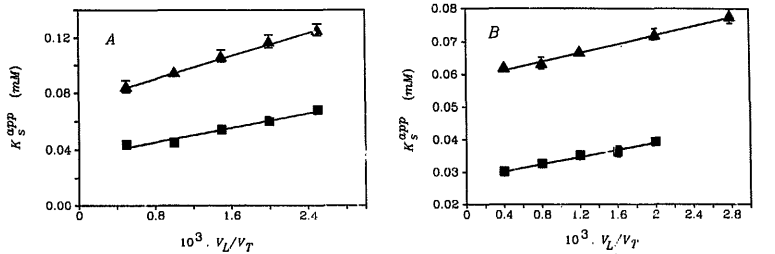

Fig. 2. Effect of the proteoliposomal lipid volume fraction α_L on the apparent spectral dissociation constants, K_s^{app}, for interaction of *N,N*-diethylaniline (A) and *S*-(−)-nicotine (B) with cytochrome P450IIB4. The K_s^{app} for *N,N*-diethylaniline were determined at pH 7.6 (■) and pH 7.0 (▲) and for *S*-(−)-nicotine at pH 8.3 (■) and pH 7.8 (▲). The vertical bars represent ± 1 S.D.

where [v] is the vesicle concentration, and $[Q_l]$ and $[Q_a]$ represent the concentration of neutral quencher in the membrane and the aqueous phase, respectively, defined with regard to the total volume V_T.

Application of the analysis requires obtaining Stern-Volmer plots at several lipid concentrations. At each particular level of I_0/I, a plot of $[Q]_T$ vs. [v] yields one pair of values for K_{eq} and ⟨Q⟩. Secondary plots of K_{eq} against ⟨Q⟩ are Scatchard plots. Partition of the quencher in the vesicles occurs when K_{eq} is independent of ⟨Q⟩, while a linear relationship with a negative slope indicates binding. If both binding and partition occur, the Scatchard plots show a decreasing dependence of K_{eq} with increasing ⟨Q⟩ asymptotically approaching a constant value.

Results

Binding measurements for the determination of liposomal membrane partition coefficients

Interaction of *N,N*-diethylaniline with reconstituted cytochrome P450IIB4 caused a type I difference spectrum with an absorption maximum and minimum at 387 nm and 421 nm, respectively, and an isosbestic point at 407 nm (Fig. 1A). Titration of reconstituted cytochrome P450IIB4 with *S*-(−)-nicotine (Fig. 1B) resulted in the formation of a type II difference spectrum with peak, isosbestic and trough wavelengths of 432 nm, 422 nm and 411 nm, respectively, which is characteristic for direct ligation to a low-spin haem iron [40]. For each ligand, apparent spectral dissociation constants were determined from the absorbance difference between the peak and trough of the respective difference spectra. The ligand saturation fitted accurately to rectangular hyperbolae. The apparent spectral dissociation constants increased linearly with increasing concentration of proteoliposomes (Figs. 2A and B) as predicted by Eqn. 11. Effective dissociation constants K_s' (calculated values ± 1 S.D.), obtained by extrapolation of K_s^{app} values to zero lipid volume fraction, amounted to 18.7 ± 0.3 μM (pH 8.3) and 22.7 ± 0.2 μM (pH 7.8) for *S*-(−)-nicotine, and 32.6 ± 1.9 μM (pH 7.6) and 55.8 ± 0.7 μM (pH 7.0) for *N,N*-diethylaniline. The membrane partition coefficients of both ligands, determined from the binding studies, are given in Table I.

pH dependence of ligand binding

In order to examine the effect of pH on the binding of the ligands to cytochrome P450IIB4, difference spectra were recorded at several pH values between pH 6.0 and 8.5. The magnitude of the type I and type II difference spectra, recorded at identical concentrations of cytochrome P450IIB4, increased with increasing pH, but no alteration of the shape of the spectra could be observed by changing the pH of the assay buffer (results not shown). The apparent dissociation constant for the binding of *S*-(−)-nicotine to cy-

Fig. 1. Spectral interactions of *N,N*-diethylaniline (A) and *S*-(−)-nicotine (B) with cytochrome P450IIB4 reconstituted into vesicles of PC/PE/DPPA (2:1:0.06, w/w). The cytochrome P450 and phospholipid concentration of the proteoliposomes were 2.0 nmol/ml and 1.0 mg/ml (*N,N*-diethylaniline binding) and 2.8 nmol/ml and 1.2 mg/ml (*S*-(−)-nicotine binding), respectively. Increasing displacements from the baseline correspond to subsequent additions of 15, 30, 45, 60, 90, 150 and 240 μM *N,N*-diethylaniline, and of 7.2, 14.5, 21.7, 29.0, 43.5, 72.4 and 115.9 μM *S*-(−)-nicotine (final concentrations) to the sample cuvette. The spectral titrations were performed in 2 ml of 0.1 M Tris-HCl buffer, pH 8.3 (*S*-(−)-nicotine binding) and 50 mM potassium phosphate buffer, pH 7.4 (*N,N*-diethylaniline binding) each buffer containing 20% (v/v) glycerol and 0.2 mM EDTA.

TABLE I

Membrane partition coefficients ± S.D. for the accumulation of S-(−)-nicotine and N,N-diethylaniline into liposomes of PC/PE/DPPA (2:1:0.06, w/w)

The K_p values were obtained from binding studies with reconstituted cytochrome P450IIB4 and from steady-state fluorescence quenching measurements, using 8-(2-anthryl)octanoic acid as membrane probe.

	K_p	
	S-(−)-nicotine	*N,N*-diethylaniline
Cytochrome *P*-450 binding	298 ± 24 (pH 8.3)	386 ± 60 (pH 7.6)
	294 ± 16 (pH 7.8)	373 ± 14 (pH 7.0)
Fluorescence quenching		391 ± 67

tochrome P450IIB4 decreased gradually as the pH was raised from 6.8 to 8.5 (Fig. 3A). K_s^{app} for *N,N*-diethylaniline binding decreased with increasing pH until around 7.5 and then leveled off (Fig. 3A). However, when the K_s^{app} values were recalculated to effective dissociation constants (Eqn. 11), the ligands' affinity for cytochrome P450IIB4 became virtually independent of the pH (Fig. 3B). This finding was in accordance with the assumption that basic lipid-soluble compounds bind in their neutral form to cytochrome *P*-450. In accordance with Eqn. 11, the dependence of K_s^{app} on the pH can then be explained by an increase of neutral ligand molecules available to the enzyme for binding upon increasing the pH, resulting in a decrease of K_s^{app}.

Fluorescence quenching studies

Time-resolved fluorescence quenching measurements revealed a mono-exponential decay for 8-(2-anthryl)octanoic acid in PC/PE/DPPA (2:1:0.06, w/w) liposomes with a lifetime $\tau_0 = 3.50 \pm 0.03$ ns, and

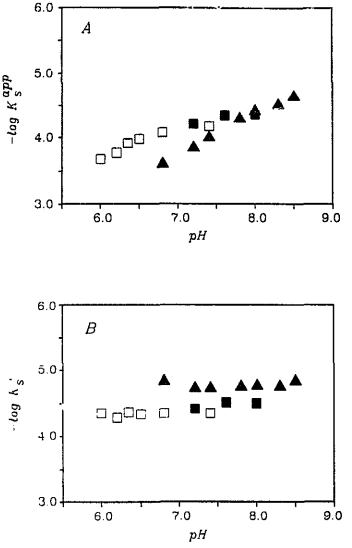

Fig. 3. pH-dependence for the binding of *N,N*-diethylaniline (■, □) and *S*-(−)-nicotine (▲) to cytochrome P450IIB4 (2 nmol/ml) reconstituted into PC/PE/DPPA (2:1:0.06, w/w) liposomes ($\alpha_L = 1.0 \cdot 10^{-3}$ and $0.85 \cdot 10^{-3}$ for the binding measurements with *N,N*-diethylaniline and *S*-(−)-nicotine, respectively). (A) Apparent dissociation constants, K_s^{app}, were determined in 0.1 M Tris-HCl buffer (■, ▲) or 50 mM potassium phosphate buffer (□) at the indicated pH in the presence of 20% (v/v) glycerol and 0.2 mM EDTA. (B) Effective dissociation constants, K_s', were calculated from K_s^{app} values according to Eqn. 11, accepting K_p values of 296 and 383 for *S*-(−)-nicotine and *N,N*-diethylaniline, respectively.

TABLE II

Apparent bimolecular quenching rate constants k_q^{app} at different lipid volume fractions α_L for the quenching of the fluorescence intensity of 8-(2-anthryl)octanoic acid by S-(−)-nicotine and N,N-diethylaniline in PC/PE/DPPA (2:1:0.06, w/w) liposomes

The quenching measurements with *N,N*-diethylaniline and *S*-(−)-nicotine were carried out in 0.1 M Tris-HCl buffer, pH 7.9 and 8.5, respectively, each buffer containing 20% (v/v) glycerol and 0.2 mM EDTA.

S-(−)-Nicotine		*N,N*-Diethylaniline	
α_L ($\times 10^3$)	k_q^{app} ($\times 10^{-8}$) ($M^{-1} s^{-1}$)	α_L ($\times 10^3$)	k_q^{app} ($\times 10^{-11}$) ($M^{-1} s^{-1}$)
0.54	4.12	0.5	4.19
1.08	3.78	1.0	3.73
1.62	3.22	1.5	3.49
2.16	3.06	2.0	3.12
3.24	2.62	3.0	2.63
		4.0	2.01

absence of excimer formation (Vermeir, M. and Boens, N., unpublished data). The occurrence of a single-exponential decay was indicative for complete uptake of the fluorophore into the membrane.

Upon addition of either *S*-(−)-nicotine or *N,N*-diethylaniline to a liposomal suspension, the probe's fluorescence intensity decreased, but no exciplex band could be observed in the fluorescence spectrum. For each quencher, the Stern-Volmer plots were linear, indicating that dynamic quenching occurred. Fig. 4 shows the plots for the quenching of 8-(2-anthryl)octanoic acid by *N,N*-diethylaniline. Apparent quenching constants k_q^{app}, obtained from the slopes of the Stern-Volmer plots, decreased with increasing concentration

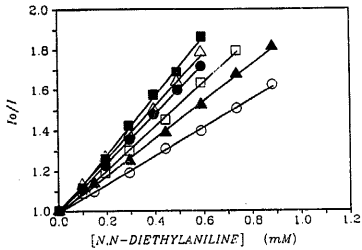

Fig. 4. Stern-Volmer plots for the quenching of the fluorescence intensity of 8-(2-anthryl)octanoic acid in liposomes of PC/PE/DPPA (2:1:0.06, w/w) by *N,N*-diethylaniline. The phospholipid concentration of the vesicles amounted to 0.5 (■), 1.0 (△), 1.5 (●), 2.0 (□), 3.0 (▲) and 4.0 (○) mg/ml. The steady-state measurements were carried out in 0.1 M Tris-HCl buffer (pH 7.9) containing 20% (v/v) glycerol and 0.2 mM EDTA.

of vesicles (Table II). From a plot of $(k_q^{app})^{-1}$ vs. the lipid volume fraction α_L of the liposomes, a value of $(1.4 \pm 0.1) \cdot 10^9$ M^{-1} s^{-1} was determined for the bimolecular quenching rate constant k_q of *N,N*-diethylaniline. Its membrane partition coefficient obtained from the quenching measurements (Eqn. 18) is given in Table I. The concentrations of *S*-(−)-nicotine necessary to cause quenching were much higher than those of *N,N*-diethylaniline. For example, at a phospholipid concentration of 1.4 mM ($\alpha_L = 1.08 \cdot 10^{-3}$), a I_0/I level of 1.4 was only obtained at a 300 mM concentration of *S*-(−)-nicotine. Such high quencher concentrations will significantly modify the structure of the bilayer membrane. Nevertheless, for *S*-(−)-nicotine the apparent quenching rate constants also decreased with increasing phospholipid concentration (Table II).

TABLE III

Average number of N,N-diethylaniline molecules per vesicle ± S.D. at various I_0/I levels for the quenching of 8-(2-anthryl)octanoic acid in liposomes of PC/PE/DPPA (2:1:0.06, w/w)

The average number of quencher molecules was calculated from Eqn. 19.

I_0/I	⟨*N,N*-diethylaniline⟩ ($\times 10^{-4}$)
1.2	1.2 ± 0.1
1.4	2.3 ± 0.2
1.6	3.5 ± 0.3
1.8	4.6 ± 0.4
2.0	5.7 ± 0.5
2.2	6.9 ± 0.6

Moreover, a plot of $(k_q^{app})^{-1}$ as a function of α_L gave a straight line from which a bimolecular quenching rate constant $k_q = (1.9 \pm 0.1) \cdot 10^6$ M^{-1} s^{-1} and a partition coefficient $K_p = 319 \pm 33$ could be calculated. However, it is questionable if the latter result relates to a 'membrane' partition coefficient because of the high quencher concentrations.

Analysis of the quenching data of N,N-diethylaniline with Scatchard plots

From the Stern-Volmer plots for the quenching by *N,N*-diethylaniline, concentrations $[Q]_T$ as a function of the vesicle concentration [v] were determined at various I_0/I levels (Fig. 5). Values for the average number of quencher molecules per vesicle ⟨Q⟩ (Table III) and the equilibrium distribution constant K_{eq} were determined according to Eqn. 19. The (K_{eq} vs. ⟨Q⟩)-plot (Fig. 6) for the interaction of *N,N*-diethylaniline with PC/PE/DPPA (2:1:0.06, w/w) liposomes was horizontal, indicating that the quencher partitions into the liposomal membranes. The value of K_{eq} at a level of $I_0/I = 2.2$ amounted to $(1.1 \pm 0.2) \cdot 10^8$ M^{-1}.

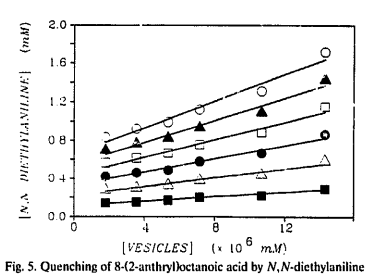

Fig. 5. Quenching of 8-(2-anthryl)octanoic acid by *N,N*-diethylaniline plotted according to Eqn. 19. The symbols correspond to I_0/I values of 1.2 (■), 1.4 (△), 1.6 (●), 1.8 (□), 2.0 (▲) and 2.2 (○).

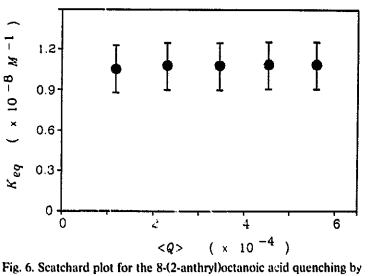

Fig. 6. Scatchard plot for the 8-(2-anthryl)octanoic acid quenching by *N,N*-diethylaniline in PC/PE/DPPA (2:1:0.06, w/w) liposomes. The error bars represent ± 1 S.D.

Discussion

In the present model, it was assumed that equilibrium binding to cytochrome *P*-450 solely occurs with the uncharged form of basic lipophilic ligands and that ionized ligand molecules are unable to accumulate within the membrane. A number of experimental data from the literature support these suppositions. Martin and Hansch [41] found a better correlation between the Michaelis constant for mixed-function oxidation of various substrates and their octanol/water partition coefficient, if one postulated that only the uncharged species of the compounds are substrates for the microsomal enzymes. The observation that fully protonated *n*-octylamine and aminoglutethimide induce type II difference spectra upon binding to cytochrome *P*-450 was explained by postulating the transfer of the amine proton to a protein ligand displaced from the haem and binding of the neutral form to the haem iron, or exclusive binding of neutral ligand to cytochrome *P*-450 within the membrane [42,43]. *N*-Demethylation of a series of *N*,*N*-dimethylamines could adequately be described by a model in which the reaction rate was related to the concentration of neutral base and in which competitive inhibition by protonation was taken into account [44]. Totani et al. [45] analyzed the binding of pyridine and *N*-methylimidazole to cytochrome P450CI (P450cam) as a function of pH. Estimates of the dissociation constants of the *N*-methylimidazolinium and pyridinium ions were significantly higher in comparison with the K_s values for the neutral form of these compounds. These data support the assumption that only the concentration of the uncharged form of basic lipid-soluble compounds has to be taken into account for the formulation of the binding equation. Our results on the pH dependence of the binding of *S*-(−)-nicotine and *N*,*N*-diethylaniline to cytochrome P450IIB4 likewise are in accordance with this hypothesis. Indeed, effective dissociation constants, defined with respect to neutral ligand concentrations, were constant at different pH-values which can be expected if only the uncharged form of basic ligands binds to the enzyme. Similarly, Sono et al. [46] investigated the binding of several exogenous ligands to chloroperoxidase, a protein with essentially identical haem iron coordination structure and spectroscopic properties as cytochrome *P*-450. Their equilibrium binding studies revealed that the 'intrinsic' dissociation constants for binding of pyridine, imidazole and *N*-phenylimidazole in their neutral form were independent of pH, indicating that also in this case the basic ligands bind in their deprotonated form to the enzyme.

For hydrophobic amine drugs [47–49] and fatty acids [50], it was demonstrated that both ionized and neutral forms interact with lipid bilayers. The two forms, however, may locate at different transverse positions within the membrane. Charged tetracaine, for example, resides in the headgroup region of phosphatidylcholine membranes, while the neutral form penetrates into the hydrophobic region of the lipid bilayer [51]. The membrane partition coefficients reported for the neutral form of tetracaine [52], chlorpromazine [53] and of certain spin-labelled local anaesthetics [54], were significantly higher than the values for the ionized form of these compounds. Further, fluorescence quenching studies showed that charged tetracaine and *N*,*N*-dimethylaniline are unable to partition into the lipid compartment of neutral lipid bilayers [55,56]. From these observations one can conclude that positively charged lipophilic molecules interact only to a small extent with neutral membranes. Hence, to simplify the model, it may be quite reasonable to assume that only the neutral form partitions into the membrane.

The accumulation of small amphiphilic and hydrophobic compounds into lipid membranes has been approached as a partition [16,53,57–59] or a binding process [60,61]. The term 'binding', however, should be reserved to describe the saturable interaction of ligands with structural entities such as the binding pockets of enzymes and binding proteins. Such specific binding sites can hardly be defined in phospholipid bilayers. The observation that, at relatively low solute concentrations, accumulation of solute in the membrane is characterized by a constant distribution ratio between its concentrations in the membrane and in the aqueous phase, indicates membrane partitioning. The present liposomal systems contained 2 mol% DPPA and 33 mol% PE. Under the experimental conditions, the vesicles were negatively charged [62] which could facilitate electrostatic interaction between the bilayers and the charged compounds. However, analysis of the binding and fluorescence quenching results revealed that the interaction of *S*-(−)-nicotine and *N*,*N*-diethylaniline with the PC/PE/DPPA bilayers can be considered as a pure partition process. Indeed, the reciprocal of the apparent quenching rate constant for the quenching of 8-(2-anthryl)octanoic acid by *N*,*N*-diethylaniline was linearly dependent on the liposomal concentration. Moreover, when the equilibrium distribution constant K_{eq} was plotted against the average number of *N*,*N*-diethylaniline molecules per vesicle, a horizontal line was obtained which also indicated membrane partitioning. Furthermore, the apparent spectral dissociation constants for the interaction of the ligands with reconstituted cytochrome P450IIB4 increased linearly with increasing lipid volume fraction of the proteoliposomes in accordance with the hypothesis that the ligands partition between the aqueous phase and the membrane (Eqn. 11). The similarity between the different K_p values of each ligand, determined at different pH, also was in accordance with the partition concept, since a partition coefficient is as-

sumed to be constant and should thus be independent of the pH of the buffer. Moreover, for *N,N*-diethylaniline, the K_p values obtained from the binding studies were in good agreement with the value calculated from the fluorescence measurements, which provides evidence for the validity of the binding model. Probably, no electrostatic interaction was observed due to the low charge density of the membranes and to the relatively high ionic strength of the buffers, reducing the negative surface potential of the vesicles. The membrane partition coefficients were determined with a sufficient level of accuracy (standard deviations amounted at most to 17%), and the values are quite realistic. For comparison, a K_p value of about 160 was found for the partition of *N,N*-dimethylaniline in dimyristoylphosphatidylcholine vesicles [56].

The quenching of the fluorescence intensity of 8-(2-anthryl)octanoic acid in liposomes by *S*-(−)-nicotine and *N,N*-diethylaniline could be described by linear Stern-Volmer plots. The bimolecular quenching rate constant k_q for the *N,N*-diethylaniline/8-(2-anthryl)octanoic acid system was of an identical order of magnitude as that reported for the quenching of *n*-(9-anthroyloxy) fatty acids by *N,N*-dimethylaniline [56]. For *S*-(−)-nicotine, however, a significantly smaller k_q value was determined (about three orders of magnitude less than that for *N,N*-diethylaniline). As the electron-acceptor capacity of 8-(2-anthryl)octanoic acid in the quenching experiments with *S*-(−)-nicotine and *N,N*-diethylaniline was identical, the lower quenching rate constant implied that *S*-(−)-nicotine is a less efficient electron-donor than *N,N*-diethylaniline. For this reason, high concentrations of *S*-(−)-nicotine were required to obtain sufficient quenching of the fluorophore. It is not clear how the 'vesicles' will be organized at such high quencher concentrations. Possibly, a linear distribution of quencher molecules occurs between the aqueous phase and undefined mixed aggregates of phospholipid and *S*-(−)-nicotine molecules. In this respect, the term 'membrane partition coefficient' probably may no longer be appropriate. Curiously, the value for the partition coefficient was comparable with the K_p values determined from the binding studies. If the bimolecular quenching rate constant of a quencher is small, steady-state fluorescence quenching may not be suitable for determination of its membrane partition coefficient.

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