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Research Paper

The Thermodynamics of the Partitioning of Ionizing Molecules Between Aqueous Buffers and Phospholipid Membranes

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Purpose. To study the thermodynamics of partitioning of eight ionising dual D_2-recepto β_2-adrenoceptor agonists between vesicles of L-α-dimyristoylphosphatidylcholine (DMPC) and aqueous buffers.
Methods. The thermodynamics of partitioning have been studied by isothermal titration calorimetry (ITC).
Results. Compounds which are predominantly cationic at pH 7.4 (designated as class 1 compounds) have a more exothermic partitioning than those which are predominantly in the electronically neutral form (designated as class 2 compounds) at pH 7.4, and less positive standard entropies of partitioning. Under acidic conditions (pH 4.0), class compounds 2 (predominantly electronically neutral at pH 7.4) are almost completely cationic and accordingly have a more exothermic partitioning than at pH 7.4. The standard entropies of partitioning also depend on the pH. When the compounds are predominantly cationic, the standard entropy change is less positive (less favourable) than under conditions where the compounds are predominantly electronically neutral.
Conclusions. The observations are consistent with the notion of there being a favourable electrostatic interaction (enthalpically) between the positively charged amino-group of predominantly cationic compounds and the negatively charged phosphate group of the vesicle.

KEY WORDS: ionizing molecules; isothermal titration calorimetry; phospholipid membranes; thermodynamics of partitioning.

INTRODUCTION

Until recently, little attention has been paid to the importance of drug–membrane interactions in the design of new drugs. Rather, focus has centered around drug–protein interactions. A useful means of studying such drug–membrane interactions is measurement of partition coefficients of compounds between aqueous buffers and a model membrane phase, usually composed of phospholipid vesicles (liposomes) (1–3). The study of charged compounds in particular is interesting and relevant as many drug molecules are ionized, to some degree, at physiologically relevant pH values.

The development of a long-acting, dual D_2-receptor and β_2-adrenoceptor agonist as a novel treatment for chronic obstructive pulmonary disease (COPD) has highlighted the importance of specific drug–phospholipid membrane interactions (4,5). The duration of β_2-adrenoceptor agonism has been found to correlate with the membrane affinity of compounds that possess either protonated or unprotonated secondary amino groups, at pH 7.4 (4); the former compounds are predominantly cationic, whereas the latter are predominantly electronically neutral. The duration of β_2-adrenoceptor agonism was shown to be governed by bulk lipophilicity, as measured by octanol/water log $D_{7.4}$, and ionization state (pK_a of the secondary amino group). Compounds that are predominantly in the cationic form at pH 7.4, as a consequence of protonation of the amino group, were shown to exhibit enhanced partitioning into model membrane systems, and correspondingly longer duration of action, compared to electronically neutral compounds (no protonation of the amino group) of comparable bulk lipophilicity (log $D_{7.4}$). This was rationalized in terms of the compounds that are predominantly in the cationic form being able to partition into a phospholipid bilayer in an ordered way. They can form an ion–pair interaction (electrostatic interaction) with the negatively charged phosphate group of the membrane phospholipids, while orienting their lipophilic groups into the hydrophobic core of the bilayer. The closer in structure the compound is to the geometry of the phospholipid membrane, the more favorable the distribution of the compounds in this interfacial way would be. This interaction was suggested to be absent for the uncharged electronically neutral compounds. The results of this work provide further evidence that drug–membrane interactions control the duration of pharmacological action of the agonists (the so-called plasmolemma diffusion microkinetic hypothesis) in contrast

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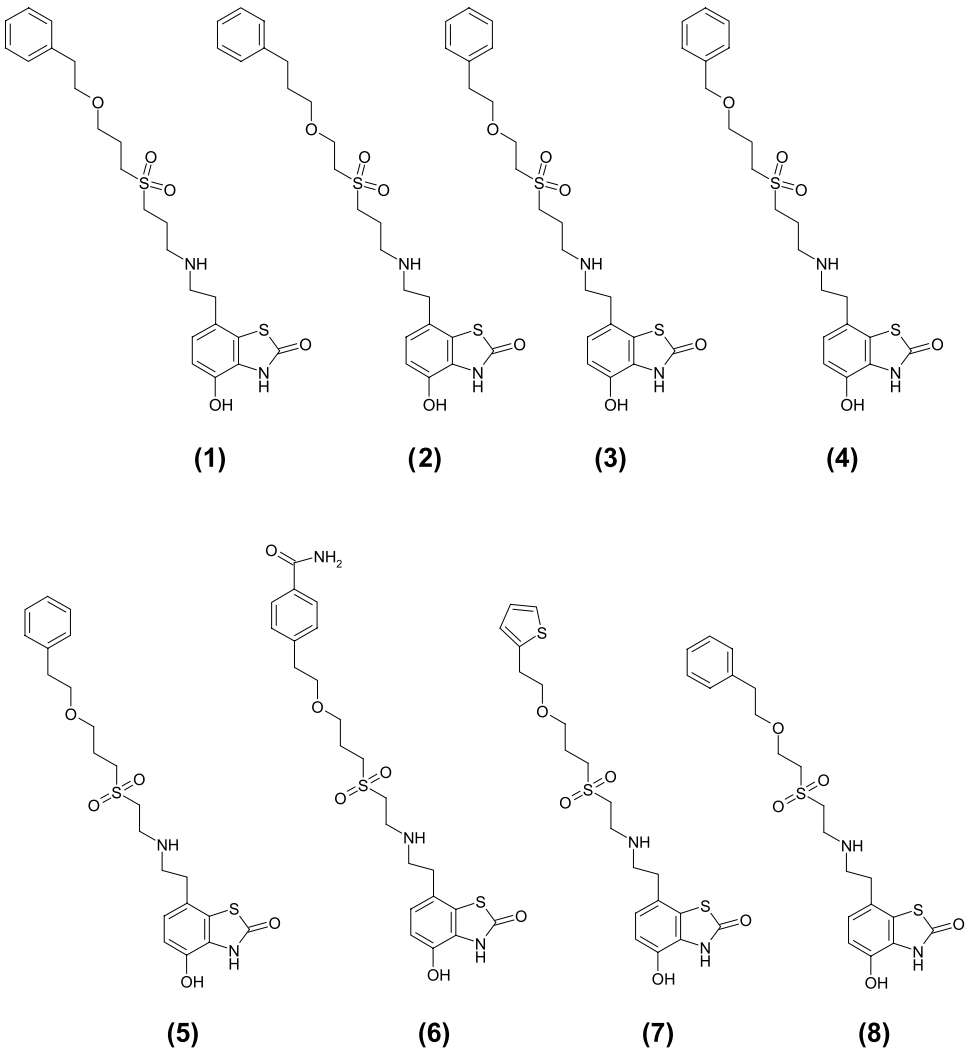

Fig. 1. Chemical structures of **1**–**8**.

to a direct drug receptor interaction (exosite hypothesis) (4). Neutron diffraction studies of amlodipine and nimodipine provide strong evidence for such an electrostatic interaction between the positively charged protonated primary amino group of amlodipine and the negatively charged phospholipid head group region (6,7). The uncharged nimodipine has been shown to reside predominantly in the hydrophobic core of the membrane, this compound not being able to benefit from ionic interactions. The hydrophobic portions of each compound are oriented into the hydrophobic membrane core.

A number of investigations have involved examining the pH dependence of the membrane distribution coefficient (D_{mem}) of compounds and comparing this to pH distribution profiles using the more traditional 1-octanol buffer system (8–14). Differences between these pH dependencies have been attributed to the charged form of some molecules being able to partition into the model membrane phases. Charged species can partition into 1-octanol as ion pairs (15). However, studies have shown that the binding of highly ionized compounds to liposomes involves little contribution from ion pairing and instead mainly arises from incorporation of the charged species alone (15,16).

It is conceivable that certain structural motifs in ionized molecules could enhance their partitioning into phospholipid bilayers. One such motif may be the amino group; measurements on amlodipine (primary amine), xamoterol, atenolol, and propranolol (secondary amines), and flunarizine (tertiary amine) have shown these compounds to have rather high values of log D_{mem} (7,17,18). To probe further the nature of interactions between ionized molecules and phospholipid membranes, studies have been carried out measuring the thermodynamic parameters of partitioning of the dual D_2-receptor and β_2-adrenoceptor agonists **1**–**8** (as shown in Fig. 1) possessing a secondary amino group, but with variable pK_a, at both physiological pH (pH 7.4) and under acidic conditions (pH 4.0), into phospholipid vesicles. Pivotal to these studies has been the use of isothermal titration calorimetry (ITC). This technique has been widely applied in studying phenomena ranging from the binding of ligands to proteins, to the binding of compounds (ionized and unionized) to membrane systems (18–22).

MATERIALS AND METHODS

Reagents

Dimyristoyl L-α-phosphatidylcholine (DMPC, 99%+) was obtained from Sigma (St. Louis, MO, USA). All the 7-(2-aminoethyl)-4-hydroxybenzothiazol-2-(3*H*)-one derivatives (**1**–**8**) were synthesized at AstraZeneca R&D Charnwood

(Loughborough, UK). Acetonitrile (HPLC gradient quality) was obtained from Fisher Chemicals, Fisher Scientific (Loughborough, UK). Trifluoroacetic acid (for HPLC gradient applications) was obtained from Fisher Chemicals, Fisher Scientific.

Preparation of Lipid Vesicles

HEPES buffer (20 mM) with added KCl (100 mM) was used for vesicle preparations at pH 7.4. Acetic acid/sodium acetate (20 mM) with added KCl (100 mM) was used for vesicle preparations at pH 4.0. Multilamellar vesicle suspensions of concentrations 1.0–5.0 mg/mL were prepared by hydrating DMPC with the required amount of the appropriate buffer and vortex mixing. Unilamellar vesicle suspensions of concentrations 3.0–5.0 mg/mL and of approximately 100 nm diameter were prepared using a standard method involving repeated extrusion of the multilamellar vesicle solution through two stacked polycarbonate filters (Isopore polycarbonate membrane filters, pore size 100 nm; Millipore, Watford, UK) encased in a pressurized extrusion apparatus (Lipex Biomembranes Inc., Vancouver, BC, Canada).

Determination of Membrane-Buffer Distribution Coefficients

Experiments were carried out with slight modification of the method we have previously described (15). In brief, this involves adding 1 mL of a solution of the relevant compound (50–200 μM) in either HEPES buffer or acetic acid/sodium acetate buffer as above) to 1 mL of the multilamellar vesicle suspension, in triplicate. A fourth solution containing 1 mL of compound solution and 1 mL of buffer was also prepared to give the initial concentration of compound before partitioning. The four solutions were then equilibrated at 30°C for 2 h before separation of the free compound from the membrane-bound compound via centrifugation (9,300×*g*, 5 min). An aliquot of the supernatant was removed (200 μL) and analyzed by HPLC. The membrane distribution coefficients (D_{DMPC}) were then calculated using Eq. (1), which assumes that the density of DMPC vesicles (ρ_{DMPC}) is 1.014 g/mL.

$$D_{DMPC} = \frac{[B]_{DMPC}}{[B]_{aq}} = \left(\frac{1.014 V_{total}(PA_{total} - PA_{aq})}{m_{DMPC} PA_{aq}}\right) \quad (1)$$

where $[B]_{DMPC}$ and $[B]_{aq}$ are the molar concentrations of compound in the membrane phase and aqueous phase, respectively, at equilibrium; V_{total} is the total volume of the partition mixture, comprising both aqueous phase and membrane phases (in mL); m_{DMPC} is the mass of DMPC in grams used; and PA_{total} and PA_{aq} are the HPLC peak areas from the reference solution and the supernatant from the partition mixture, respectively. This equation is valid when $D_{DMPC} \gg 1$ as is the case for all compounds in the current study. All data are represented as the mean of triplicate measurements.

HPLC Analysis

A Waters modular HPLC system consisting of a 600 s controller, a 996 photodiode array detector, a 616 pump, a 717 autosampler and the Millennium software system was used with a Waters Symmetry C8 3.5-μm column (4.6 × 50 mm) at 25°C (Waters Corp., Milford, MA, USA). For analyses of all compounds, the eluent was run as a linear gradient comprising 5–95% acetonitrile, the remaining portion being 0.1% trifluoroacetic acid/water over a period of 5 min, with subsequent reequilibration at 5% acetonitrile:95% water (0.1% trifluoroacetic acid) for 2 min.

Preparation of Compound Solutions

Solutions of compounds for ITC measurements were prepared by dissolving approximately 0.1 mg/mL of compound in the appropriate buffer and sonicating for 30 min. The solutions were then filtered to yield a stock solution of compound ready for use in calorimetric experiments. The concentration of compound was determined by HPLC analysis with reference to a standard solution of compound dissolved in DMSO of known concentration.

Measurement of Binding/Partition Enthalpies

Measurements were conducted with a MicroCal Inc. Micro Calorimetry System (MCS) Isothermal Titration Calorimeter (ITC) coupled to an MCS Controller Unit and dedicated PC employing Microcal Observer software. Analysis of the calorimetric data was carried out using Microcal Origin Version 2.9 (Microcal Software, Inc., Northampton, MA, USA). The sample cell (1.3548 mL) was filled with unilamellar vesicles (3.0 mg/mL, 4.42 mM or 5.0 mg/mL, 7.37 mM DMPC). Injections (10 μL) of compound solution (40–220 μM in the appropriate buffer) were made from an injection syringe rotating at a speed of 400 rpm (to allow thorough mixing of sample and titrant) coupled to a digital stepping motor. Measurements were made at 30°C. In a typical experiment, a total of ten injections were made. The observed data were corrected for the fraction of compound bound to the liposome bilayer under the given conditions, this being calculated using Eq. (2).

$$X_{DMPC} = 1 \Big/ \left[1 + \left(\frac{1,014\, V_{aq}}{m_{DMPC} D_{DMPC}}\right)\right] \quad (2)$$

where X_{DMPC} is the fraction of drug bound to the membrane, V_{aq} is the volume of the aqueous phase (mL), D_{DMPC} is the membrane distribution coefficient, and m_{DMPC} is the mass of

Table I. Macroscopic pK_as for Compounds **1–8**

Compound	pK_1	pK_2
1	7.8[b]	8.7[a]
2	7.9[b]	8.7[a]
3	7.8[b]	8.8[a]
4	7.9[b]	8.8[a]
5	6.1[a]	7.9[b]
6	6.4[a]	8.2[b]
7	6.8[a]	8.3[b]
8	6.8[a]	8.2[b]

Standard deviation in pK_a determinations is ±0.10.
[a] Determined potentiometrically.
[b] Determined spectroscopically.

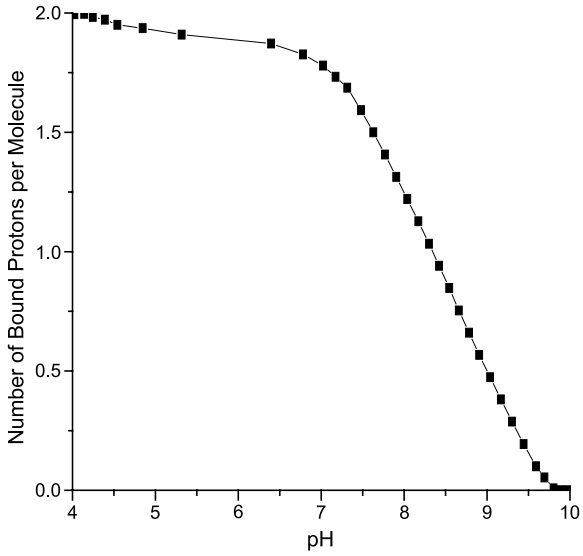

Fig. 2. Example Bjerrum plot for **3**. This is a plot of the average number of bound protons per molecule of sample *vs.* pH, derived from titration data.

DMPC (mg). The enthalpy derived from the last injection was never significantly different from that derived from the earlier injections, indicating that the phospholipid remained in large excess.

Thermodynamic Parameters

All Gibbs free energies and entropies referred to in this paper are standard Gibbs free energies and standard entropies with the standard state being a 1 M solution.

Table II. Estimated Microscopic Constants for **1**–**8**

Compound	pK_a	pK_b	pK_c	pK_d
1	8.15	8.43	8.43	8.15
2	8.15	8.50	8.50	8.15
3	8.15	8.59	8.59	8.15
4	8.15	8.64	8.64	8.15
5	7.90	6.10	6.10	7.90
6	8.20	6.40	6.40	8.20
7	8.30	6.80	6.80	8.30
8	8.20	6.80	6.80	8.20

Measurement of pK_a Values

Measurements were determined by potentiometric titration using a GLpK_a instrument and by UV spectrophotometry using an accompanying dip probe absorption spectrophotometer attachment (DPAS) (both manufactured by UK Sirius Analytical Instruments, Sussex, UK).

RESULTS AND DISCUSSION

The compounds in the current study (**1**–**8**) were chosen to have essentially the same membrane affinity, as measured by the membrane distribution coefficients at 30°C (log D_{DMPC}) and pH 7.4. This results in similar Gibbs free energies of partitioning ($\Delta G°_{part}$) for all the compounds at pH 7.4 and 30°C. We then wished to determine if there were statistically significant differences in either or both the enthalpy of partitioning (ΔH_{part}) and the entropy of partitioning ($\Delta S°_{part}$) of the compounds into phospholipid membranes, resulting from the different ionization state of the compounds leading to different modes of interaction with the model membranes.

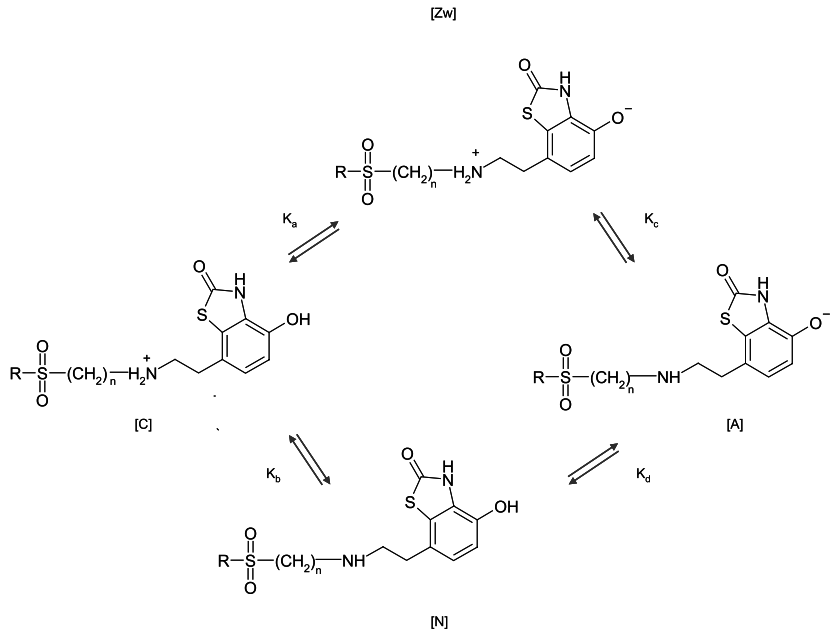

Fig. 3. Ionization scheme for **1**–**8**.

Table III. Estimated Fractions of Species for **1**–**8** Present at pH 7.4 and 4.0

Compound	$f_N^{7.4}$	$f_C^{7.4}$	$f_A^{7.4}$	$f_{Zw}^{7.4}$	$f_N^{4.0}$	$f_C^{4.0}$	$f_A^{4.0}$	$f_{Zw}^{4.0}$
1	0.07	0.78	0.01	0.14	0.00	1.00	0.00	0.00
2	0.06	0.79	0.01	0.14	0.00	1.00	0.00	0.00
3	0.05	0.80	0.01	0.14	0.00	1.00	0.00	0.00
4	0.05	0.80	0.01	0.14	0.00	1.00	0.00	0.00
5	0.72	0.04	0.23	0.01	0.01	0.99	0.00	0.00
6	0.79	0.08	0.12	0.01	0.00	1.00	0.00	0.00
7	0.71	0.18	0.09	0.02	0.00	1.00	0.00	0.00
8	0.69	0.17	0.11	0.03	0.00	1.00	0.00	0.00

Subscripts refer to the following: N, neutral species; C, cationic species; A, anionic species; Zw, zwitterionic species. The superscripts refer to the pH.

IONIZATION STATE OF THE COMPOUNDS

Pivotal to interpretation of the results in the current study is an understanding of the ionization state of the compounds. Consequently, the following section presents an in depth analysis of this aspect.

Table I lists the pK_a values of all the compounds, determined potentiometrically and spectroscopically, at 25°C, and at a constant ionic strength (I = 0.10 M). Figure 2 shows an example experimental Bjerrum plot for **3**. Two values are listed for each compound, pK_1 and pK_2, and these refer to the first and second macroscopic dissociation constants. Within the pH range of a titration experiment (pH 2–11), two functional groups can ionize within the molecules; the amino group and the phenolic group. For **1**–**8** below pH 4, the amino group is fully ionized; when pH is increased, this becomes deprotonated and the phenolic group ionizes. Four different species can, in principle, coexist in solution at any pH, in varying proportions; the cationic form [C], the neutral form [N], the zwitterionic form [Zw] and the anionic form [A], as shown in Fig. 3. To calculate the fractions of the individual species present in solution for **1**–**8**, at any given pH, knowledge of the microscopic pK_a values—pK_a, pK_b, pK_c, and pK_d—in Fig. 3, is required. For **5**–**8**, it is possible to estimate these values by making a series of approximations. The macroscopic pK_a values, pK_1 and pK_2, are well separated by >1.5 log units, and the secondary amino group and phenol ionizing centers are distally remote. The ionization of the secondary amino group can therefore be assumed to have a small influence on the ionization of the phenolic group, and vice versa. This is supported by $\sigma_{para}-$ values for $-CH_2CH_2NH_2$ and $-CH_2CH_2-NH_3^+$, these being −0.06 and 0.17, respectively (23,24). The Hammett equation referring to the ionization of phenols is pK_a = 9.92−2.23 $\sum\sigma_{para}-$ (24). Therefore, the substituent effects on the phenol group ionization are small and similar. Hence, pK_2 ≈ pK_a ≈ pK_d (phenolic group ionizations) and pK_1 ≈ pK_b ≈ pK_c (secondary amino group ionizations). These designations are based upon pK_2 for **5**–**8** being experimentally determined spectroscopically; the phenolic group has a UV chromophore, whereas the secondary amino group, being remote from the aromatic group, does not.

For **1**–**4**, the microscopic pK_a values referring to the ionization of the phenolic group are approximately equal, pK_a ≈ pK_d, and can be estimated by taking the average value of the estimated pK_a or pK_d ≈ pK_2 for **5**–**8**, i.e., 8.15, because the phenolic group in **1**–**4** is remote from the secondary amino group and in a similar environment to that in **5**–**8**. In addition, for **1**–**4**, pK_b ≈ pK_c, $K_1 = K_a + K_b$, and $1/K_2 = 1/K_c + 1/K_d$ (25). For **1**–**4**, pK_a and pK_d are fixed to a value of 8.15. The values of pK_b = pK_c were then estimated by an iterative least squares fitting procedure. That is, pK_b (= pK_c) was allowed to vary until the value of $\{(pK_{1(fit)} - pK_{1(measured)})^2 + (pK_{2(fit)} - pK_{2(measured)})^2\}$ is minimized. Here, p$K_{1(fit)}$ and p$K_{2(fit)}$ are the values obtained by substituting the values of K_a and K_d (fixed as $10^{-8.15}$) and K_b and K_c (varied iteratively) into the equations for K_1 and K_2 described above, whereas p$K_{1(measured)}$ and p$K_{2(measured)}$ are the experimentally determined values.

The thus estimated microscopic pK_a values and fractions of cationic (f_C), neutral (f_N), zwitterionic (f_{Zw}), and anionic

Table IV. Membrane Distribution Coefficients for **1**–**8** at pH 7.4 and 4.0

Compound	log D_{DMPC} (pH 7.4)	log D_{DMPC} (pH 4.0)
1	2.62	2.51
2	2.96	2.77
3	2.55	2.45
4	2.61	2.57
5	2.90	2.60
6	2.52	2.20
7	2.93	2.61
8	2.51	2.09

Standard deviation in log D_{DMPC} determinations is ±0.10.

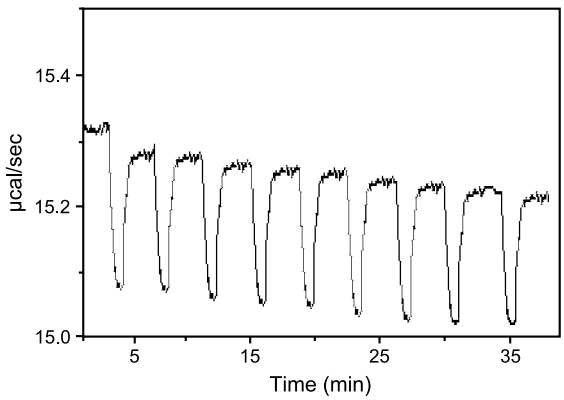

Fig. 4. ITC trace for the partitioning of a 120-µM solution of **1** into unilamellar vesicles of DMPC at 30°C (HEPES buffer, pH 7.40).

Table V. Thermodynamic Data for **1–8** at 30°C (HEPES Buffer, pH 7.4)

Compound	$\Delta G°_{part}$ (kJ mol^{-1})	ΔH_{part} (kJ mol^{-1})	$\Delta S°_{part}$ (J mol^{-1} K^{-1})
1	−15.2	−10.7	15
2	−17.2	−15.5	6
3	−14.7 (−14.7)	−10.1 (−9.7)	15 (17)
4	−15.1	−10.9	14
5	−16.8 (−17.0)	−7.1 (−7.7)	32 (31)
6	−14.6	−4.1	35
7	−17.0	−8.2	29
8	−14.6	−4.5	33

Values in parentheses refer to pH 7.4 (0.02 M phosphate, 0.10 M KCl). Standard deviation in ΔG_{part} is ±1 kJ mol^{-1}, ΔH_{part} is ±2 kJ mol^{-1}, ΔS_{part} is ± 5 J mol^{-1} K^{-1}.

(f_A) forms for **1–8** are listed in Table II and Table III. Inspection of Table III reveals that **1–4** are present predominantly in the cationic form [C] at pH 7.4 (and at pH 4.0), where both the secondary amino group and the phenolic group are protonated, i.e., f_C is > 0.5 at pH 7.4 and f_C = 1.0 at pH 4.0. These will be referred to as class 1 compounds from this point on. For **5–8**, the compounds are predominantly present in the cationic form at pH 4.0, where both the secondary amino group and the phenolic group are protonated, but not at pH 7.4; at the higher pH, f_C is low. For these compounds the predominant form is electronically neutral [N] at pH 7.4 (f_N > 0.5). These will be referred to as class 2 compounds from this point on.

ISOTHERMAL TITRATION CALORIMETRY (ITC) STUDIES

Distribution coefficients of **1–8** (D_{DMPC}) between multilamellar vesicle preparations of DMPC and HEPES buffer (pH 7.4) were measured at 30°C and pH 7.4. In addition, measurements of D_{DMPC} were made at pH 4.0 employing acetic acid/sodium acetate buffer; values are listed in Table IV. It has been shown that standard Gibbs free energies of partitioning of charged and uncharged compounds into multilamellar vesicles are equal to the free energies of partitioning of the same compounds into large unilamellar vesicles, produced by the extrusion method; although the corresponding enthalpies and standard entropies of partitioning may differ substantially (9,26). This potential for divergence in the latter parameters did not matter to the current study, as liposome distribution coefficients were used only to obtain values for the standard Gibbs free energy of partitioning. The compounds studied had relatively similar lipophilicities, so the same concentration of phospholipid was used for all measurements (1 mg/mL). The DMPC concentration was set such that ($PA_{initial}$ − PA_{part}) was readily measurable. The concentration of compound was chosen to ensure that the molar ratio of DMPC to bound compound was always greater than 60. This phospholipid/compound ratio should ensure that any perturbation of the bilayer structure is small and that the partitioning of charged species is not significantly diminished by the surface charge added to the bilayer by the associated compound. Studies involving the ionized forms of flunarizine, bibucaine and etidocaine have shown that the concentration dependence of phospholipid binding of these species is well modeled by the Gouy–Chapman theory, and indicate that a lipid/compound ratio greater than 60 is sufficient to ensure any concentration dependence of the partitioning will be insignificant (18,27). It is important to note that all of the compounds used in the current study were stable at both pH 7.4 and 4.0 over the time course of all experiments.

Table VI. Thermodynamic Data for **3–8** at 30°C (Acetic Acid/ Sodium Acetate Buffer, pH 4.0)

Compound	$\Delta G°_{part}$ (kJ mol^{-1})	ΔH_{part} (kJ mol^{-1})	$\Delta S°_{part}$ (J mol^{-1} K^{-1})
3	−14.8	−11.2	12
5	−15.1	−14.7	1
6	−12.8	−11.1	5
7	−15.1	−16.8	−5
8	−12.1	−10.7	5

Standard deviation in ΔG_{part} is ±1 kJ mol^{-1}, ΔH_{part} is ±2 kJ mol^{-1}, ΔS_{part} is ±5 J mol^{-1} K^{-1}.

The observed enthalpies for the partitioning (ΔH_{obs}) of **1–8** into unilamellar vesicle preparations of DMPC at pH 7.4 and at pH 4.0 for **3** and **5–8** were measured by using a high-sensitivity isothermal titration microcalorimeter (ITC). Fig. 4 shows an example of one such experiment. A series of ten injections of 10 μl of a 120-μM solution of **1** (in HEPES buffer) were made. The partitioning process for **1** is exothermic and is essentially constant for each injection, signifying that DMPC is in sufficiently large excess over added compound. The heat of dilution was determined by injecting the same compound solution into the sample cell containing only HEPES buffer; it was found to be only a small value for all compounds studied, less exothermic than −0.5 kJ mol^{-1}. This was subtracted from the observed enthalpy to give an apparent enthalpy, ΔH_{App}. The mem-

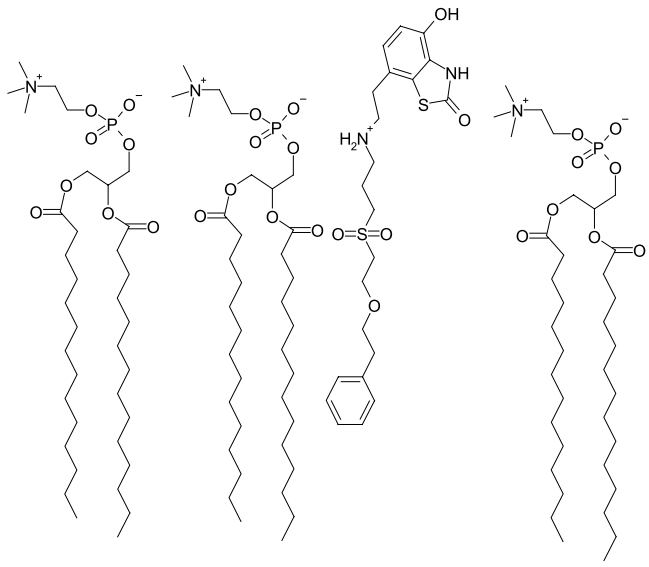

Fig. 5. Schematic representation of the electrostatic interaction between the phosphate head-group and the protonated amino-group of **3** at pH 4.0 and 7.4.

brane distribution coefficients determined for each of the compounds studied at 30°C were used to correct for the fraction of the compound bound to the liposomes (X_{DMPC}) using Eq. (2). This correction factor value was used to yield a true enthalpy of partitioning, ΔH_{Part}, at 30°C using Eq. (3). The fraction of bound compound was sufficiently high to minimize errors in the corrected enthalpies of partitioning.

$$\Delta H_{Part} = \left(\frac{\Delta H_{App}}{X_{DMPC}}\right) \tag{3}$$

Tables V and VI show the thermodynamic parameters of partitioning of **1**–**8** at pH 7.4 and of **3**, **5**–**8** at pH 4.0; the standard Gibbs free energy of partitioning, $\Delta G°_{part}$; the enthalpy of partitioning, $\Delta H°_{part}$, and the standard entropy of partitioning, $\Delta S°_{part}$. Measurements at pH 4.0 were only carried out for one compound (**3**) from class 1, as the extent of protonation will be very similar at pH 7.4 and 4.0. In addition, for compounds **3** and **5**, thermodynamic data are given at pH 7.4 employing phosphate buffer to see if there is any major buffer contribution to the measured enthalpies (contributions from the enthalpy of ionization of the buffer). Phosphate is known to have a very small enthalpy of ionization under these conditions, whereas HEPES has a much larger value (9). It can be seen that a change of buffer system has no significant affect on the thermodynamic parameters of partitioning. The standard Gibbs free energies of partitioning were calculated from the measured membrane distribution coefficients of **1**–**8** using Eq. (4). The standard entropies of partitioning were obtained from the thermodynamic relationship, $\Delta G°_{part} = \Delta H_{part} - T\Delta S°_{part}$.

$$\Delta G_{Part=-2.303\,RT\,\log\,D_{DMPC}} \tag{4}$$

The most salient features of the thermodynamic data of partitioning at pH 7.4 of **1**–**8** are that the enthalpies are all negative (an exothermic partitioning), whereas the standard entropies are positive. In addition, it is apparent that the enthalpies of partitioning are more negative for the class 1 compounds (**1**–**4**) than for the class 2 compounds (**5**–**8**), and the entropy changes are more positive for class 2 compounds than for class 1 compounds. For the thermodynamic data under acidic conditions (pH 4.0), the enthalpies of partitioning of class 2 compounds (**5**–**8**) are significantly more negative than at pH 7.4, resulting from the compounds being almost completely in the cationic form at pH 4.0. In addition, the entropies of partitioning are less positive for class 2 compounds at pH 4.0 than at pH 7.4.

In order to more rigorously determine whether there is statistically significant differentiation between the two compound classes employed in the current study, a two-tailed *t* test, for unpaired data and assuming equal variances in the thermodynamic data, was conducted on the data obtained at pH 7.4. In terms of the enthalpy of partitioning (at pH 7.4), the data from the class 1 compounds are significantly different from class 2 compounds ($p = 0.011$). The two compound classes also give significantly different entropies of partitioning ($p = 0.0002$) at pH 7.4. The standard Gibbs free energy of partitioning of the two compounds classes are not significantly different ($p = 0.82$). This is expected because the compounds were chosen at the outset to have similar membrane distribution coefficients, and hence similar standard Gibbs free energies of partitioning. It is therefore possible to say with statistical significance that there are differences, thermodynamically at pH 7.4, between class 1 and class 2 compounds, which is most clearly manifested in their standard entropies of partitioning and to a slightly lesser degree in their enthalpies of partitioning.

The observations are consistent with the notion of there being a favorable electrostatic interaction between the positively charged protonated secondary amino group of class 1 compounds at pH 4.0 and 7.4 (and for class 2 compounds at pH 4.0), and the negatively charged phosphate group of the DMPC molecules constituting the membrane bilayer. It is postulated that an ion–pair interaction is formed with the negative-charged phosphate group of membrane phospholipids, while orienting their lipophilic groups into the hydrophobic core of the bilayer. The closer in structure the compound is to the geometry of the phospholipid membrane, the more favorable the distribution of the compound in this interfacial way would be. This is shown schematically in Fig. 5. This interaction is not possible at pH 7.4 for the class 2 compounds **5**–**8**, and consequently they have a less exothermic value of ΔH_{part}. In addition, such an electrostatic interaction for compounds that are predominantly cationic might be expected to lead to a less favorable (less positive) entropy of partitioning compared to compounds that are predominantly electronically neutral because both the partitioned molecules and the phospholipid molecules would be in a more ordered state than for the case when such an electrostatic interaction is absent. In addition, it is evident that the thermodynamic driving force for partitioning of class I compounds at pH 7.4 is largely enthalpic, whereas for class 2 compounds at pH 7.4 it is mainly entropic. This pattern is consistent with a fairly simplistic interpretation that the class I compounds have a large electrostatic, and therefore enthalpic contribution to the free energy change. Conversely, the partitioning of class 2 presumably into the hydrophobic core of the membranes is largely due to the hydrophobic effect and is therefore dominated by an increase in entropy arising from removal of the hydrophobic compounds from water. However, the main aim of this study was to demonstrate that the two classes of compounds have different modes of interaction with the model membrane phase, which is evident in the different balance of enthalpic and entropic contributions; moreover, a rigorous mechanistic interpretation of these differences in not really possible with the observed enthalpy and entropy changes alone.

This interpretation is in agreement with an earlier study of the partitioning of amlodipine into phospholipid membranes (20). This study was conducted under conditions (pH 7.4 and 27°C) where the amino group is completely protonated, i.e., the cationic form of the molecule dominates. ^{2}H NMR indicated that amlodipine adopts a well-defined position in the membrane bilayer. In particular, the positively charged ethanolamine side group is located near the water–lipid interface interacting with the dipoles of the head-group region, according to a nonspecific electrostatic mechanism and inducing a reorientation of the phosphocholine dipoles toward the water phase. An analogous mode of binding to that proposed in the current study has also been postulated

for the partitioning into dioleylphosphatidylcholine (DOPC) of propanolol, lidocaine, and procaine at pH values where these molecules are protonated (28).

In a previous study, the thermodynamic parameters for the partitioning of a series of phenols and anisoles into DMPC have been determined (29). The compounds studied were chosen to investigate the effects of alkyl chain length, size of the substituent groups, substituent position on the aromatic ring, and the effect of hydrogen bonding groups within the molecules. The measured free energies, enthalpies, and entropies of partitioning were rationalized in terms of disruption of the ordered phospholipid membrane gel phase by the partitioned compounds, hydrogen bonding interactions between the phenolic group (and other polar groups within the molecules) and the phospholipid head-group region, and hydrophobic interactions between the liposome core and portions of the molecules that penetrate into this region. However, experiments were conducted below the phase transition temperature of 22°C (30), whereas data for the current study were determined above this temperature. Hence the two data sets are not comparable. In addition, the thermodynamic parameters in an earlier study were not obtained directly via a calorimetric technique; rather, the temperature dependence of the membrane distribution coefficients was used to generate van't Hoff plots. This gives considerably more error in the derived enthalpies and entropies of partitioning.

A number of studies (18–22) on the binding/partitioning of drugs into membrane bilayers indicate that at pH values where the compounds are charged, the enthalpy is negative, and conclude that enthalpy is the main driving force. Isothermal titration calorimetry was used in all of these studies, providing very accurate thermodynamic data. The current study is in agreement with the general observation of an exothermic partitioning process, i.e., a negative enthalpy, for compounds that are charged. It is evident that $\Delta H_{\text{part}} < 0$ for class I compounds at pH 7.4 and 4.0, and for class 2 compounds at pH 4.0, and additionally constitutes the main component of the thermodynamic driving force under these conditions.

CONCLUSIONS

It is apparent that there are significant differences in the thermodynamics of partitioning into phospholipid vesicles between compounds which, under a given set of conditions, are predominantly present in the cationic form and those which are predominantly present in the neutral form. These thermodynamic differences may relate to differences in the duration of action of compound classes in acting as β_2-adrenoceptor agonists and further support the plasmolemma microkinetic hypothesis for the duration of action of β_2 agonists (4).

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