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REVIEW

Model-free analysis of binding at lipid membranes employing micro-calorimetric measurements

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Abstract Based on universal thermodynamic principles (Schwarz in Biophys Chem 86:119–129, 2000) it is shown how measured enthalpy changes can be utilized to determine the relevant binding isotherm as well as the variation of the molar enthalpy change. This is carried out in a novel way involving multiple titration experiments whose evaluation requires no beforehand assumptions or models whatever. An appropriate specific model mechanism may be discussed afterwards and developed in view of the given experimental results. The pertinent procedure is demonstrated using micro-calorimetric data obtained in the case of the local anesthetic dibucaine as it associates with POPC liposomes. Mutual interactions of the bound ligand molecules could be described in terms of repulsive enthalpic and entropic activity coefficients. Apparently these are induced by electrostatic forces and by the finite size of binding sites, respectively.

Keywords Association isotherm · Binding enthalpy · Novel approach · Dibucaine · Molecular interactions · Activity coefficients

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Introduction

The availability of ultra-sensitive micro-calorimeter instruments rendered thermodynamic measurements popular in biophysics and biochemistry [2]. For example, successful areas are drug or peptide binding to liposomes. It was also very tempting to study protein binding. Concerning the data analysis, however, most of the researcher benefit from the built-in software. The latter requires titration over the entire range from very dilute to complete saturation. Often the entire range is not accessible for a number of technical or physical reasons, among others is the limited solubility, concentration dependent conformational changes of the binding site, oligomerization. The software allows data fitting according to several built-in binding models and provides an apparent binding constant which later has to be interpreted by the experimentalist. While this approach is mostly satisfactory it will lead to discrepancies in as much the degree of binding r deviates from a linear relation. For example, binding of a ligand to liposomes could be described in general by an association isotherm $r = f(C_f)$ where $r = C_{bound}/C_{lipid}$ and C_f stands for concentration of the free, C_{bound} for bound ligand and C_{lipid} for lipid concentration. Only in the concentration range where a linear relationship is observed the slope is called binding constant. Below we develop an approach to obtain the association isotherm requiring only titrations in a limited concentration range. We demonstrate how to analyze pertinent micro-calorimetric data so that the association isotherm as well as the functional course of $\Delta H_m(r)$ can be determined solely from measured data in a model free manner. In case of microcalorimetry the molar binding enthalpy ΔH_m had to be introduced as a function of the degree of binding r, which is needed to determine the thermodynamic association isotherm.

In order to demonstrate the overall procedure we have applied it to lipid membrane binding of dibucaine, a local anesthetics. Local anesthetics are known to block signal conduction in nerves. Apparently this is accomplished by suppressing the ion flux through sodium channels [3, 4]. The tertiary amine dibucaine belongs to this class of drugs. There is an extended hydrophobic chain that provides for a pronounced affinity with the core of lipid bilayers. Its amino head group has a pK of 8.85 [5] so that at a physiological pH of 5.5 a fully developed positive charge is formed (see Fig. 1). This cationic form was argued to be the more active state of the drug [6]. There is evidence that the phase transition temperature of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline (POPC) is reduced upon drug binding. Hence an anesthesia was suggested to result from an increase of liquid crystalline lipid causing a conformational change of Na^+-channels [7]. In view of a possible molecular mechanism basic research regarding the binding of dibucaine on phospholipid bilayers, especially liposomes, has been conducted for long in many laboratories. In a thermodynamic view the binding is actually an unspecific association, i.e., there are no specific binding sites. Such a process should rather be described in terms of a partitioning equilibrium of the substrate between the lipid and aqueous bulk phases, which can quantitatively be characterized by an appropriate partition coefficient. As a simple model one may employ a Scatchard plot approach [8]. This would be equivalent to a presentation of measured data by means of a Langmuir adsorption isotherm [5, 9]. It must be noted, however, that a model like that implicitly assumes a fixed number of specific binding sites as well as a lack of interactions between bound ligand molecules. Certainly such properties do not hold generally true for the kind of association being considered here. Electrostatic interactions of the charges on bound dibucaine were discussed with the Gouy–Chapman model applied to data obtained by dialysis [9].

Adsorption studies of membrane active molecules on liposome bilayers can easily be carried out exploiting the technical progress in the construction of micro-calorimeters. Thus very small heat quanta in a titration experiment

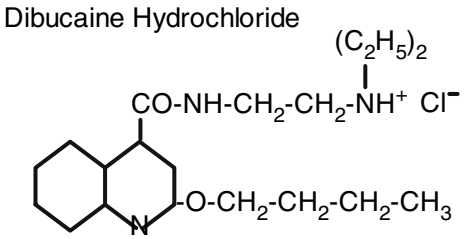

Fig. 1 Chemical structure of dibucaine hydrochloride at pH 5.5

of the binding process may be recorded. The measured data have been evaluated assuming that the molar binding enthalpy ΔH_m remains invariable in the course of increasing the surface density of bound ligand when adding more and more of the substrate to a given amount of lipid material [10]. Actually, however, one must expect molecular interactions, which result in changes of ΔH_m. Ignoring this point would lead to incorrect adsorption isotherms. In another study [11] this problem had been realized but was resolved by assuming a specific model implying a linear dependence of $\Delta H_m(r)$ on the molar fraction of bound ligand in the lipid phase.

In the present article we propose an extended experimental procedure and its pertinent quantitative evaluation, which is based on a universal theoretical conception [1]. It provides sufficient additional thermodynamic information to determine the association isotherm without using any presumptive properties of the underlying reaction mechanism. In other words, our approach does not require certain model specific knowledge whatever. In particular, also the variation of ΔH_m upon a progressive crowding of bound substrate can be ascertained directly. Furthermore quantitative results regarding activity coefficients of the lipid solvated ligand are available. We shall demonstrate a detailed version of the whole procedure here with the dibucaine-POPC liposome system. It could be applied most generally to other binding/adsorption reactions as well.

A molecular binding model that fits the observed data can possibly be elaborated afterwards. Activity coefficients may be interpreted in terms of the evolving molecular interactions of bound ligand.

Theoretical basis

We consider the given lipid bilayer phase in the course of binding a ligand through some kind of association. This takes place as a partitioning process out of an adjoining non-lipid (usually aqueous) phase. Our experiments are done at constant pressure and temperature. The resulting enthalpy change ΔH as an extensive thermodynamic function depends on the extensive variables n_L (mol of total lipid) and n (mol of bound ligand). Thus for a small change due to an increase of n (at constant n_L) one has

$$dH = \Delta H_m \cdot dn$$

where $\Delta H_m = \frac{\partial H}{\partial n} = f(n_L, n)$ is the molar enthalpy of binding.

Under the present circumstances both molar amounts are the only variables of state being sufficient to describe the equilibrium properties of the given binding system. As

ΔH_m is an intensive function of state, it only depends on the ratio of n and n_L. In other words $\Delta H_m = f(r)$ where $r = \frac{n}{n_L}$ is the appropriate binding ratio.

Now the above dH can easily be integrated while keeping this r constant, resulting in the fact that any enthalpy change can be described as

$$\Delta H = \Delta H_m \cdot n. \tag{1a}$$

When dividing this relation by n_L one obtains a quantity

$$Q(r) = \frac{\Delta H}{n_L} = \Delta H_m(r) \cdot r \tag{1b}$$

which proves to be the product of so far unknown terms, namely r and $\Delta H_m(r)$, respectively. In order to calculate r one must know $\Delta H_m(r)$!

Apparently such Q can be experimentally obtained in a titration experiment with added ligand at a fixed value of n_L on the basis of the measured ΔH and the known value of given n_L. This variable Q is to be recorded as a function of C_{Lig} while paying attention to the gradually diluted C_{Lip} after each injection of more ligand (C_{Lig} and C_{Lip} standing for actual total concentrations of ligand and lipid in the whole measuring cell system). Such titration routines are to be repeated for a number of sufficiently different fixed n_L.

Subsequently one may take advantage of the mass conservation principle, namely

$$C_{Lig} = r \cdot C_{Lip} + C_f \tag{2}$$

where C_f denotes the free ligand concentration in the non-lipid phase (usually an aqueous buffer). Accordingly various plots of C_{Lig} versus C_{Lip} are to be drawn from titrations with different n_L but the same value of Q. Those plots must result in straight lines since constant Q implies invariant r and C_f because of their functional relationship according to the existing partitioning isotherm $r = f(C_f)$. Based on such linear mass conservation plots the variable r can be determined from the slope and C_f from the intercept on the ordinate axis [1]. In the range of available Q the partitioning isotherm (''binding curve'') can so be determined without knowing any other information. In particular, no specific assumptions regarding $\Delta H_m(r)$ are required.

Furthermore one has

$$\Delta H_m(r) = \frac{Q(r)}{r} \tag{3}$$

(see Eq. 1b) so that one obtains the molar enthalpy change as it possibly varies upon an increasing degree of binding.

Materials and methods

A dibucaine (Sigma, Taufkirchen, Germany) stock solution C^o_{Dib} = 6 mM was prepared in 50 mM phosphate and 100 mM NaCl, and buffered at pH of 5.5. POPC in chloroform was obtained from Avanti Polar Lipids (Birmingham, AL). Chloroform was removed from the POPC stock solution by evaporation under a stream of nitrogen gas. The resulting thin film was dried under vacuum for several hours and then hydrated with a 50 mM phosphate, 100 mM NaCl pH 5.5 solution. To ensure a homogeneous liposome population, freeze–thaw cycles and extrusion on a 200 nm cut-off membranes were carried out.

The ITC experiments were performed with a VP-ITC micro-calorimeter (Microcal Northampton, MA, USA). The sample cell (starting volume V_o = 1.437 ml) was filled with the phospholipid solution while the titration syringe (296 µl) contained the dibucaine stock solution. The binding was monitored at 27°C. A given preparation was used in order to generate a number of basic lipid concentrations C^o_{Lip} to be filled initially in a measuring cell. Then the system contains a fixed lipid amount of $n_L = V_o \cdot C^o_{Lip}$ in the course of a given titration.

Three titration series were completed with five different starting concentrations C^o_{Lip}. Each of the three stocks was differentiated only by their preparation history, namely

1. Stock A1: Being titrated without delay on the day of extrusion with C^o_{Lip} = 11.11, 15, 16.65, 25, 33.33 mM, respectively.
2. Stock A2: An aged mode of A1, titrated 2 days later with C^o_{Lip} = 10, 15.385, 20, 22.20, 33.33 mM, respectively.
3. Stock B: Another independently extruded stock, also studied right after preparation, with C^o_{Lip} = 10, 15.385, 22.20, 33.33, 50 mM, respectively.

This did evolve from the suspicion that according to the stock preparation history the binding properties may be different. We surmised that the extruded lipid bilayer would be generally generated in structurally somewhat diverse metastable states.

In any of these cases the total ΔH was determined after an injection of a total volume ΔV (in successive steps) from a dibucaine stock solution C^o_{Dib} = 6 mM. Apparent equilibration did occur after less than 2 min in each single injection.

The dilution resulting from the injection procedure caused only negligible heating. Thus all the measured ΔH is due to binding. On the other hand, the relevant total dibucaine and lipid concentrations in the measuring cell have been recalculated by means of the equations

$$C_{\mathrm{Dib}} = (\Delta V/(V_0 + \Delta V)) \cdot C^{\mathrm{o}}_{\mathrm{Dib}}$$
$$C_{\mathrm{Lip}} = \{\ 1\ -\ (C_{\mathrm{Dib}}/C^{\mathrm{o}}_{\mathrm{Dib}})\}\ \ C^{\mathrm{o}}_{\mathrm{Lip}}.$$

In a titration series with a given lipid amount all the observed enthalpy changes have turned out to be negative. For the sake of simplicity we have therefore chosen to consider a pertinent positive version $Q = -\Delta H/n_{\mathrm{L}}$.

Results

Titrations and mass conservation plots

Five titration experiments at different lipid concentrations were completed for each lipid stock solutions. Those related to stock A1 are shown in Fig. 2 as an example.

For a constant value of Q (see selected dashed horizontal lines in Fig. 2), where r as well as C_{f} is invariant, the interpolated C_{Dib} are then plotted versus C_{Lip}. According to Eq. 2 this is expected to result in a straight line with a slope of r and an ordinate intercept of C_{f}. Such mass conversation plots for a number of Q are displayed in Fig. 3. They are indeed exceedingly linear as is observed in all our measurements of relevant data no matter which stock is considered.

The reverse version of titration, namely small amount of lipid being injected into a dibucaine solution is also possible. This can quite analogously be analyzed to determine $Q(r)$-quantities leading to the same results. Then plots of Q versus C_{Lip} display a decreasing functional course instead of an increase versus C_{Dib} as observed in Fig. 2. This does not really offer any significant advantage over the version, which we have preferred.

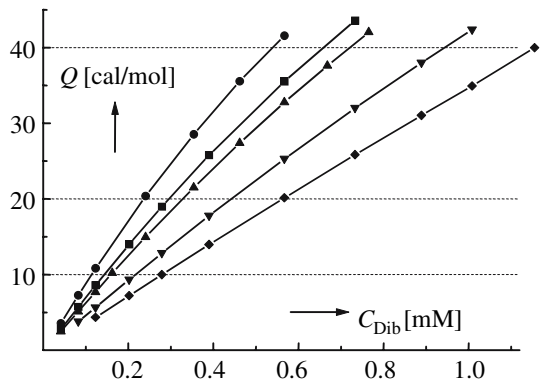

Fig. 2 Plots of the parameter Q (cal/mol) for a total amount of lipid derived from various $C^{\mathrm{o}}_{\mathrm{Lip}}$ (mM) = 11.11 (*filled circle*), 15 (*filled square*), 16.65 (*filled triangle*), 25 (*filled inverted triangle*), 33.3 (*filled diamond*) versus the established total dibucaine concentrations as measured with lipid stock preparation A1

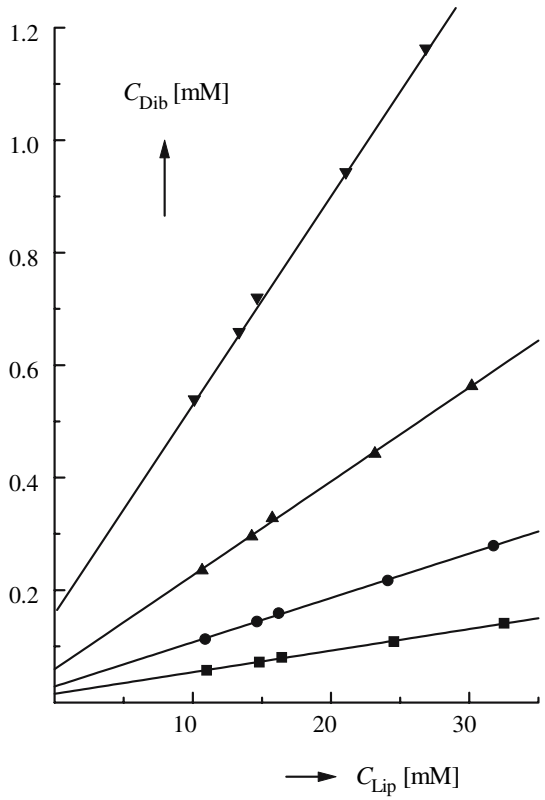

Fig. 3 Mass conservation plots of total C_{Dib} versus actual C_{Lip} for various selected parameters Q (cal/mol) = 5 (*filled square*), 10 (*filled circle*), 20 (*filled triangle*), 40 (*filled inverted triangle*) in the case of lipid stock preparation A1

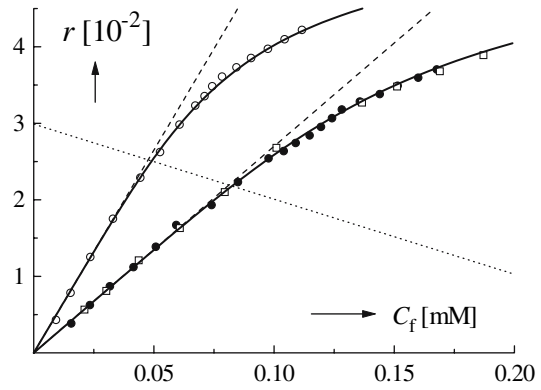

Fig. 4 Apparent association isotherms (binding ratio vs. free dibucaine concentration) for each of the three lipid stock preparations: A1 (*filled circle*), A2 (*open circle*), B (*open square*)

The association isotherm

Applying linear regression analysis to the mass conservation plots the association/partitioning isotherm (resp. ''binding curve'') could be evaluated. The results are depicted in Fig. 4. In case of the aged stock A2 (open circles) the binding turned out to be much more effective than for

the fresh stock A1 (solid circles). However, the findings with the other freshly prepared stock B (open squares) do practically reproduce those of stock A1. In any event there is an initially linear course through the origin. Without further relevant information such observation might perhaps be attributed to an ideal partitioning isotherm, namely $r = K_p \cdot C_f$ with a partition coefficient K_p (see ''Discussion''). That quantity would be equal to the slope indicated by dashed lines in the figure. In this way one obtains a $K_p = 270\ M^{-1}$ (A1, B) and $530\ M^{-1}$ (A2), respectively.

Deviations from linearity being observed at higher binding ratios may in the first place be generally expressed by an activity coefficient $\alpha = K_p\ (C_f/r)$ (see Eq. 6 in ''Discussion''). It arises from mutual interactions of bound ligand molecules. The obvious downward curvature beyond about $r = 0.022$ in Fig. 4 results in an increasing $\alpha > 1$ indicating more and more mutual repulsion of bound ligand when the molecules become increasingly crowded upon a growing binding ratio. The activity coefficients as they have been determined in this way from the present partitioning data are presented in Fig. 5. They have shown practically the same dependence on r for each of the stocks studied in spite of the fact that two of the K_p are considerably different. The various results can quite satisfactorily be fitted in an empirical way by the functional relation

$$1/\alpha = 1 - 9.3 \times 10^4 \cdot r^4.$$

The r^4-dependence being involved in the fit strongly points to a failure of the Langmuir isotherm model where a linear course of $1/\alpha$ should apply. The present considerably larger exponent apparently reflects the absence of defined specific binding sites. This implies that in approaching the maximum binding capacity it needs more and more thrust to squeeze a drug molecule between the already bound ones that have largely exhausted the open membrane area being still accessible to further binding.

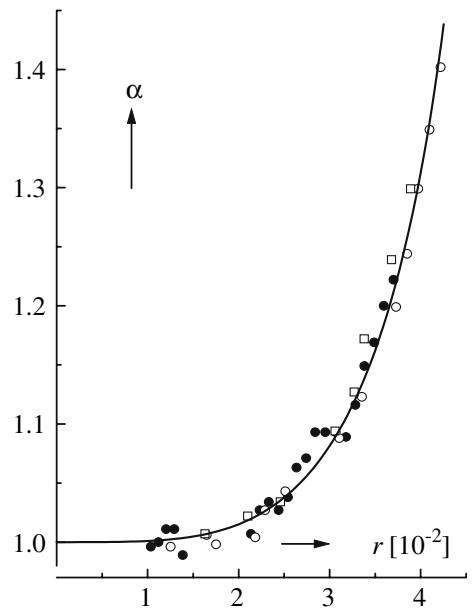

Fig. 5 Apparent activity coefficients versus binding ratio for each of the three lipid stock preparations: A1 (*filled circle*), A2 (*open circle*), B (*open square*)

Now we have to realize that the present evaluation of binding parameters can only be taken as a preliminary interpretation. The additional information supplied by our findings as for ΔH_m (see below) call for an improved analysis concerning K_p and α. A more detailed argumentation is given in ''Discussion'' and ''Appendix''.

Calculation of binding strength

Nevertheless, with the so far tentative results regarding K_p and α the actual binding properties can be determined for a given pair of total C_{Dib} and C_{Lip}. One has to take into account the mass conservation relation according to

$$r = r_o - C_f/C_{Lip} \quad \left(r_o = C_{Dib}/C_{Lip}\right).$$

This implies a straight line in the r, C_f coordinate system between the two intercepts r_o (the largest possible binding ratio) and C_{Dib} on the ordinate and abscissa axes, respectively. Its intersection point with the binding curve amounts to the actual values of r, C_f. A practical example for $C^o_{Dib} = 0.3$ mM and $C_{Lip} = 10$ mM is demonstrated in Fig. 4 where the condition of mass conservation is indicated by a dotted line.

Naturally the appropriate r, C_f can also be calculated numerically based on those two functional relationships. In particular, one obtains the mol fraction of bound ligand as

$$C_b/C_{Dib} = x_b = K_p C_{Lip}/\left(\alpha + K_p C_{Lip}\right)$$
$$\left(\text{where } C_b = C_{Dib} - C_f\right).$$

Thus the highest binding activity at a given C_{Dib} will be encountered here below $r \approx 0.02$ where $\alpha = 1$. This ideal binding accordingly takes place with stocks A1, B up to about $C_{Dib} = 0.02\ C_{Lip} + 0.075$ mM, and $C_{Dib} = 0.023 \cdot C_{Lip} + 0.045$ mM for stock A2.

In case of $C_{Lip} = 10$ mM and $C_{Dib} < 0.275$ mM it results in 73/84% bound dibucaine, respectively.

With 50 mM lipid the ideal case does extend to $C_{Dib} \approx 1.1$ mM resulting in 93.1/96.4% binding.

Once one injects a total of 2 mM dibucaine a repulsive non-ideal $\alpha = 1.21/1.3$ at $r = 0.036/0.039$ has to be taken into account. This does, however, reduce the relative amount of bound dibucaine merely a little to 91.8/95.3%.

Generally one can conclude that in the present experimental concentration ranges the binding proves to be rather

strong. In other words, only comparatively low free ligand concentrations are left after the binding equilibrium has been established.

The molar enthalpy of binding

Applying Eq. 3 to the present results one obtains $-\Delta H_m(r) = Q/r$ as a function of the actual binding ratio. A pronounced practically linear growth was obtained starting at an initial ΔH_m^o in the limit of low r as illustrated in Fig. 6. It is expressed by a fit according to

$$\Delta H_m = \Delta H_m^o + \beta \cdot r \tag{4}$$

where $\Delta H_m^o/\beta$ (kcal/mol) = 1.325/6.92 (A1), 1.19/5.98 (A2), 0.907/7.00 (B).

The solvation energetics apparently changes upon more and more binding. Furthermore the present results clearly indicate that a non-ideal component has to be considered right from the low binding range. Otherwise a constant value of ΔH_m should be observed there. This point is not apparent in the measured binding curve as such. It will be investigated in some detail in ''Discussion''.

Implication of preparation history

As mentioned before in the ''Materials and methods'' we have repeated the present measuring routine with three slightly diverse liposome stock solutions that had been subject to a different treatment though originally prepared in the same way with uniform amounts of the various material components.

In each case the experimental measuring procedure could be carried out smoothly according to the procedures described above. The respective mass conservation plots always proved to be excellent straight lines perfectly in line with those shown in Fig. 3. Furthermore, the binding properties such as the association isotherms and molar enthalpy changes being evaluated from the pertinent data reflect analogous qualitative features. There are, however, differences in a certain sense regarding the relevant quantitative results.

In this context one has to bear in mind that the lipid bilayer product is not delivered in a thermodynamically stable equilibrium. One must expect variations of a metastable structural state depending on peculiar preparation coincidences and aging processes. Thus it appears to be quite reasonable to encounter a certain scattering of binding parameters when comparing the results obtained with liposome stock solutions having gone through a somewhat different past history.

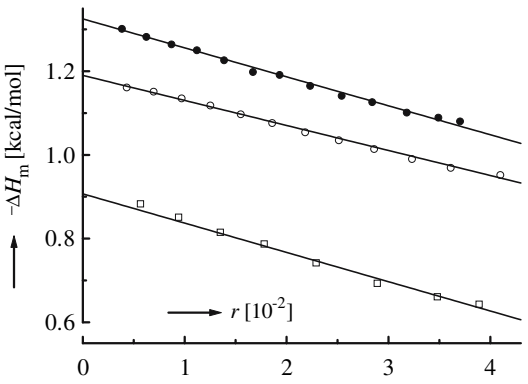

Fig. 6 Molar binding enthalpies as a function of the binding ratio for each of the three lipid stock preparations: A1 (*filled circle*), A2 (*open circle*), B (*open square*)

Discussion

The present system is considered to comprise two phases in a metastable thermodynamic equilibrium. In other words, there may be structural changes owing to aging or a repeated fresh preparation.

Bound dibucaine is solvated in the lipid moiety whereas the free molecule is dissolved in an aqueous medium (buffer). In the latter phase the concentration is low enough to permit a thermodynamically ideal characteristics. Thus the appropriate chemical potential can be expressed as

$$\mu_f = \mu_f^{\varnothing} + RT \cdot \ln\ C_f \tag{5a}$$

($\mu_f^{\varnothing}$ is relevant constant standard potential). In the lipid phase mutual interaction of bound ligand must be taken into account as quantitatively described by an average activity coefficient α depending on r so that

$$\mu_b = \mu_b^{\varnothing} + RT \cdot \ln(\alpha\, r). \tag{5b}$$

In the established partitioning equilibrium one has $\mu_f = \mu_b$ leading to a general relation for the association isotherm, namely $\alpha\ r = K_p \cdot C_f$ with an ideal partition (''binding'') constant

$$K_p = \exp(-\Delta\mu^{\varnothing}/RT) \tag{6}$$

($\Delta\mu^{\varnothing} = \mu_b^{\varnothing} - \mu_f^{\varnothing}$). A value of $\alpha = 1$ (at sufficiently low r) indicates ideal partitioning. This implies a partition isotherm (i.e., r versus C_f) that runs as a straight line through the origin. Mutual repulsion of bound ligand is reflected in $\alpha > 1$ whereas attraction results in $\alpha < 1$. The latter would especially occur in the event of aggregation. Such was, however, not observed here.

The increase of binding enthalpy due to the repulsion exerted by electrical charges on the bound ligand can be

discussed in terms of the Gouy–Chapman model. This leads to a respective activity coefficient

$$\alpha_E = \exp\left\{2v \cdot \sin\ h^{-1}(vb \cdot r)\right\}. \quad (7a)$$

It involves the effective charge number per molecule v, which according to previous experience usually turns out to be somewhat smaller than the actual physical value. In addition we have here $b = 3.54/(I/\mathrm{M})^{1/2}$, a factor depending on the ionic strength I of the buffer. With our $I = 0.15$ M we have $b = 9.14$. A detailed account of these fundamentals together with practical applications has been given in the literature [12, 13]. In the present case of one positive charge on dibucaine where $v < 1$ and $r < 0.04$ one may simply set

$$\alpha_E = \exp\left(2v^2 \cdot b \cdot r\right). \quad (7b)$$

Indeed we have observed a gradual increase of enthalpy upon more binding that implies a pertinent activity coefficient

$$\alpha_H = \exp\left(\frac{\beta}{RT} \cdot r\right) \quad (7c)$$

(see Eq. A2b in ''Appendix''). Therefore we can set the enthalpic $\alpha_H = \alpha_E$ with a charge number $v = 0.77$ when taking our $\beta \approx 6.5$ kcal/mol (see data of Eq. 4). This value of v is quite reasonable under the given circumstances. Thus we attribute the present enthalpic non-ideality to electrostatic repulsion. This leads to a modified association isotherm, namely

$$\alpha_H(r) \cdot r = K_p \cdot C_f \quad (8)$$

taking into account those non-ideal interactions that are apparently effective already in the low r-range.

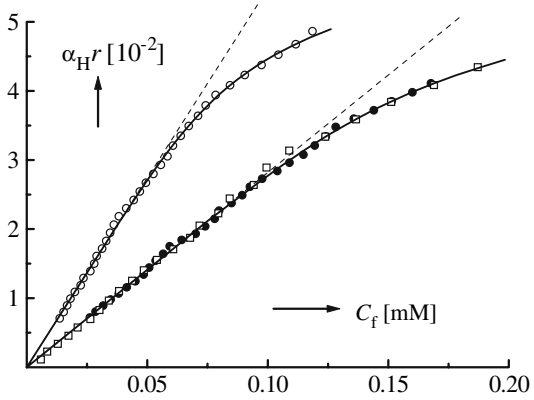

Fig. 7 Modified association isotherms taking into account the enthalpic activity coefficient for each of the three lipid stock preparations: A1 (*filled circle*), A2 (*open circle*), B (*open square*)

Thus the actual value of K_p has to be determined from a plot of $\alpha_H \cdot r$ versus C_f as demonstrated by the dashed slopes in Fig. 7. The now improved $K_p = 282\ \mathrm{M}^{-1}$ (A1, B) and $K_p = 548\ \mathrm{M}^{-1}$ (A2) are found to be slightly larger than the previous tentative values. On the other hand the curves clearly demonstrate the existence of another repulsive activity coefficient that comes into play at a larger degree of binding.

Possibly this results from the finite size of a bound ligand molecule. It implies a restriction of free area for further binding when there is already some area not accessible for binding due to previously bound ligand. It reduces the binding entropy ΔS generating a specific $\alpha_S(r)$ so that the total $\alpha = \alpha_H(r)\ .\ \alpha_S(r)$. Then our data lead to

$$\alpha_S(r) = K_p \cdot C_f/(\alpha_H(r) \cdot r) \quad (9)$$

as displayed in Fig. 8. There seems to be no significant difference between the three stocks.

We have investigated this problem in terms of $\sigma(r)$, the reduced fraction of available binding area. The general approach resulting in a pertinent activity coefficient α_S is described in ''Appendix'' (see Eq. A4). Choosing a special function

$$\sigma = 1 - (r/r_\infty)^6$$

we obtain

$$\alpha_S = \frac{1}{\alpha} \cdot \exp\left\{6 \cdot \left(\frac{1}{\sigma} - 1\right)\right\} \quad (10a, b)$$

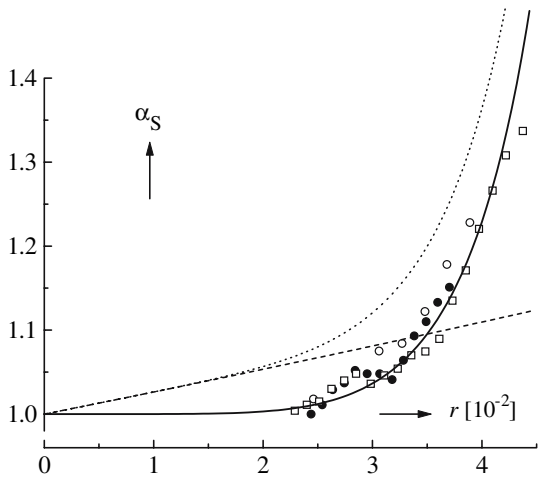

Fig. 8 Entropic activity coefficient versus binding ratio for each of the three lipid stock preparations: A1 (*filled circle*), A2 (*open circle*), B (*open square*). The *dashed line* indicates the enthalpic (Gouy–Chapman) activity constant. The *dotted curve* stands for the total combined $\alpha = \alpha_H\ \alpha_S$

in order to fit the very steep increase of α_S close to $r \rightarrow r_\infty$. The points in Fig. 8 are fitted with $r_\infty = 0.072$. Thus an average binding site actually can be extrapolated to cover roughly 14 lipids on the whole, i.e., about 7 of them on the outer layer.

Certainly our ''apparent size of a binding site'' is an empirical parameter. It simply gives an idea how many lipid molecules do on the average tally with a bound dibucaine molecule under saturating conditions.

As far as the apparent loss of binding area upon the increase of binding degree is concerned we have on a trial basis quantitatively discussed an entropically founded α_S that does fit our data. This is of course a formal measure since an underlying molecular model mechanism is so far missing.

At this point we note some divergent findings having been reported in the literature regarding binding isotherms in similar cases. A study of practically the same dibucaine-POPC system though using different methods (ultracentrifugation, UV-spectroscopy) [14] has presented partitioning isotherms looking like ours and reflecting as well a K_p of about the same level. However, there is a peculiar distinction as far as the concentration ranges are concerned. The relevant C_f are by one order of magnitude larger. Furthermore the binding capacity r_∞ comes up to values around 0.5. In other words, there must be a potential of two lipid molecules binding one dibucaine. This observation is actually confirmed by data obtained with the related phospholipid DMPC [5]. Apparently the application of much larger dibucaine concentrations than were used in the present study causes a structural change of the liposomes, possibly micellation, which permits markedly more binding capacity than a simple bilayer. In this view we believe that under our comparatively low drug concentrations dibucaine still associates with an essentially intact membrane.

Appendix

As mentioned above the lipid associated dibucaine is evidently subject to thermodynamically non-ideally conditions owing to mutual molecular interactions. This can be described in terms of an activity coefficient α in the formulation of the pertinent chemical potential. The latter is defined as

$$\mu_b = \frac{\partial H}{\partial n} - T \cdot \frac{\partial S}{\partial n} \quad (\text{at constant } T,\ n_L) \tag{A1}$$

arising from enthalpic and entropic sources, respectively.

The first one will produce a non-ideal part that manifests itself in a dependence of the molar enthalpy change on the binding ratio r. In the present case we have according to Eq. 4

$$\frac{\partial H}{\partial n} = \Delta H_m^o + \beta \cdot r = \Delta H_m^o + RT \cdot \ln\ \alpha_H$$
$$\text{with } \alpha_H = \exp\left(\frac{\beta}{RT} \cdot r\right). \tag{A2a, b}$$

Actually this α_H has already been shown to coincide with the activity coefficient emerging from the Gouy–Chapman model describing electrostatic repulsion of charged molecules.

In the second place we note that the gradual decrease of available free binding sites in the course of more and more bound ligand does affect the entropy S of the system. This implies a repulsive activity coefficient $\alpha_S \rightarrow \infty$ that finally precludes binding above a certain maximum capacity r_∞. For a quantitative analysis one may start from Boltzmann's formula regarding the small increase of entropy in the event of one ligand molecule being associated with a lipid area covering one mol of lipid, i.e., $dS = k\ \ln\omega_o$. The quantity ω_o is the factor by which the degree of degeneration Ω (''the number of equivalent quantum states'') is enhanced in the first basic binding step. If one deals with an amount of n_L mol lipid that factor will be proportional to the actually available binding area. This amounts to $\omega = \sigma\ n_L\omega_o$ where $\sigma \leq 1$ is to account for the fraction of lipid area that is actually open to binding. Now the increase of entropy owing to N bound molecules which are indistinguishable can be calculated as

$$\Delta S = k \cdot \ln\ \left(\omega^N/N!\right) = n \cdot R \cdot \left\{\ln\left(\frac{e\omega_o}{N_A}\right) + \ln\frac{\sigma}{r}\right\} \tag{A3}$$

when taking advantage of Stirling's formula (N_A: Avogadro's number). By differentiation of ΔS one eventually arrives at

$$\frac{\partial S}{\partial n} = \Delta S^\varnothing - R \cdot \ln\left(\alpha_S \cdot r\right) \tag{A4a}$$

with a standard molar entropy of binding

$$\Delta S^\varnothing = R \cdot \ln\left(\frac{\omega_o}{N_A}\right). \tag{A4b}$$

An entropically originated activity coefficient thus becomes

$$\alpha_S = \frac{1}{\sigma} \cdot \exp\left\{-\frac{d\ln\sigma}{d\ln r}\right\}. \tag{A4c}$$

With our chosen version of $\sigma(r)$ according to Eq. 10a this leads to Eq. 10b.

Taking into account the above enthalpy and entropy contributions the chemical potential of bound ligand turns out as

$$\mu_b = \mu_b^{\varnothing} + RT \cdot \ln(\alpha_H\, \alpha_S \cdot r) \quad \left(\mu_b^{\varnothing} = \Delta H_m^{o} - T \cdot \Delta S^{\varnothing}\right). \tag{A5a}$$

Now the equilibrium condition $\mu_b = \mu_f$ results in

$$\alpha_H \alpha_S \cdot r = K_p \quad C_f. \tag{A5b}$$

This means we have the apparent overall activity coefficient α split up in terms of the two factors α_H and α_S where the first one is largely linearly dependent on the binding ratio whereas the latter one becomes especially momentous when the number of available free binding sites comes close to nil.

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