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Interaction of amphiphiles with integral membrane proteins. II. A simple, minimal model for the nonspecific interaction of amphiphiles with the anion exchanger of the erythrocyte membrane

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In a previous paper we have reported on the structural perturbation of the erythrocyte membrane anion exchanger by a regular series of model amphiphiles, as shown by differential scanning calorimetry (Gruber, H.J. and Low, P.S., Biochim. Biophys. Acta, preceding article). Now the data are interpreted by a model in which the effects of amphiphile structure upon buffer-membrane partitioning are well separated from the dependence of the intrinsic potencies of membrane-bound amphiphiles upon amphiphile structure. The buffer-membrane partitioning situation was demonstrated to regularly change between extremes within a series of homologous amphiphiles, i.e. from a negligible to a predominant fraction of total amphiphile in the sample residing in the membrane. Based upon this demonstration a large number of reports on the chain length dependence of apparent potency could be reinterpreted in terms of chain length profiles of intrinsic potency, allowing for a comparison of the responses of various membrane proteins to homologous series of amphiphiles. The response patterns for chain length variation could be divided into three distinct classes: the intrinsic potency (i) can be independent of chain length over a very wide range of length, (ii) it can be rather independent up to a critical length where a sudden cut-off in potency occurs, or (iii) it can drop monotonically over a wide range of chain length. The intrinsic potency values of saturated fatty acids in destabilizing the anion exchanger were interpreted by very simple assumptions: only direct interactions between amphiphiles and target proteins and a simple amphiphile partition equilibrium between a pool of equivalent low affinity sites on the protein and the bulk lipid matrix. The observed monotonic decay of the intrinsic potency of saturated fatty acids with increasing chain length from C_8 to C_{20} was translated into a constant increment of free energy by which each additional CH_2 favors the transfer away from sites on the protein towards the bulk lipid matrix. Arguments were presented suggesting that the direct interaction between amphiphiles and target protein is completely nonspecific for alkyl chain length while the residual specificity for shorter over longer amphiphiles is due to the higher tendency of longer chains to preferentially bind in the bulk lipid matrix. Thus a completely new role of the lipid as a competitor, rather than a mediator, was postulated.

Introduction

Small amphiphilic molecules produce numerous effects in biological membranes by directly or

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indirectly affecting both protein and lipid components in structure and function. Only in those few cases where a distinct receptor site with high affinity for amphiphilic drugs has been identified has the mechanism of amphiphile action be elucidated [1–5]. The majority of amphiphile effects in biomembranes, however, occurs at rather high membrane concentrations of amphiphiles which fall into the range of the Meyer-Overton rule for anesthesia, i.e. with several mol% of anesthetic being dissolved in the membrane [6–17]. Due to the low affinities of amphiphiles for most of their potential target sites in membranes their mechanism of action has remained a matter of considerable debate. Two types of hypotheses have coexisted for a long time, which claim that the action of amphiphiles occur either directly on the perturbed proteins [8,9,12,13,17–26] or that amphiphile actions on proteins are generally indirect, with the lipid phase acting as a mediator [6,27–29].

A particular experimental strategy has frequently been employed to resolve this question; it is best described by the term 'target site mapping' and involves the use of regular series of amphiphiles to probe the nature of the hypothetical target site for steric and other characteristics [30]. Apart from charge [18,19,23], the variation of chain length and double bonds have proven to be useful to 'map' target sites of amphiphilic drugs [5,7,8-17,21,31–33].

In a preceding study we have reported the structural perturbation of the erythrocyte membrane anion exchanger by regular series of model amphiphiles, all of which are alkane derivatives. Now a model is presented to quantitatively evaluate the role of alkyl chain length in determining the intrinsic potency of homologous amphiphiles. The model separates the 2-fold consequence of chain length variation into the effect on buffer-membrane partitioning and the effect on intrinsic potency. Furthermore, intrinsic potencies are interpreted in a quantitative way. The underlying assumptions are extremely simple: only direct drug–protein interaction and a simple drug partition equilibrium between equivalent sites on the protein and the bulk of the membrane. Within this concept the lipid is assigned a new role in determining the 'intrinsic' potency patterns of the target sites, although as a competitor with the protein for amphiphile binding, rather than as a mediator.

Theoretical

Our model is devised to explain the structural perturbation of an integral membrane protein as a consequence of the presence of amphiphiles in the membrane. As shown in Fig. 1A, it is assumed that the total amount of amphiphilic drug (D) is distributed between the buffer and the membrane phase, indicated by the subscripts b and m, respectively. Furthermore, it is proposed that the membrane-bound drug population is distributed between generic sites on the target protein (subscript a) and other sites in the bulk lipid matrix (including sites on other membrane proteins, subscript m). The model assumes that both distribution equilibria can be described by simple partition constants.

Buffer-membrane partitioning

The data to which this model has been applied allow for a separate treatment of the buffer-membrane partitioning without having to simultaneously analyze the situation within the membrane [34]. The reason is the following: the drug effect which we have monitored is the shift in the protein's denaturation temperature (ΔT). This

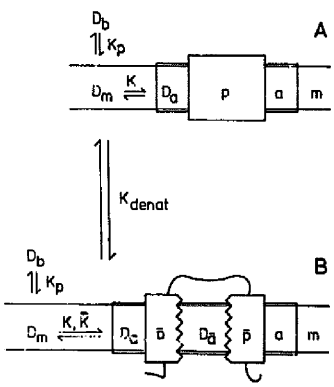


Fig. 1. Partition equilibria of amphiphilic drugs (D) between the buffer phase (b), the bulk lipid matrix (m), and 'annular' or similar sites (a) on the protein of interest (p). (A) Native state; (B) denatured state.

temperature shift has regularly been found to linearly depend on the concentration of amphiphile within the membrane. The slope of the linear titration curve can thus be identified with the intrinsic potency (P) of any particular amphiphile. Combination of this empirical result with the concept of simple buffer-membrane partitioning (partitioning constant K_p) yields

$$\Delta T = PK_p \frac{N_t}{V_t + V_m(K_p - 1)} \quad (1)$$

where N and V mean moles of amphiphile and volume, respectively, and the subscripts t and m refer to total sample and to membrane, respectively (for a derivation see Ref. 34). With this equation it is possible to calculate K_p and P while P can be analyzed further in a separate step, due to the concentration independence of P.

For several reasons discussed below we found it important to demonstrate the strong influence of the membrane/buffer ratio on the apparent effectiveness of amphiphiles. A good graphical representation thereof is provided by the simulation of the 'classical plot' of apparent drug potencies versus chain length of homologous amphiphiles. Commonly 'apparent potency' is used as a sloppy term for 'equieffective drug concentration' which actually is the reciprocal of the former. We arbitrarily defined equieffective amphiphile concentrations as those which lowered the membrane protein denaturation transition by 1 C°. Eqn. 1 can be rearranged to calculate these equieffective concentrations from the model parameters P and K_p:

$$\frac{N_t}{V_t}(\Delta T = -1\,\mathrm{C}^\circ) = \frac{1}{-P} \frac{1 + (V_m/V_t)(K_p - 1)}{K_p} \quad (2)$$

From the definition of intrinsic potency it follows that the corresponding equieffective concentration within the membrane is given by

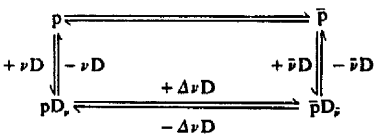
$$\frac{N_m}{V_m}(\Delta T = -1\,\mathrm{C}^\circ) = \frac{1}{-P} \quad (3)$$

In order to visualize the effect of partitioning alone the relative amount of membrane-bound amphiphiles can be plotted against chain length for identical experimental conditions. The corresponding expression is obtained by rearrangement of Eqn. 1 in Ref. 34:

$$\frac{N_m}{N_t} = \frac{K_p}{(V_t/V_m) + K_p - 1} \quad (4)$$

Model for the shift in the denaturation temperature of an integral membrane protein caused by amphiphiles

Data analysis by a fit to Eqn. 1 allows one to calculate the buffer-membrane partition constant K_p and the intrinsic potency P for a particular amphiphile. In a second step the so-derived potency values can be evaluated further by the subsequently proposed, more interesting part of our model. It is the main principle of this model that the destabilizing effect upon the target protein is solely due to direct protein–drug interaction. Therefore the following scheme is used for the analysis of the interdependence between protein denaturation and drug binding [20,35]:



(5)

Here, p stands for the native and $\bar{p}$ for the denatured protein molecule, ν and $\bar{\nu}$ for the average binding number in the native and denatured state, respectively, and $\Delta\nu$ denotes the differential binding number upon the denaturation of the protein.

Of the two types of equilibria involved in this scheme the vertical direction shall be considered first. Amphiphile binding towards membrane proteins has frequently been calculated from inhibition data under the model assumptions of multiple binding sites with equal binding constants and with a definite drug/protein ratio in the inactive complex [20,24]. The apparent drug/protein stoichiometry has been deduced from the saturation effect in the concentration dependence of inhibition. In contrast to the inhibition assay the titration curves of the depression in the denaturation temperature of the same protein by the same amphiphiles were observed to be completely [19,34] or nearly linear [36]. This means that drug binding

proceeds beyond that drug/protein stoichiometry which is reported by the inhibition assay.

The observed linear titration curves, in other words the absence of even partial saturation, is best accounted for by simple partitioning of the amphiphilic perturbants between binding sites on the protein and the rest of the membrane. This is only possible if all potentially occupied binding sites on the protein have a very similar affinity for a particular amphiphile, i.e. all transfer free energies (Δg) of individual amphiphiles are practically equal, and if the affinities are so low that the total pool of binding sites on the protein does not get enriched very much with drug molecules in comparison to the bulk lipid matrix. As a consequence, the bulk lipid matrix should not become depleted significantly upon the presence of an amphiphile-binding integral membrane protein. Therefore, both the molar drug amount in the total membrane and in the lipid matrix (including other proteins not of interest) can be denoted by N_m. Based on a similar approximation, the membrane volume and the bulk lipid matrix volume are both symbolized by V_m. Because of equal, and low affinity of all binding sites on the protein not even partial saturation of binding can occur within reasonable levels of amphiphile concentrations in the membrane, and binding can be described by a partitioning equilibrium instead. The partition constant K is defined by

$$\frac{N_a}{V_a} = K\frac{N_m}{V_m} \tag{6}$$

for the native state of the protein. When defining a molar annular volume per mole of protein (V_a/n_p) it is possible to formally convert 'annular' (subscript a) concentrations (N_a/V_a) into average drug binding numbers (ν)

$$\nu = \frac{N_a}{n_p} = \frac{V_a}{n_p}K\frac{N_m}{V_m} \tag{7}$$

The partition constant is related to the transfer free energy in the usual way:

$$\Delta g^\circ = -RT \ln K \tag{8}$$

In order to describe binding to the denatured form of the protein it is necessary to offer a model for the denaturation event itself. Two limiting cases are considered, one of which is shown in Fig. 1B. The corresponding model is provisionally called the 'cryptic site model' since the major effect of protein denaturation is thought to be the creation of newly exposed drug binding sites on the protein. This should typically occur when denaturation is accompanied by protein unfolding or helix segregation. Only the newly created sites are taken into account; in other words, the additional annular volume (ΔV_a), and partitioning ($\bar{K}$) between these sites and the lipid matrix lead to a certain number of drug molecules additionally bound per mole denatured protein monomer:

$$\Delta\nu = \frac{\Delta V_a}{n_p}\bar{K}\frac{N_m}{V_m} \tag{9}$$

So far the drug binding numbers in the native and the denatured states have been derived from the proposed model assumptions. In order to predict the destabilizing effect (ΔT) on a membrane protein from the membrane concentration of an amphiphile (n_m/V_m) it has to be shown how such binding numbers can shift the midpoint temperature of protein denaturation. The denaturation process is commonly treated by a simple two-state equilibrium [20,35] as indicated in Eqn. (scheme) 5 and the midpoint temperature is given by

$$T = \frac{\Delta H}{\Delta S} \tag{10}$$

The presence of amphiphiles shifts T, therefore the thermodynamic parameters of denaturation must be altered by drug binding:

$$T' = \frac{\Delta H + \Delta H'}{\Delta S + \Delta S'} \tag{11}$$

and the net effect is

$$T' - T = \Delta T = \frac{\Delta H'\Delta S - \Delta H\Delta S'}{(\Delta S + \Delta S')\Delta S} \tag{12}$$

The term $\Delta S'$ in the denominator can likely be neglected (vide infra) and rearrangement of Eqn. 12 gives

$$\Delta T = \frac{1}{\Delta S}(\Delta H' - T\Delta S') = \frac{\Delta G'}{\Delta S} \tag{13}$$

Thereby $\Delta G'$ is equivalent to the denaturation free energy brought about by protein–amphiphile in-

teraction and from thermodynamics it follows that this term is identical to the differential free binding energy of amphiphiles towards the denatured versus the native form of the protein. At this point it is possible to link protein destabilization to drug binding numbers.

The differential free energy $\Delta G'$ can now be attributed to the cumulative action of all amphiphiles that bind to the newly exposed sites:

$$\Delta G' = \Delta\nu\Delta\bar{g}^\circ \tag{14}$$

Combination of Eqns. 8, 9, 13, 14, and 15 yields the final expression

$$\Delta T = P\frac{N_m}{V_m};\ P = \text{constant} \tag{15}$$

wherein P contains only terms which are thought to be constant for the interaction of a particular amphiphile with a particular membrane protein. For the above proposed cryptic site model P is given by

$$P = \frac{1}{\Delta S}\frac{\Delta V_a}{n_p}\Delta\bar{g}^\circ \exp\left(-\frac{\Delta\bar{g}^\circ}{RT}\right) \tag{16}$$

The limiting case opposite to the cryptic site model can be called the 'differential affinity model'. Thereby it is assumed that the major effect of denaturation is not the creation of new amphiphile binding sites on the protein but a change in the affinity of the existing sites, leading to a change in the binding number.

$$\nu = \frac{V_a}{n_p}\bar{K}\frac{N_m}{V_m} \tag{17}$$

The differential binding free energy $\Delta G'$ is then composed of contributions both from permanently bound molecules and from molecules which bind additionally upon denaturation:

$$\Delta G' = \nu(\Delta\bar{g}^\circ - \Delta g^\circ) + (\bar{\nu} - \nu)\Delta\bar{g}^\circ \tag{18}$$

Combination of Eqns. 7, 8, 13, 15, 17, and 18 gives:

$$P = \frac{1}{\Delta S}\frac{V_a}{n_p}\left\{(\Delta\bar{g}^\circ - \Delta g^\circ)\exp\left(-\frac{\Delta g^\circ}{RT}\right) + \Delta\bar{g}^\circ\left[\exp\left(-\frac{\Delta\bar{g}^\circ}{RT}\right) - \exp\left(-\frac{\Delta g^\circ}{RT}\right)\right]\right\} \tag{19}$$

A major objective is to analyze which structural elements of an amphiphile have which influence on the amphiphile's ability to interact with the protein. In the example to follow, homologous series of amphiphiles which are alkane derivatives have been chosen as model cases. It has invariably been found that such homologous molecules show partition constants between an aqueous phase and a hydrophobic phase or microphase which are exponentially dependent upon their chain length [7,13,26,34]. In other words, their transfer free energies are linearly dependent upon the number of methylene groups. By analogy, the same principle can be applied to the membrane-internal partition equilibria which have been the basis for the model of amphiphile action proposed above:

$$\Delta g^\circ = \Delta g^\circ(i) = \Delta g_o^\circ + i\Delta g_m^\circ$$

$$\Delta\bar{g}^\circ = \Delta\bar{g}^\circ(i) = \Delta\bar{g}_o^\circ + i\Delta\bar{g}_m^\circ \tag{20}$$

Here, i denotes the number of methylene groups plus the single methyl group and the subscripts o and m stand for head group and methylene/methyl group, respectively. Eqn. 16 or 19 can thus be extended to account for the chain length dependence of the intrinsic potency P(i) by substituting Eqn. 20 for the corresponding terms. The expected alkyl chain length dependence of amphiphile partitioning between target protein and lipid matrix has therefore served to introduce the chain length dependence into the predicted intrinsic potency values of homologous amphiphiles.

Results and Discussion

It has been the objective of this study to characterize the primary target site of amphiphiles, and thereby to elucidate the mechanism by which amphiphiles can perturb a membrane protein. The strategy adopted is commonly called 'target site mapping' [30] which means that the site of amphiphile action is distinguished by its response patterns towards regular series of model amphiphiles. In particular, we have reported the destabilization of the anion exchanger of the erythrocyte membrane by saturated fatty acids in a preceding study [34], whereby alkyl chain length was the structural parameter being varied.

According to the model in Fig. 1 the effect of structural features of amphiphiles upon their effectiveness has to be decomposed quantitatively into two independent components: those affecting buffer-membrane partitioning and others affecting the intrinsic potency of the membrane-bound fraction. This separation is particularly important since we have regularly found structural features of amphiphiles that enhance partitioning to lower intrinsic potency at the same time. As will be discussed below, much confusion has arisen in the literature from not separating these two components and dealing with apparent potencies instead. The more correct characterization of drug efficacy by intrinsic potency values and their quantitative interpretation by our model will be shown to provide an insight into a hitherto unpostulated role of membrane lipid as a competitor with the protein for the interaction with small amphiphilic molecules.

Separation of buffer-membrane partitioning from intrinsic potency

Most previous studies have compared amphiphiles in their effectivity on the basis of equieffective concentrations in the total sample. In this study equieffective concentrations were not determined by interpolation within a set of experimental data. Instead, they were calculated via Eqn. 2 in the reverse direction from a higher level of data analysis, namely from the model parameters P and K_p which had been extracted from experimental data via Eqn. 1 [34]. In Fig. 2A the equieffective concentrations of saturated fatty acids, all of which are predicted to lower the thermal transition of the erythrocyte membrane anion exchanger by 1 C°, have been plotted versus amphiphile chain length in the usual semilogarithmic way (closed and open circles). Two very different experimental situations with respect to the fractional volume of membranes in the sample were chosen in these simulations: V_m/V_t was held constant at either 10^{-2} (solid lines and closed symbols) or 10^{-4} (dashed lines and open symbols). In each case the semilogarithmic chain length profile of equieffective fatty acid concentrations exhibits four regions of interest: a steep linear region at short chain length, an intermediate behaviour at intermediate chain length, a rather flat,

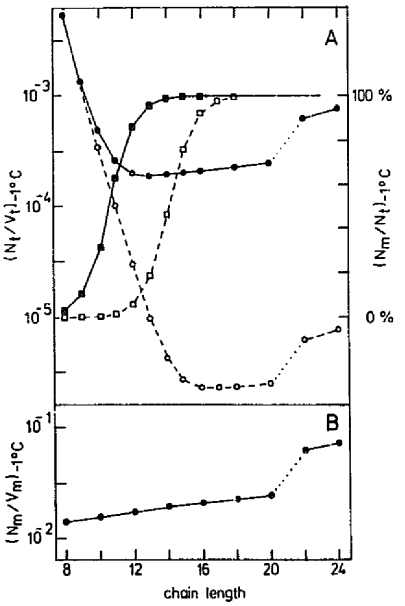


Fig. 2. (A) Classical plot of equieffective sample concentrations (N_t/V_t) of homologous saturated fatty acids versus the total chain length n (●, ○). These equieffective concentrations, all of which lower the denaturation temperature of the erythrocyte membrane anion exchanger by 1 C°, were calculated according to Eqn. 19. Potencies P were taken from Fig. 6A in Ref. 34, values for odd chain length were obtained by interpolation. Partition constants were calculated from the linear least-squares fit shown in Fig. 6B of Ref. 34. Relative membrane volumes in the sample (V_m/V_t) were held constant at 10^{-2} (———, ■, ●) or 10^{-4} (— — —, □, ○). V_m was defined by assuming a density of 1 mg/μl of membrane. The ordinate on the right hand side shows the partitioning situation (■, □); the percentage of the total fatty acid pool which resides in the membrane was calculated according to Eqn. 4. (B) Plot of equieffective concentrations of homologous fatty acids within the membrane. The values of (N_m/V_m) were calculated according to Eqn. 3 and are independent of the relative volume of membrane in the sample (●).

linear part at long chain length, and a sudden step towards higher equieffective concentrations, or lower apparent potencies, for extremely long fatty acids. Comparisons of the two plots for different membrane/buffer ratios shows that the classification of short, intermediate, and long alkyl chains strongly depends on the experimental conditions and that the equieffective concentrations of long chain fatty acids drop by a factor of 10^2 when the

relative volume of the membranes in the sample is lowered from 10^{-2} to 10^{-4}.

A quantitative explanation for this behaviour is given by the corresponding plots of the membrane-bound fractions of fatty acids (closed and open squares in Fig. 2A). At short chain lengths only a negligible fraction of amphiphile has partitioned into the membrane and, therefore, the equieffective sample concentrations are virtually independent of the membrane/buffer ratio. The steep and nearly linear slope of $\log(N_t/V_t)$ versus chain length is mainly due to the linear increase in $\log K_p$ versus chain length (factorial increase of 3.7 in K_p per CH_2 group). In the long chain region of Fig. 2A the situation is characterized by complete partitioning of amphiphile into the membrane. Obviously, the still-existing relationship between K_p and chain length is now irrelevant and any change in equieffective concentrations with chain length reflects the chain length profile of intrinsic potency, as exemplified by the sudden change between eicosanoic and docosanoic acid. At intermediate chain length values the influence of K_p upon the chain length profile gradually drops from predominant to negligible as the hydrophobicities of fatty acids are increased and the membrane-bound fractions approach 100% Concomitantly the membrane/buffer ratio will start to gain influence on the magnitude of the equieffective concentrations.

This data simulation served to illustrate the reason for the biphasic shape of the chain length profile of so-called 'apparent potencies', which contrasts with the monotonic chain length dependence of the equieffective membrane concentrations, the reciprocal values of the intrinsic potencies of homologous fatty acid from C_8 to C_{20} (Fig. 2B). Obviously intrinsic potency is distinguished as a much more general measure of drug activity than apparent potency.

The unexpected finding of a monotonic decrease in the chain length profile of the intrinsic potency of fatty acids provoked our interest to compare with literature data on the chain length dependence of amphiphile potency. Unfortunately however, in nearly all of the studies on the role of amphiphile chain length the effectivities of amphiphiles have been given in terms of apparent rather than intrinsic potency [9,10,12–17,21,31, 32,37], while the complicating influence of partitioning upon apparent drug potency was explicitly analyzed only in a minority of studies [5,7,8,11]. Yet, based on expectations derived from Fig. 2 it was possible to qualitatively 'reconstruct' the corresponding chain length profile of the intrinsic potency. Three classes of chain length patterns could be distinguished:

(i) In a small number of cases amphiphile potency appears to be completely independent of alkyl chain length. This can be deduced from the inhibition data on erythrocyte membrane Na^+/K^+-ATPase by homologous alkylamines and their *N,N,N*-trimethyl and *N,N,N*-triethyl derivatives [13]: they appear to be intrinsically equipotent for all chain lengths between C_2 and C_{18}. The plots of equieffective sample concentration versus chain length given in this study closely resemble that in Fig. 2A, except that all long chain regions were horizontal. While the authors have repeatedly interpreted the apparent discontinuity around C_{12} to C_{14} in terms of an as yet unknown constriction of the hypothetical target site [13,38] there can be no doubt that it is a mere 'artifact' of this type of plot as explained above. The same explanation seems to apply to the inhibition of Na^+,Ca^{2+}-exchange in cardiac sarcolemma by alkanamines and quarternarized analogues between C_8 and C_{16} [14] and to the block of the voltage-dependent Na^+ channel by saturated fatty acids [32].

(ii) The contrasting type of chain length pattern is one in which a rather constant intrinsic potency at short chain lengths suddenly drops to nearly zero at an intermediate chain length. The best known example thereof is the much-discussed cut-off in the intrinsic potency of alkanols which occurs at C_{13} with respect to both general anesthesia [9,25,26,37,39] and blockade of the voltage-gated Na^+ channel [21,31]. Although neither intrinsic potencies nor equieffective membrane concentrations have been reported to date, it is clear from the data that the cut-off is one in intrinsic, not just in apparent potency. It is very interesting to note that even quarternarized alkanamines show a very strong chain length dependence in both the mechanism and the potency of Na^+ channel inhibition, with the C_{16} derivative being totally inactive [37]. This contrasts the chain

length independence of the same series with respect to Na^+/K^+-ATPase and Na^+,Ca^{2+}-exchange as stated above. This diversity in the chain length profiles of one and the same amphiphile series necessarily leads to a pluralistic view of membrane perturbation by amphiphiles in which the mechanism of action varies for different membrane proteins. This view is further supported by the observed cut-off effects in the intrinsic potency of alkanols with respect to further membrane proteins which occur at various chain lengths much shorter than C_{13} [5,8,9,11].

(iii) The monotonic decrease in intrinsic potency with increasing chain length depicted in Fig. 2B represents a third type of pattern which is intermediate between the two extremes discussed above. Surprisingly the same chain length dependence seems to occur in the perturbation of several ion transport proteins besides band 3 by the same series of amphiphiles, i.e. by saturated fatty acids. In particular we have deduced this from the data on the inhibition of rat brain Na^+/K^+-ATPase [10,16], inhibition of sarcoplasmic reticulum ATPase [12], stimulation of Na^+,Ca^{2+}-exchange in cardiac sarcolemma [14,15], and stimulation of calmodulin-independent, as well as inhibition of calmodulin-dependent Ca^{2+}-ATPase of the erythrocyte membrane [17]. The monotonic chain length profile in all examples of the latter group certainly does not automatically imply that all of these proteins are perturbed by fatty acids via the same mechanism. Yet it can be suspected that a constant mechanistic element occurs in all of these examples. Below it will be shown that the role of the bulk lipid matrix would be a good candidate for this element, even if the amphiphile effect on the proteins is direct and not communicated through the lipid.

It can be summarized that the reinterpretation of apparent potency data has opened up the possibility to directly compare a large body of data. Up until now the discussion of the role of chain length has solely focused on the cut-off in the general and local anesthetic potency of alkanols at C_{13} which is so dramatic that it could even properly identified from apparent potency data. From the compilation of chain length patterns given above it can be seen that a broad spectrum of such patterns exists, that they are strongly dependent on the target protein and that chain length profiles should provide one of several criteria which allow assignment of a particular mechanism of amphiphile action.

Interpretation of intrinsic potency and of its chain length dependence by partitioning within the membrane

Above, it has been shown that an explicit analysis of buffer-membrane partitioning is necessary in order to obtain an unequivocal characterization of amphiphile effectivity. Subsequently the so-derived intrinsic potency values will be analyzed further with respect to the mechanism of the model depicted in Fig. 1.

The first point of agreement between the model and the data can be recognized without doing any calculation. Empirically it has been found that the shift in the denaturation temperature of band 3 caused by fatty acid, fatty alcohols, fatty amines, as well as lidocaine, a tertiary amine anesthetic, is practically linearly dependent upon their concentration within the membrane, up to at least 100 mmol of amphiphile per kg membrane [19,34]. This means that Eqn. 15 has been confirmed as an empirical rule, in other words that intrinsic potencies are not themselves concentration-dependent. According to both versions of the model this is predicted on the basis of the simple concept that membrane-bound amphiphiles partition between the bulk lipid matrix (m) and a large pool of binding sites (a) on the target protein, as shown in Fig. 1.

Under which circumstances would the titration curves of amphiphile effects be expected to deviate from linearity? Using the terminology of our model this should happen (i) if the total pool of potential binding sites on the target protein consists of classes of sites with different affinities and two or more such classes actually become occupied or (ii) if all potential binding sites are of equal affinity, yet the total pool of sites is so limited and/or the affinity for the sites is so high that partial saturation can occur in the experimentally used concentration range. An attempt was made to estimate for which number and which affinity of binding sites we should have observed saturation effects in the drug titration curves. A Langmuir-type adsorption model was used to

calculate binding numbers ($\Delta\nu$) versus membrane concentration of drug. Thereby it was assumed that 70% of the membrane volume in the clipped and stripped inside-out vesicles consisted of lipid (estimated from a comparison between protein concentrations and gravimetrically determined membrane concentrations), that practically all of the protein consisted of the 55 kDa fragment of band 3, and that 30 new potential binding sites were exposed upon denaturation. From the results (data not shown) it was concluded that only $\bar{K}$ values smaller than 5 were acceptable because larger $\bar{K}$ values would lead to titration curves with saturation effects above those observed in the experiments. Between 10 and 100 newly exposed sites per protein could be assumed without much change in the upper limit for $\bar{K}$. This upper limit of 5 for $\bar{K}$ compares very well with data on the binding of fatty acids, fatty amines, and fatty alcohols to a large number of 'annular' sites and to fewer, but similar 'nonannular' sites on reconstituted ATPase from sarcoplasmic reticulum [23]. From the effective binding constants in this study the relative affinities for protein over lipid can be seen to vary between 0.1 and 2.5. Thus our estimate for $\bar{K}$ is in accordance with these spectroscopically determined partition constants. In addition, the interpretation of 'nonannular' sites as those inbetween helices given in Ref. 23 is consistent with the cryptic site model for amphiphile effects on protein denaturation shown in Fig. 2B.

Besides the concentration independence of potency, another aspect of our data on the perturbation of band 3 by fatty acids could be interpreted by the above proposed model: the monotonic decrease of intrinsic potency with increasing chain length. The nearly monoexponential chain length profile could be simulated both by the 'cryptic site' model and by the 'differential affinity' model, as shown in Fig. 3. Thus a selection between these two denaturation models is not possible on the basis of the available data. Because of its simplicity (only two model parameters instead of four) and plausibility the latter model will be used in all further discussions. The solid line shown in Fig. 3 connects the relative potency values calculated for $\Delta\bar{g}_o^\circ = -3000$ J/mol and $\Delta\bar{g}_m^\circ = 61$ J/mol. As can be seen from Table I, parameter sets with smaller negative values for

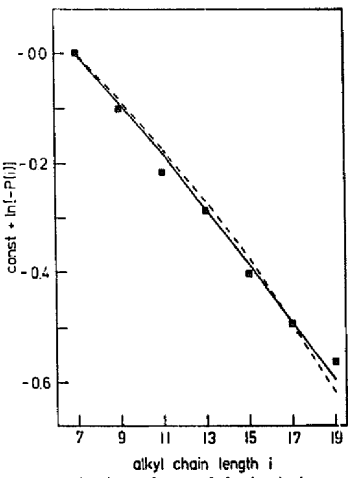


Fig. 3. Chain lenght dependence of the intrinsic potency $P(i)$ of saturated fatty acids in destabilizing the erythrocyte membrane anion exchanger in a semilogarithmic representation: ■, experimental vales; ———, cryptic site model ($\Delta\bar{g}_o^\circ = -3000$ J·mol^{-1}, $\Delta\bar{g}_m^\circ = +61.0$ J·mol^{-1}), — — —, differential affinity model ($\Delta g_o^\circ = +1800$ J·mol^{-1}, $\Delta g_m^\circ = +2.7$ J·mol^{-1}, $\Delta\bar{g}_o^\circ - \Delta g_o^\circ = -37.5$ J·mol^{-1}, $\Delta\bar{g}_m^\circ - \Delta g_m^\circ = +1.1$ J·mol^{-1}). The lines connect the calculated points in order to guide the eye.

$\Delta\bar{g}_o^\circ$ gave worse predictions for the chain length profile while going to extremely large negative values for $\Delta\bar{g}_o^\circ$ caused further, yet insignificant

TABLE I

PARAMETER SETS FOR THE STIMULATION OF THE CHAIN LENGTH PROFILE OF THE INTRINSIC POTENCIES OF SATURATED FATTY ACIDS BY THE CRYPTIC SITE MODEL ACCORDING TO EQNS. 16 AND 20

The predicted matrix-'annulus' partition constant K of octadecanoic acid was calculated according to Eqn. 8. As an average value for the experimental range of denaturation temperatures T was kept constant at 65 °C.

$\Delta\bar{g}_o^\circ$ (J·mol^{-1})	$\Delta\bar{g}_m^\circ$ (J·mol^{-1})	Error in P [a] (%)	K (C_{18})
−300	+8.7	4.1	1.1
−3000	+61.0	2.7	2.0
−8000	+97.0	2.1	10.0

[a] The quality of the fit is indicated by the square root of the mean of the relative prediction error squares, $[(P_{calc} - P_{exp})/P_{exp}]^2$, multiplied by 100%.

improvements in the quality of the fit. Obviously the appropriate numbers for the model parameters $\Delta\bar{g}_o^\circ$ and $\Delta\bar{g}_m^\circ$ cannot be pinned down by this kind of data analysis as long as the protein- and membrane-dependent terms ΔS and $\Delta V_a/n_p$ are not known from an independent source of information. Both as an illustration of $\Delta\bar{g}_o^\circ$ and $\Delta\bar{g}_m^\circ$ and as help for the selection of the more appropriate parameters sets, the matrix-protein partition constant $\bar{K}$ was calculated for $i = 17$, i.e. for the 18 : 0 acid according to Eqns. 8 and 20. From a comparison with the estimate of $\bar{K}$ given above it follows that the parameter set $\Delta\bar{g}_o^\circ = -3000$ J/mol and $\Delta\bar{g}_m^\circ = 61$ J/mol used to calculate the solid line in Fig. 3 seems to be a realistic one.

In spite of the failure to arrive at an unequivocal parameter set for the simulation of the chain length profile of intrinsic potency, several very useful results of the above presented analysis can be pointed out. First of all, the two-step partition model depicted in Fig. 1 has turned out to be sufficient for a mechanistic interpretation of the experimental data. Second, the partitioning of fatty acids between bulk lipid matrix and target protein apparently depends on the balance between the effects of the polar, or charged head group, favoring amphiphile–protein interaction ($\Delta\bar{g}_o^\circ < 0$), and the effect of the alkyl chain which favors partitioning into the bulk lipid matrix ($i\Delta\bar{g}_m^\circ > 0$). Furthermore, the relative preference of the target site for shorter chains is best described by a nonspecific model in which each methylene group contributes with the same incremental free binding energy $\Delta\bar{g}_m^\circ$ to the unfavourable role of the alkyl chain.

We have found literature on amphiphile–lipid interaction that provides the clue to a very plausible and interesting explanation for the linear increment $\Delta\bar{g}_m^\circ$ by which each additional methylene group favors partitioning into the bulk lipid matrix, rather than towards the target protein. Semiempirical calculations for mixed fatty alcohol-dipalmitoylphosphatidylcholine (DPPC) bilayers have predicted the alcohol–alcohol interaction energies to decrease and the alcohol–lipid interactions to increase linearly with alkanol chain length, such that alkanols with more than 8 carbons should preferentially become dispersed in the bilayer rather than becoming clustered as predicted for the shorter ones [40]. In a theoretical analysis of the measured dose-dependent nonlinear response of the main transition of DPPC to homologous alcohols very similar conclusions were reached [41]. Thus there is reason to believe that the linear loss $\Delta\bar{g}_m^\circ$ per methylene group in the relative affinity of amphiphiles for protein over lipid is partly or wholly determined by the graded specificity of lipid–amphiphile interactions, while the protein–amphiphile interaction might be totally nonselective for chain length. We feel confronted with the paradoxical interpretation that lipid matrix, while not being the primary 'target site' responsible for protein perturbation, nevertheless might partly or fully determine the apparent drug specificity of the hypothetical target site. In other words, the lipid matrix is seen as a competitor with the protein (instead of a mediator!) in the protein-lipid-amphiphile system in all those cases where the model proposed in this study is formally applicable. The lipid, therefore, not only complicates target site mapping studies via buffer-membrane partitioning effects, rendering apparent potency patterns meaningless for membrane systems, but its modulating influence should 'contaminate' even intrinsic potency patterns, even when the primary target site is on the amphiphile-perturbed protein itself.

Criticism of the model

The concept of buffer-membrane partitioning of amphiphilic drugs is quite trivial, and indeed data analysis and discussion in terms of actual membrane concentration and intrinsic potency, or efficacy, has been quite common [7,9,26,29,42,43]. So the question may be raised why this point was emphasized in the present study and why it was made an integral part of our model. The reason for doing so was given by the fact that in most studies on amphiphile chain length the buffer-membrane distribution was neither evaluated nor even mentioned. Consequently many chain length profiles of apparent potencies reported in the literature produce an unclear, or misleading impression of the role alkyl chain length and do not allow for a comparison between different studies. We have shown that such data can be reassessed at least qualitatively in terms of intrinsic potency and that a so-derived compilation of chain length patterns leads to a very pluralistic view of

amphiphilie action, even if only amphiphile 'targets' with low affinity are included.

The method by which buffer-membrane partitioning has been determined also deserves comment. Sometimes lipid-free model systems have been chosen in order to circumvent the complications associated with the presence of lipid [25,30,36,44]. Occasionally, however, the complicating influence of the membrane/buffer ratio upon the net effect of amphiphiles has been exploited to determine effective partition constants [19,43]. We have used exactly this method to simultaneously calculate partition constants and intrinsic potencies for DSC experiments. In its favor it can be said that it yields relevant partition constants even under those circumstances where it would be very difficult to determine K_p by an independent experiment. This was the case in our calorimetric experiments where the temperature was 65–70°C. Its weakness lies in the fact that only a very simple partition model can be used to avoid a situation with too many 'adjustable parameters' and that it is therefore not possible to account for the possible influence of a change in surface potential upon the magnitude of K_p. While surface potential changes with increasing drug concentration cannot be excluded for our experiments we felt compelled to resort to the simplest possible model that could fit the data, in other words to Eqn. 1.

The more important part of the above proposed model deals with the fate and action of amphiphiles within a membrane, rather than with buffer-membrane partitioning. The corresponding model assumptions are in good accordance with the findings of others: (i) We have proposed that amphiphiles simply partition between the bulk lipid matrix and multiple binding sites on integral membrane proteins. Based on mostly spectroscopic evidence others have arrived at very similar models with various degrees of further sophistication [22–24,45–47]. Furthermore, the concept of a very large number of thermodynamically equivalent amphiphile binding sites on a protein has been proposed before, whereby the degree of partial (!) inhibition of any single enzyme molecule has been demonstrated to be linearly related to the number of bound anesthetics [44]. In exactly the same way we have assumed the thermal destabilization of the erythrocyte membrane anion exchanger to be linearly proportional to the number of differentially bound molecules as stated in Eqn. 14. (ii) Thermal protein denaturation and the modulating influence of anesthetics thereupon has already been modeled by equilibria like those in Eqn. (scheme) 5 [20,35]. In a study on the functional inhibition and the denaturation temperature shift of membrane-bound cytochrome-*c* oxidase by butanol, tetracaine, or dibucaine it has also been shown that the enzyme's response pattern to the different amphiphiles could hardly be explained by other than direct interactions and that the enthalpy and entropy terms of drug–protein interaction are small compared to those for protein denaturation itself [20]. Neglecting $\Delta S'$ in the denominator of Eqn. 12, therefore, seems to give a valid approximation.

Which insights have been gained by the application of the model proposed in this study to the data on amphiphile action reported in the preceding study [34]? It has been shown to what extent target site mapping by monitoring the protein's response to amphiphiles can be evaluated to characterize the hypothetical target site, or sites. The hypothetical site of fatty acid action on the erythrocyte membrane anion exchanger has turned out to exhibit a graded preference for shorter over longer alkyl chains and a comparison with the literature has shown this behaviour to be fairly common among ion transport proteins. It has further been shown that only additional, independent information on amphiphile–lipid interaction allows one to separate out the mechanistic influence of the bulk lipid matrix even for those cases where the lipid has no direct role in communicating amphiphile effects.

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