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ANALYSIS OF MEMBRANE-BOUND ACCEPTORS

A CORRECTION FUNCTION FOR NON-SPECIFIC ACCUMULATION OF POORLY WATER-SOLUBLE HYDROPHOBIC OR AMPHIPATHIC LIGANDS BASED ON THE LIGAND PARTITION CONCEPT

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Abstract—Non-specific ligand accumulation into membrane material, which may contribute considerably to the experimental signal obtained in binding studies with labelled amphipathic and hydrophobic ligands, may be accounted for by linear partition of the ligands into the membrane phase. For application to binding data obtained at a single membrane-lipid concentration, a fitting procedure is proposed which allows one to correct for non-specific ligand partition. If the assumption is met that the amount of acceptor-bound ligand is small compared to the total amount present in the system, one can validly interpret the data in terms of total ligand concentrations. The apparent dissociation constants K_d^{app} thus obtained should be corrected for the often large effects of the size of the partition compartment(s), by performing assays at several membrane-lipid concentrations. The importance of the latter correction is stressed and an approach for obtaining the characteristic effective dissociation constants K'_d is indicated. The procedure also yields estimates of the ligand/membrane partition coefficients.

In analysing the binding parameters of labelled ligands interacting with membrane-bound acceptor proteins, accurate correction for non-specific ligand accumulation by structures other than the acceptor sites is of crucial importance [1–5]. The commonly used procedure of estimating non-specific accumulation as the radioactivity remaining in the sample in the presence of an excess of cold ligand has been claimed to be inadequate [3, 5] and may yield results mimicking acceptor heterogeneity [1, 5] where none exists. According to Mendel and Mendel [3], the solution to the problem simply consists of fitting the appropriate mathematical model (including one or several terms for specific binding of ligand and a term for non-specific accumulation) directly to the binding data by non-linear regression analysis. This permits the data to be separated into (at least) two components, one of which represents non-specific ligand acumulation. For critical evaluation of the mathematical model chosen, the ligand concentration range explored should ideally be extended from $0.1 \times K_d$ (for the highest affinity sites, if multiple sites are present) up to $10 \times K_d$ (for the lowest affinity sites) [5–7]. If the range is extended considerably beyond $10 \times K_d$, the saturation range itself may be covered inadequately [8] or the specific response may be too small to be noticed [9]. The major problem remaining thus would appear to be to define a descriptive function for non-specific ligand accumulation.

Based on an analysis of the mechanisms of accumulation of poorly water-soluble hydrophobic and amphipathic substances into membrane material, a correction function is derived. Application to experimental binding data and the consequences for their analysis are discussed.

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THEORY

Membrane partition of ligands

The observation that ligand accumulation into bilayer membranes is apparently hyperbolic [10–13] has given rise to its interpretation in terms of saturable binding to a limited number of phospholipid binding sites. Analysis of the available evidence indicates that the primary phenomenon to be considered is linear ligand dissolution into the membrane lipids [4, 14, 15], a number of secondary phenomena subsequently giving rise to non-linear accumulation at relatively high ligand concentrations.

For a number of cationic, neutral and anionic amphipaths, the membrane partition coefficient P_A^m is constant at low ligand concentration [10, 14–26]. In a number of cases [18–26], biphasic curves showing a clear discontinuity between linear (at relatively low ligand concentrations) and hyperbola-like accumulation (at relatively high ligand concentrations) were found, e.g. for partitioning (i) of cationic amphipaths [20, 21] and of teniposide and etoposide [25] into a variety of natural and synthetic phospholipid vesicles and (ii) of dipicrylamine into black lipid films [26]. Also note that for the anionic substance bilirubin-IXα, hyperbola-like membrane saturation [12] has been shown to be preceded by linear pigment accumulation [17, 24]. The binding picture definitely does not apply to accumulation of apolar ligands by bilayer membranes. For example, transfer of *n*-alkanes from the surrounding aqueous medium to bilayer lipid is driven primarily by an

increase in entropy, the interaction between the alkanes and the acyl chains of the phospholipids being extremely weak [15].

The concept that ligand accumulation into bilayer membranes can primarily be described by a constant membrane partition coefficient—as observed for several ligands—implies that the membrane-lipid phase and the surrounding aqueous phase are sufficiently dilute so that interaction between ligand molecules is minimal and significant modification of the solvents does not occur. This condition generally applies to the aqueous phase of membrane-containing systems used for experimental work. For example, consider an aqueous microsomal suspension from rat liver. According to DePierre and Dallner [27], the weight ratio of protein to lipid is 7:3. Taking 800 as the average molecular mass of the lipids and 30,000 as that of the protein subunits, a microsomal suspension at protein concentration 3 mg/mL is 55.6 M with respect to water and only about 1.5 and 0.1 mM with respect to phospholipid and protein, respectively. The ligand concentrations are usually much lower. With regard to the intramembranal concentrations that are to be expected under physiological and pharmacological conditions a clue is provided by the behaviour of general anaesthetics. These agents, which represent a wide variety of chemical structures with partition coefficients covering about five orders of magnitude, provoke anaesthesia of 50% of a group of animals at intramembranal concentrations x_A^m (expressed as mole fractions) equal to about 0.02 [28, 29]. This corresponds to already fairly dilute ligand solutions in the lipidic phase. Under physiological conditions, the intramembranal concentration of A (x_A^m) thus will be well below 0.01. Excluding specifically such amphipaths as cholesterol [30] which are involved in the formation of bilayer structures, we submit the working hypothesis that, similarly as for general anaesthetics, other xenobiotics and natural ligands generally occur at low intramembranal concentrations. This is obvious for natural substances such as ethanol and a number of steroids, acting as general anaesthetics [29, 31]. Observations with bilirubin-IXα suggest that linear accumulation of natural ligands into membranes may even apply to pathological states characterized by relatively high steady-state concentrations. At an aqueous concentration of unbound bilirubin-IXα equal to 1.25 μM—which is within the range of linear membrane accumulation [17]—already more than 90% of the bilirubin-IXα binding sites on the intracellular binding protein ligandin are occupied [32]. The value of 1.25 μM bilirubin-IXα is far above the unbound concentration in liver cytosol of normal and congenetically jaundiced male rats [32].

Non-linear accumulation at relatively high ligand concentrations may be due to several causes. In certain cases, the non-linearity has been explained by postulating 'saturation of phospholipid binding sites'. It should be noted that apparent saturation was reached only when the ligand/phospholipid molar ratio was as high as 0.1–0.35 [10, 12]. This represents concentrated solutions, quite different from the initial phospholipid solvent. In such cases, ligand accumulation is accompanied by continuous changes in the activity coefficient of the ligand (ligand–ligand interaction) [33–37] and by significant alteration of the physical properties of the phospholipid solvent. Aggregation of amphipaths at relatively high ligand concentration [20, 36] and, in some cases, limitingly low solubility in the aqueous phase [19] may contribute to the observed non-linearity. Increasing repulsive interaction between charged ligands and the surface charge of the membrane might further add a non-linear component to the accumulation curves [38, 39]. For negatively charged bilayer membranes, increasing the ionic strength of the solvent increases the membrane accumulation of anionic ligands [39] and decreases the accumulation of cationic ligands [38].

Fitting function

Consider ligand binding to two classes of independent binding site present on a membrane preparation. On the assumption that the total amount of ligand is large compared to that bound by the acceptor, the fitting function at fixed membrane-lipid concentration is

$$C_t = C_1 \cdot \frac{A}{K_{d1}^{app} + A} + C_2 \cdot \frac{A}{k_{d2}^{app} + A} + f_c^{app} \cdot A \tag{1}$$

where C_t is the total concentration of ligand present in the membrane material and A the total concentration of ligand. C_j is the concentration of binding sites and K_{dj}^{app} the apparent dissociation constant for the binding class j, respectively. The last term of Eqn 1 corrects for ligand partition into the membrane material. The apparent dissociation constant(s) K_{dj}^{app} and the correction function f_c^{app} which both depend on the extent of ligand partition through the ligand distribution function F_A [4], are given by

$$K_{dj}^{app} = K'_{dj} \cdot F_A \tag{2}$$

$$f_c^{app} = LP_A^m/F_A \tag{3}$$

where L is the concentration of membrane lipid. K'_{dj}, the effective dissociation constant [4] of the binding sites in class j, is given by

$$K'_{dj} = K_{dj} \text{ for an aqueous-faced hydrophilic site} \tag{4}$$

$$K'_{dj} = K_{dj}/P_A \text{ for a hydrophobic site} \tag{5}$$

where P_A is the incremental binding site partition coefficient P_A^{bs} (for aqueous-faced amphipathic sites) or the membrane partition coefficient P_A^m (for lipid-faced and mixed sites) [4]. For a non-ionizing ligand, the distribution function [4] F_A is

$$F_A = 1 + P_A^m \cdot L \qquad F_A \geq 1. \tag{6}$$

Similar expressions that increase linearly with the membrane-lipid concentration L are found for ionizable ligands for which only the neutral form partitions significantly into the membrane lipid.* If

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saturation curves are obtained at several membrane-lipid concentrations, the fitting function is

$$C_t = C_1 \cdot \frac{A}{K'_{d1}F_A + A} + C_2 \cdot \frac{A}{K'_{d2}F_A + A} + (L \cdot P_A^m/F_A) \cdot A \quad (7)$$

where F_A should be replaced by the expression appropriate for the ligand used. The modelling procedure [4] yields the parameters K'_{dj} and P_A^m in mole fraction units, the total concentrations A and L being given by the mole ratios n_A/N^{aq} and n_L/N^{aq}. N^{aq} is the total amount (in moles) of the aqueous phase and n_A and n_L the amount of ligand and membrane lipid, respectively. Equations 1–7 remain valid when the molarities of A and L are used instead of mole-ratio concentrations, the parameters K'_{dj} and P_A^m also being in molarity units.

If the assumption is not satisfied that the amount of acceptor-bound ligand is small compared to the total amount of ligand, the following formula applies

$$C_t = C_1 \cdot \frac{x_A^{aq}}{K'_{d1} + x_A^{aq}} + C_2 \cdot \frac{x_A^{aq}}{K'_{d2} + x_A^{aq}} + f_c^{app} \cdot A \quad (8)$$

where x_A^{aq} is the local concentration in the aqueous phase.

DISCUSSION

Analysis of the available evidence supports the concept that for hydrophobic and amphipathic ligands an experimental concentration range can generally be found over which the partition coefficient P_A^m is constant. If the accumulation curves are non-linear, it is of interest to further explore a range of low ligand concentrations since precisely the linear partition range may be relevant for the functioning of natural and xenobiotic ligands. Denotation of the non-specific accumulation of ligands by membranes as "non-saturable binding" or "non-specific binding" [3, 5] is inappropriate. Although solvation of ligand molecules involves the binding of solvent molecules, the term binding should be reserved, in a biological context, to describe the selective and saturable transfer of a ligand to a localizable structure such as a binding pocket present on an enzyme or binding protein. The forces driving ligand accumulation into membranes clearly are non-specific. The available evidence further supports the view that the accumulation process is non-saturable over physiologically and pathologically relevant ranges of ligand concentration. We therefore prefer such terms as "non-specific accumulation" or "partition".

Provided the amount of ligand bound to the acceptor is small, non-linear regression of Eqn 1 to binding data obtained at a single membrane-lipid concentration corrects for non-specific ligand accumulation and yields the concentration of binding site C_j and the apparent dissociation constant K_{dj}^{app} for each binding class ($j = 1, 2$). For accurate characterization of the binding sites, in particular in comparing results obtained for the same preparation assayed at different membrane-lipid concentrations or for different preparations such as whole cells, an organelle-bound or a further purified acceptor, the apparent dissociation constants K_{dj}^{app} should be corrected for the extent of ligand partition so that values of the effective dissociation constants K'_{dj} are obtained. Indeed, F_A may cover several orders of magnitude as shown e.g. for binding of danazol to microsomal cytochrome P450 [40]. Also note that, even when the membrane-lipid concentration L is small, the distribution function F_A may be important since the membrane partition coefficient P_A^m of hydrophobic and amphipathic ligands is expected to increase approximately in parallel with the effective affinity constant $1/K'_{dj}$ (Eqn 5). Fitting Eqn 7 to binding data obtained at several concentrations of membrane lipid yields the binding parameters C_j and K'_{dj} and also provides an estimate of the membrane partition coefficient P_A^m. As a check, one may verify whether the values of K_{dj}^{app}, obtained by fitting Eqn 1 to the individual saturation curves, increase linearly with the membrane-lipid concentration [4]. When the amount of acceptor-bound ligand cannot be neglected compared to the total amount of ligand, Eqn 8 should be used. The same correction term $f_c^{app} \cdot A$ applies as in the former case but the procedure now yields the effective dissociation constants K'_{dj}. Equations 1, 7 and 8 are easily extended to systems containing several lipidic compartments and more than two classes of specific binding site. However, already fitting the Michaelis–Menten equation to binding data obtained with a system containing only two classes of binding site may require fairly large numbers of accurate data before any confidence can be placed in the fitted parameters [41, 42].

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