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INTERPRETATION AND ANALYSIS OF RECEPTOR BINDING EXPERIMENTS WHICH YIELD NON-LINEAR SCATCHARD PLOTS AND BINDING CONSTANTS DEPENDENT UPON RECEPTOR CONCENTRATION

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Abstracts—Receptor-binding assays with radiolabelled ligands are widely used to evaluate the biological activity of drugs and hormones. The affinity, usually expressed as the dissociation constant (K_d), and the capacity (B_{max}) of the receptor preparation for various ligands are determined in order to compare quantitatively various agonists and antagonists. Experiments with the same ligand and receptor, however, often yield rather disparate values for these binding parameters. One obvious reason for variability in K_d is that straight lines are fitted to data that are clearly curved. Another and more serious reason is that a ligand's apparent dissociation constant decreases when the receptor preparation is diluted and so experiments done at different receptor concentrations do not give identical results. We demonstrate that both of these observations, i.e. the effect of receptor concentration and the curvature of Scatchard plots, can be explained by the presence of a competitive inhibitor in the receptor preparation, a possibility which is not normally considered in the analysis and interpretation of receptor binding assays. We show that the apparent K_d obtained by the conventional one- or two-site analysis may be several orders of magnitude larger than the true dissociation constant and the affinity is therefore seriously underestimated. Application of a model, which assumes that an inhibitor is present in the receptor preparation, will improve the quantitative determination of K_d and B_{max} significantly. As a simple alternative method we explain how the apparent binding parameters obtained by the conventional method should be interpreted and how they can be used to estimate the true affinity, provided sufficiently low concentration data are available.

Key words: receptor binding, competitive inhibitor, competing ligand, binding parameters, receptor heterogeneity, curved Scatchard plots

Measurement of the binding of ligands to crude membrane preparations from various tissues ("grinding and binding") is now a firmly established way to study ligand–receptor interactions at the molecular level and receptor binding assays which use radiolabelled ligands of high specific radioactivity are widely used to evaluate the biological activity of drugs and hormones. This type of assay has several advantages: it is relatively simple, economical with tissue (animal or human) and a large number of compounds can be tested rapidly. The usual aim of receptor binding experiments is to characterize the affinity (as the association constant (K_a) or more commonly its reciprocal, the dissociation constant (K_d)) and the binding capacity (B_{max}) of the receptor system. Much effort has been expended to maximize the efficiency of the practical aspects of these assays with, for example, robotic liquid handling systems and cell harvesters, but there has not yet been a commensurate improvement in the analysis of the experimental data [1].

Two particular problems concerning this aspect are apparent from reading the current literature on receptor binding: firstly straight lines are fitted to Scatchard plots [2, 3] of experimental data which are obviously non-linear (the following papers include just some examples [4–8]) and secondly, the affinity of the ligand often varies widely with the receptor concentration [9]. It has been observed that dilution of the receptor preparation decreases K_d and may also increase B_{max}.

The usual approach with ligand binding experiments is to use a fixed concentration of receptor preparation and vary the ligand concentration. This applies to investigations with both a relatively pure soluble protein such as albumin and also to the ligand–receptor work which has grown rapidly over the last decade or so. This experimental approach will not therefore reveal any effect of receptor concentration upon K_d and B_{max}. Nevertheless, although there has been little systematic investigation of the effect of receptor concentration upon the affinity of a ligand for its receptor there are now a substantial number of reports which provide evidence that dilution of the acceptor (receptor preparation or albumin) increases the affinity K_a (i.e. K_d decreases) and/or the number of binding sites. This phenomenon has been observed with a wide variety of ligands and receptor preparations that include: histones [10], uterine cytosol [11], striatal homogenates [12, 13] and see also the eight references

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quoted by Seeman *et al.* [9] for insulin receptors, muscarinic receptors, β-adrenergic receptors and dopamine D_2 receptors.

The literature for this effect of dilution on affinity for albumin and several other proteins has also been discussed [14] and in many cases the effect of protein/receptor concentration emerged incidentally during the investigation, with little or no attempt being made to explain it. This phenomenon has not yet been documented so well with tissue receptors although [^{3}H]spiperone, a ligand for dopamine D_2 receptors in the brain, has been considered in some detail [9]. The K_d values for spiperone vary more than 120-fold, from 13 to 1600 pM and they show a consistent dependence upon receptor concentration, with more dilute receptor preparations having higher affinities, i.e. smaller K_d values [9].

We show below that the receptor binding data and the constants (especially K_d), which depend upon the receptor preparation and its concentration, and which are sometimes interpreted as heterogeneity of binding sites, can be explained by a binding model [15, 16] which assumes the presence of a competitive inhibitor (or a contaminant) in the receptor preparation. This model explains why the apparent binding affinities and number of receptor sites appear to vary with receptor concentration in a systematic way and it also offers a better approach to the quantitative analysis of data from ligand–receptor binding experiments. We also show how the apparent binding parameters obtained by the conventional method should be interpreted and how they can be used to estimate the true affinity, provided sufficiently low concentration data are available.

METHODS

Binding model. The binding of a ligand, L, to a single set of independent and identical receptor sites, R, is described by the usual binding equation:

$$b = b_{\max}\frac{K_1F}{1 + K_1F},$$

where F (= [L]) is the concentration of free or unbound drug and K_1 is the association constant. The amount of drug bound to the receptor is defined as

$$b = B/C_R, \qquad (2)$$

where B is the concentration of bound ligand and C_R is the concentration of the receptor. The binding capacity of the receptor $b_{\max}$ is equal to the number of sites per receptor molecule (n) if C_R is given in molar units which, however, requires the molecular weight of the receptor to be known which is seldom the case. The association constant will be used throughout in this section in order to obtain the simplest possible equations.

In the so-called Scatchard plot (b/F versus b) the binding curve Eqn (1) is a straight line which intersects the b/F-axis at a value equal to $K_1b_{\max}$ and the b-axis at a value equal to $b_{\max}$. Site heterogeneity will give rise to a curvature in a Scatchard plot. If the receptor has two sets of independent sites with different association constants K_1 and K_2, then the binding equation has the form

$$b = b_{1max}\frac{K_1F}{1 + K_1F} + b_{2\max}\frac{K_2F}{1 + K_2F}, \qquad (3)$$

where $b_{1\max}$ and $b_{2\max}$ are the binding capacity of the two sets. The curvature increases with increasing difference between the association constants K_1 and K_2 and a limiting appearance of a superposition of two straight lines may be seen.

If the receptor system contains a competitive inhibitor, I, then the binding of the ligand, L, to a receptor with a single set of sites is given by the well-known equation

$$b = b_{\max}\frac{K_aF}{1 + K_aF + K_iI}, \qquad (4)$$

where K_a and K_i are the association constants for the ligand and the inhibitor, respectively. Note that the true association constant for the ligand is called K_a since in the following K_1 and K_2 will be used to denote apparent association constants obtained by an analysis based on Eqn (3). In most treatments of the effect of an inhibitor it is assumed that the free concentration of inhibitor, I, is constant, i.e. independent of the free ligand concentration. For this simple case the product term K_iI is just a constant and the only effect of the inhibitor is that the association constant is replaced by an apparent association constant equal to $K_1/(1 + K_iI)$. In a Scatchard plot a straight line would again be observed and if a series of experiments with different concentrations of inhibitor were performed, then a series of straight lines with different slopes and intersects on the b/F-axis, but with the same intersect at $b_{\max}$ on the b-axis, would be observed. This, however, is not what is observed experimentally.

If the total concentration rather than the free concentration of inhibitor is constant (i.e. independent of the concentration of the ligand) then a completely different scenario is observed. Such a situation would result, for example, if the inhibitor were associated with the receptor preparation, in which case the inhibitor could also be called a contaminant [16]. Obviously the inhibition will be larger the higher the concentration of inhibitor with respect to the receptor concentration, and hence the apparent binding affinity will be lower. Also since the ligand and the inhibitor compete for the same sites it is clear that the free concentration of inhibitor will increase with an increase in the concentration of ligand. Thus the effect of the inhibitor on the apparent binding affinity of the ligand will appear to depend on the free ligand concentration and be greatest for high ligand concentrations. The combined effect, however, is still given by Eqn (4) but the product K_iI now depends on the free ligand concentration F. This dependence has been derived previously [16] and can be written as

$$K_iI = \frac{1}{2}(\alpha\gamma + 1 + K_aF) + \frac{1}{2}\sqrt{(\alpha\gamma + 1 + K_aF)^2 + 4\beta\gamma(1 + K_aF)}, \qquad (5)$$

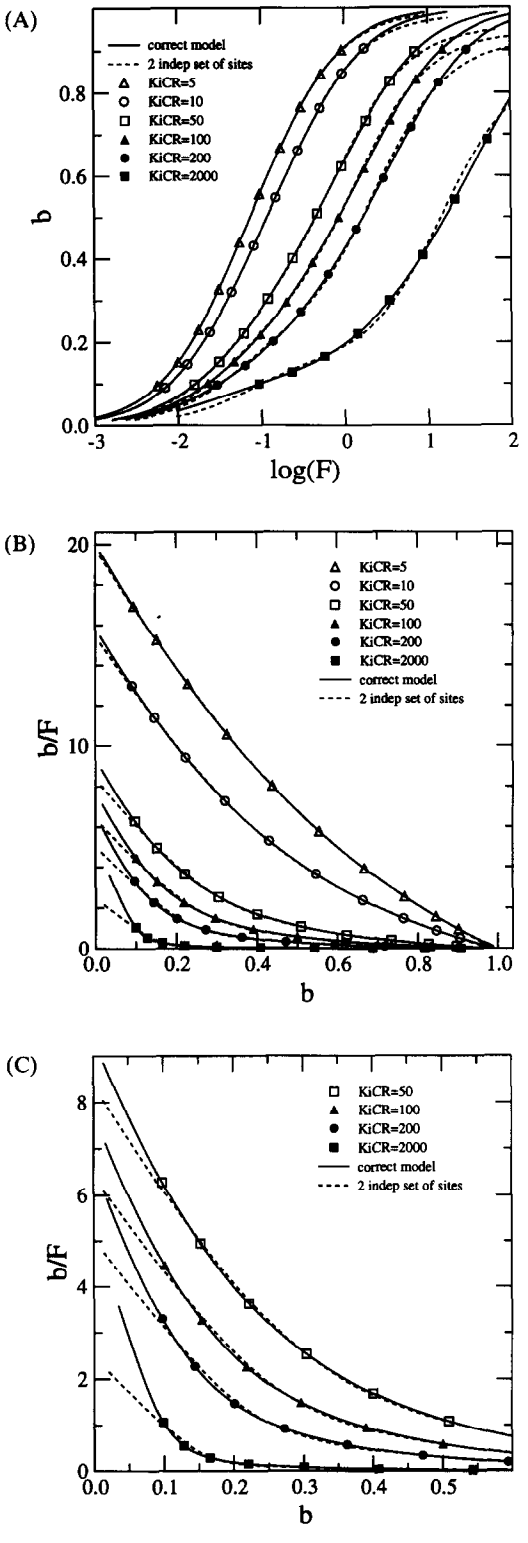

Fig. 1. Illustration of the binding of a ligand to a receptor contaminated by a competitive inhibitor. The data are displayed both as a semi-logarithmic plot (A) and as Scatchard plots (B and C). B and C are identical except that a narrower range of concentration values are displayed in C. The effects of varying the affinity of the inhibitor and/or the concentration of receptor protein were investigated with a large range of values of K_iC_R. The correct binding curve is shown by the full line drawn through the data points. The approximate binding curve obtained by fitting to Eqn (3), i.e. assuming two independent sets of sites, is shown as a broken line. The association constant for the ligand is 50 reciprocal concentration units and the ratio of inhibitor was set at 90% ($\beta = 0.90$). The unit of the amount of bound ligand b was chosen such that the maximum binding capacity $b_{max} = 1$.

where the dimensionless quantities γ and β are defined as

$$\gamma = K_iC_R \tag{6}$$

$$\beta = C_I/C_R. \tag{7}$$

This concentration ratio of inhibitor to receptor (β) is generally not known, but it is assumed to be smaller than and close to one for reasons given below. The other parameter α is simply equal to $1 - \beta$ and as in previous publications [14, 16] β represents the fraction of contaminated receptors sites in the sample and α is the free or uncontaminated fraction.

The binding curves corresponding to the model Eqns (4) and (5) are illustrated as full lines in Fig. 1. The upper part of the figure (1A) is a standard semi-log plot presentation of the data while the two lower figures (1B and C) are Scatchard plot representations. Figure 1C is identical to Fig. 1B except for a narrower view of values which corresponds more closely to the sort of experimental data that is often published. It is clear that the quantity $\gamma = K_iC_R$ has a very pronounced effect on the binding curve. The curves, especially those in a Scatchard plot, show a strong similarity to those corresponding to site heterogeneity where two sets of binding sites are present. This relationship has been investigated previously [16] and it was shown that the binding curve given by Eqns (4) and (5) can be approximated by

$$b = \alpha b_{max}\frac{K_aF}{1 + K_aF} + \beta b_{max}\frac{(K_a/\gamma)F}{1 + (K_a/\gamma)F}. \tag{8}$$

This approximate equation is formally equivalent to the binding of a ligand to two independent sets of sites, cf. Eqn (3), with the apparent high affinity association constant K_1 equal to the true ligand association constant K_a, the apparent low affinity association constant K_2 equal to K_a/γ, the capacity of the high affinity sites $b_{1max} = \alpha b_{max}$ and the capacity of the low affinity sites $b_{2max} = \beta b_{max}$.

Thus, a competitive inhibitor present at a fixed concentration ratio to the receptor will give rise to a binding curve that is similar to one produced by a heterogeneity of binding sites. There is an important difference, however, which relates to the dependence on the receptor concentration. Obviously the binding curve for a receptor with two or more independent sets of sites cannot depend on the receptor concentration. On the other hand, the apparent low affinity binding constant (K_2) in Eqn (8) is equal to K_a/γ and thus depends inversely on receptor concentration since $\gamma = K_iC_R$. A more detailed investigation shows that the apparent high affinity constant (K_1) also depends on the receptor

concentration but to a much weaker extent, see below.

Data. Data resembling experimental results for the binding of a ligand to a macromolecule in the presence of a competitive inhibitor under a variety of experimental conditions were generated from the exact binding Eqns (4) and (5). Since most assay designs are such that the percentage binding of the ligand is in the range 10–50%, we generally used data points that had a uniform distribution of bound saturation values (b) in the range 0.1–0.9. However, we studied explicitly the effect of the range of b values and in particular the minimum value of b. In all cases a value of 50 in arbitrary units was employed for the association constant (K_a) of the ligand for the receptor site although the actual value is really not critical because a different value merely corresponds to a different scale (unit) for the concentration. Only examples of receptor preparations with a high proportion of inhibitor were considered and data were generated for cases of $\beta =$ 0.7, 0.9 and 0.99. The value of $\gamma = K_iC_R$ is of prime importance for the shape of the binding curve and so a whole range of values (5–2000) was used to cover all cases. The data are displayed in Fig. 1 with various symbols according to the different values of K_iC_R. The full curve passing through the data points is the corresponding correct binding curve.

Analysis of data. The majority of receptor binding experiments are analysed in terms of the model for one or two independent sets of sites and so the data generated to simulate the binding of a ligand to a receptor in the presence of a competitive contaminant were fitted to the binding equation for two sets of independent sites, Eqn (3), by a non-linear, least squares regression method. This allowed us to investigate the significance of the parameters usually calculated in the literature. The commonly employed decomposition of the data in a Scatchard plot into two straight lines is also based on the assumption of two sets of independent sites. It should be noted, however, that the geometrical method based on the Scatchard plot is only applicable when the data are a clear superposition of two straight lines and that there are several pitfalls and misuses of the method, see for example a recent note [17], the articles by Kermode [18] and by Klotz [19] who illustrate how the binding capacity in particular will be poorly estimated. On the other hand, the direct computer fit, which treats the data as b versus F in order to minimize error propagation, can always be applied.

RESULTS

Figure 1 displays the generated data which resemble the binding of a ligand to a receptor in the presence of a competitive contaminant under a variety of experimental situations. Both a semi-log [19, 20] and a Scatchard plot are used to display the data. We believe that a Scatchard plot is superior to the semi-log plot with respect to illustrating the effects of an inhibitor and the following discussion refers to this representation of the data, i.e. Fig. 1B and C. Furthermore, it is the most commonly used presentation of receptor binding data and the figures can thus be directly compared with the experimental data in the literature. It should be noted, however, that both the following discussion and the interpretation of the apparent binding parameters are independent of how the data are presented graphically. The correct binding curve is illustrated by a full line drawn through the data points. Several values for the ratio of inhibitor to receptor were investigated, but for the sake of argument receptor preparations with 90% of the sites occupied by a competitive inhibitor (i.e. $\beta = 0.90$) were considered (Fig. 1 and Table 1) and also $\beta = 0.99$ (Table 2).

The curvature increased with increasing values of the K_iC_R parameter (Fig. 1). A value of the order of 1 caused a very small degree of curvature while a very large value of the order of 1000 produced a pronounced curvature such that the curve looks almost like a superposition of two straight lines with very different slopes. A high value of K_iC_R indicates a strongly bound inhibitor and/or a high concentration of receptor. Note that the curvature observed experimentally may depend on the range of values measured. This is especially so for high values of K_iC_R where it may be possible to observe only one (the lower) of the two branches of the curve. The importance of making experimental measurements over a wide range, and especially at low values of b must therefore be stressed.

Table 1. Fitted parameter values of Eqn (3) for data generated by the competitive contaminant model with 90% of the binding sites occupied by a competitive inhibitor, i.e. $\alpha = 0.1$ and $\beta = 0.9$. The association constant $K_a = 50$ and the various values of the parameter K_iC_R which were studied are indicated in the first column

K_iC_R	K_1	K_2	$K_a/(K_iC_R)$*	b_{1max}	b_{2max}
5	44	8.0	10	0.35	0.63
10	34	4.0	5.0	0.39	0.59
50	28	1.3	1.0	0.28	0.67
100	27	0.78	0.50	0.24	0.69
200	27	0.48	0.25	0.20	0.70
2000	35	0.09	0.13	0.13†	0.68†

* The expected theoretical value of K_2.

† Note that b_{1max} and b_{2max} only approach α and β, respectively, at high values of K_iC_R.

Table 2. Fitted parameter values of Eqn (3) for data generated by the competitive contaminant model with 99% of the binding sites occupied by a competitive inhibitor, i.e. $\alpha = 0.01$ and $\beta = 0.99$. The association $K_a = 50$ and the various values of the parameter K_iC_R which were studied are indicated in the first column. The lowest value of b studied was $b = 0.10$

K_iC_R	K_1	K_2	$K_a/(K_iC_R)$*	b_{1max}†	b_{2max}†
5	33	5.9	10	0.44	0.56
10	29	3.6	5.0	0.38	0.60
50	18	1.0	1.0	0.29	0.67
100	14	0.60	0.50	0.25	0.69
200	9.9	0.33	0.25	0.23	0.71

* The expected theoretical value of K_2.

† The unit of b was chosen such that $b_{max} = 1$.

The intercept of the Scatchard plot on the b/F-axis was seen to decrease with increasing values of K_iC_R. The approximate binding Eqn (8) is not accurate enough to describe this dependence. A more detailed examination of the exact binding Eqns (4) and (5) shows that the intercept decreases from K_a for small values of K_iC_R (≤ 1) to αK_a for large values of K_iC_R (>1000). Thus, dilution of the receptor preparation will give rise to an increase in the intercept on the b/F-axis and therefore to a corresponding increase in the apparent high affinity association constant. Figure 1B and C illustrates the dependence of the intercepts versus K_iC_R. It is seen that the main variation of the intercepts occurs for values of K_iC_R in the range 0–20. The relative change of the intercept is smaller for larger values of K_iC_R. Since the curvature of the Scatchard plot increases with increasing values of K_iC_R the extrapolated intercept based on data where the very low concentration results are missing will cause an increasingly greater underestimation of the true intercept, cf. Fig. 1. This artificial effect will tend to enlarge the true effect which should therefore be easily observed and together with the change of curvature should assist the recognition of the presence of a competitive inhibitor; particularly if experimental data are available or accessible for different concentrations of the receptor.

The fits of the data to the binding equation for two independent sets of sites, Eqn (3), are displayed in Fig. 1 as broken lines. In general, it was possible to obtain quite a good fit although the fits were progressively poorer the more curvature the data showed, especially at lower values of b. The apparent binding parameters obtained by the fitting procedure are given in Table 1. The true binding parameters, referring to the competitive contaminant model [16], which were used to generate the data are also given.

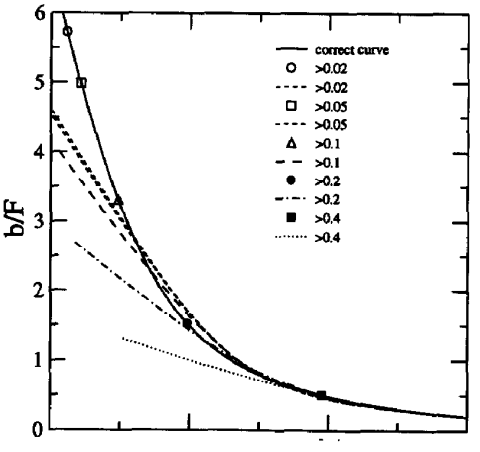

Fig. 2. Variation of the binding parameters, estimated with the two site model, which results from differences in the distribution of experimental data. All data correspond to a ligand association constant $K_a = 50$ and the inhibition was determined with $\alpha = 0.10$ and $K_iC_R = 200$. The largest value of b was in all cases equal to $0.9\,b_{max}$ while the smallest value of b varied and was 0.02, 0.05, 0.1, 0.2 and 0.4 times b_{max}, respectively. The full line is the correct binding curve given by Eqns (4) and (5) and incorporates the effect of the inhibitor. The broken lines are the best fits of the data to the two site model, Eqn (3). The corresponding estimated binding parameter values are displayed in Table 3.

Table 3. Dependence of fitted binding parameters on the range of data points

min(b)*†	K_1	b_{1max}	K_2	b_{2max}
0.05	16	0.26	0.31	0.70
0.1	14	0.27	0.30	0.69
0.2	9	0.32	0.26	0.65
0.4	4	0.43	0.19	0.56

* The 10 data points were distributed from min (b) to 0.90. In all cases the data correspond to a ligand association constant of $K_a = 50$ and the inhibition is determined by $\alpha = 0.1$ and $K_iC_R = 200$.

† The unit of b was chosen such that $b_{max} = 1$.

The effect on the fit of the presence or absence of low concentration data is already evident in Fig. 1 but it is more clearly demonstrated in Fig. 2 and Table 3. Figure 2 shows explicitly how the fit (and thus the estimated binding parameters—in particular K_1) changes with the magnitude of the lowest concentration data point. The fit is dramatically improved by adding low concentration data until the smallest b value becomes smaller than $0.5\,\alpha b_{max}$ from which point onwards no further improvement was observed.

Tables 1 and 2 show that the apparent binding parameters, i.e. the fitted values K_1 and K_2, were quite close to the true association constants K_a and to $K_a/(K_iC_R)$ as predicted by the approximate binding Eqn (8). In general, the values agree to within a factor of five and the agreement is best for low values of K_iC_R. In all cases it was found that $K_1 < K_a$, i.e. the value of K_1 obtained by the computer fit was an underestimation of the true association constant (K_a) of the ligand for the receptor site. The agreement was poorer for a higher degree of inhibition, see below. If the proportion of inhibitor is substantial ($\beta > 0.9$) the data points at high b/F values are very difficult to obtain experimentally and are also likely to be ignored as being simply in error, with the consequence that the data may only be analysed in terms of a single set of receptor sites. Since it is the low concentration data that are missing the estimated association constant would correspond to the apparent low affinity sites which have an affinity constant equal to $K_a/(K_iC_R)$. This would result in an underestimation of the affinity by one or two orders of magnitude or even more, depending on the magnitude of K_iC_R.

The fractional numbers of receptor sites estimated by the standard fitting procedure as b_{1max}/b_{max} and b_{2max}/b_{max}, where $b_{max} = b_{1max} + b_{2max}$, were in general, not usable estimates of α and β as predicted by the simplified Eqn (4). It appears as though a value around 0.3–0.5 for α is obtained for the weakly curved cases irrespective of the underlying true value (Tables 1 and 2). Only for a strongly bound inhibitor ($K_iC_R > 1000$) are the values of these parameters

good estimates of the degree of contamination β and this requires that the experimental points cover the low concentration region, i.e. there should be experimental values of b smaller than αb_{max}. Table 2 exemplifies this for a case where $\beta = 0.99$ ($\alpha = 0.01$) where the smallest value of b/b_{max} is 0.10, i.e. significantly larger than 0.01. It can be seen that as the parameter K_iC_R was increased, the estimates of K_1 became rapidly poorer.

DISCUSSION

Competitive inhibitors are an inescapable part of the experimental methods used to study receptor binding [21] and so the aim has been to point out that the effects of a competing ligand are likely to cause errors in the estimation and interpretation of the receptor binding constants. This, of course, is true whichever graphical form (Scatchard, semi-logarithmic or other) is used to present the data. For example a radiochemical impurity associated with the ligand could be an effective inhibitor. Radiochemical impurities are common in ^{3}H-labelled compounds and can become significant during just a few months of storage [22]. Furthermore, a radiochemical inhibitor will be present in a fixed ratio to the receptor when the experimental design is such that the total amount of radioactivity remains constant over the range of ligand concentrations.

One of the manifestations of a competitive inhibitor is an apparent site heterogeneity and therefore an associated non-linear Scatchard plot (upwardly concave). Other possible explanations for this observation are: (1) site heterogeneity, i.e. the receptor has more than one set of sites with different intrinsic binding constants; (2) interaction between identical sites on the receptor; and (3) cross-linking of the receptor by a bivalent ligand. None of these models has the characteristics of the competitive contaminant model, which is an inverse dependence of the affinity (K_a) and b_{1max} upon the protein concentration. Explanations (1) and (2) do not give rise to any dependence of the binding parameters upon protein concentration. Explanation (3) does give rise to a protein concentration dependence but the different binding curves in a Scatchard plot obtained by variation of the protein concentration intersect at the point $b = b_{max}/2$ [23], i.e. a dependence which is very different from that caused by a competitive inhibitor, cf. Fig. 2. None of the alternative explanations can thus account for all the experimental observations which show that apparent affinity (K_a) increases as the protein concentration decreases.

Seeman *et al.* [9] investigated the effect of protein concentration on the binding of [^{3}H]spiperone to human tissues and also assembled values of K_d for the interaction of [^{3}H]spiperone with its receptor, principally in rat striatum, from 35 publications. Both their own experimental results and the literature values were obtained over a wide range of protein concentrations. In both cases the K_d values increased linearly with protein concentrations (figs 4–7 in [9]) in exactly the way predicted by the presence of a competitive inhibitor (highly contaminated receptor) where the estimated binding constant is the apparent low affinity constant. No other obvious explanation for this concentration dependence is available apart from the partition phenomenon [9].

The effect of a contaminant was shown to increase with the value of the product K_iC_R and a value larger than one is needed to see an effect. It is not immediately clear whether this would require the contaminant to have a higher affinity than the ligand for the site. That this is not the case can be seen as follows. Most assay designs use a low concentration of receptor to restrict the percentage binding to 10–30%. If the receptor is highly contaminated, i.e. $\beta > 0.9$, then this implies that the observed binding is described by the low affinity binding term in Eqn (8) and thus $K_2F = (K_a/K_iC_R)F \approx 0.1$–$0.3$. As an example assume that the ligand and the contaminant have the same or similar affinity ($K_a \approx K_i$) then it follows that the apparent low affinity binding constant K_2 is equal to $1/C_R$, i.e. independent of the true affinity constant K_a! This rather surprising result means that if the ligand and the contaminant are similar then the apparent low affinity constant, which is the one usually stated, has little or no relation to the true association constant, but is only a measure of the receptor concentration used in the experiment. For this case the condition $K_iC_R > 1$ can be rewritten to $K_1 > K_2$, i.e. the Scatchard plot is curved and the low affinity constant depends inversely on the receptor concentration; which is exactly what is observed. In a more direct way it appears quite reasonable that $K_iC_R > 1$ for a contaminant which is similar to a high affinity, site-specific ligand.

The situation for a contaminated receptor is thus quantitatively different from that found in gold–albumin binding [15]. In the latter case the sample was only partially contaminated ($\beta \approx 0.3$) but the contaminant had a much higher affinity than the ligand. In the receptor case the site is probably highly contaminated ($\beta \approx 1$) but the affinity of the contaminant may be equal to or smaller than the affinity of the ligand.

Experimental receptor data with the above characteristics are analysed best by a computer fit to the correct binding equation for the competitive contaminant model, i.e. Eqns (4) and (5) above or Eqns (3) and (9) in Pedersen and Pedersen [16]. The model is not yet, however, included in the available curve-fitting programs and thus an alternative, although less accurate, method is desirable. We have shown that useful estimates of the binding constants for the interaction of the drug and a competitive inhibitor with the receptor can be obtained by the usual method of analysis, which assumes two independent sets of sites, provided that the apparent binding parameters are interpreted as stated above and that data at sufficiently low concentration are available.

If the presence of a competitive inhibitor is suspected, then K_d and b_{max} should be determined at several different receptor concentrations to see if the affinity increases as the protein concentration decreases. If this is the case then very low concentration data are needed to accurately determine two (rather than one) apparent binding constants because the apparent high affinity binding constant, which is equal to the true binding constant,

can only be accurately estimated for values of b smaller than αb_{max}, cf. the bottom curve in Fig. 2. This precaution will help to minimize, for example, the ambiguities that arise in receptor classifications [24] which are based mainly on binding data.

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