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Interpretation of Binding Curves Obtained with High Receptor Concentrations: Practical Aid for Computer Analysis

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SUMMARY

Typical equilibrium binding experiments cannot be quantitatively analyzed on the basis of classical mathematical equations when the receptor concentration is so high that a significant fraction of the added radioligand concentration is in the bound form. In this paper, the appropriate equations are derived and used in a commercial graphics package to estimate the binding parameters, by applying nonlinear regression to pseudo-experimental data. The analysis of saturation and homologous displacement curves obtained with high receptor concentrations reveals that the empirical determination of nonspecific binding by addition of an excess of unlabeled ligand is incorrect.

The binding of ligand to a specific receptor may be characterized by the equilibrium dissociation constant (K_d) and the maximal binding or total receptor concentration (R_{tot}). The determination of these two parameters can be achieved on the basis of saturation or displacement binding curves obtained at equilibrium. The numerical treatment of such binding data generally requires that the free ligand concentration does not significantly differ from the total added ligand concentration. To meet this constraint, the receptor concentration must be rather low, compared with the K_d value.

The rapidly developing techniques in the field of molecular biology have led to the cloning and sequencing of a number of receptors. It has thus become possible to produce a large number of recombinant receptors in transfected cell lines, such that the receptor concentration could easily exceed the K_d value. In this case, a significant fraction of the added ligand is bound to the receptor, and the free ligand concentration cannot be approximated by the total ligand concentration. To circumvent this problem, the experiment could be repeated with less tissue or fewer cells, and thus with a lower receptor concentration. However, the experimenter could be interested in analyzing data obtained when the fraction of bound ligand is not negligible, either by accident or because the receptor concentration cannot be reduced for some experimental reason. A practical example of such a situation is the study of the correlation between binding characteristics and constitutive activity of different mutated receptors. Activity and binding assays should be performed in parallel and under the same conditions. A low level of constitutive activity could require the use of a high receptor concentration, leading to a high fraction of bound ligand.

When a significant fraction of the ligand is bound, the binding data plotted against the added ligand concentration cannot be described by the usual mathematical equations defining binding curves as functions of free ligand concentration. Two methods have been proposed to estimate the binding affinity in this situation, but they can be applied only when nonspecific binding is virtually absent (1, 2). The aim of this paper is to provide the correct equations describing the total binding (specific binding plus nonspecific binding) as a function of the added ligand concentration and not of the free ligand concentration. These equations can be easily implemented in a commercial software program that estimates the binding parameters by fitting the data according to the nonlinear regression method (3).

Rationale

Model. The binding model assumes that the ligand L binds to a single class of receptors and to nonspecific sites. The specific binding obeys Michaelian kinetics characterized by the two parameters K_d and R_{tot}. Nonspecific binding linearly depends on ligand concentration and is characterized by the parameter α, defined as the ratio between nonspecifically bound ligand and free ligand. The total concentration of bound radioligand is referred to as B^*_{tot}.

Saturation curve. In this case, the total concentration of bound ligand can be expressed as a function of the free radioligand concentration, L^*

$$B^*_{tot} = \frac{R_{tot}L^*}{K_d + L^*} + \alpha L^* \qquad (1)$$

To fit experimental data on the basis of this model, eq. 1 must be transformed, because the measured experimental variable is the total amount of bound radioligand, expressed, for instance, in cpm. If

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β is the specific radioactivity expressed in cpm/molar units, the total amount of bound radioligand and the maximal specific binding expressed in cpm are given by $b^*_{tot} = \beta B^*_{tot}$ and $r_{tot} = \beta R_{tot}$, respectively. Thus, multiplying both members of eq. 1 by β, one obtains

$$b^*_{tot} = \frac{r_{tot}L^*}{K_d + L^*} + \alpha\beta L^* \quad (2)$$

Because the experimental independent variable is the total ligand concentration, L^*_{tot}, L^* is replaced by $L^*_{tot} - (b^*_{tot}/\beta)$ in eq. 2, leading to a quadratic equation that defines the relationship between the variables b^*_{tot} and L^*_{tot} and the parameters of the system

$$b^{*2}_{tot}(1+\alpha) - b^*_{tot}[\beta(K_d + L^*_{tot})(1+\alpha) + r_{tot} + \alpha\beta L^*_{tot}] + \beta L^*_{tot}[\alpha\beta(K_d + L^*_{tot}) + r_{tot}] = 0 \quad (3)$$

If the experimental system fulfills the constraints of this model, then the analytical solution of eq. 3 can be used (as shown in Table 1a) to fit a saturation curve by nonlinear regression.

Homologous displacement curve. In this case, it is assumed that the total concentration of radioligand, L^*_{tot}, is constant and that the total concentration of the homologous unlabeled ligand, L_{tot}, increases. According to the competitive inhibition model, the total concentration of bound radioligand is given by

$$B^*_{tot} = \frac{R_{tot}L^*}{K_d + L^* + L} + \alpha L^* \quad (4)$$

and, multiplying both members by β, one obtains

$$b^*_{tot} = \frac{r_{tot}L^*}{K_d + L^* + L} + \alpha\beta L^* \quad (5)$$

Because labeled and unlabeled ligands are homologous molecules, the ratio between free and total concentrations is the same for both ligands, and thus

$$L = \frac{L_{tot}}{L^*_{tot}}L^* \quad (6)$$

Replacing B^*_{tot} by $L^*_{tot} - L^*$ in eq. 4, and using eq. 6, one obtains the quadratic equation

$$L^{*2}(1+\alpha)\left(1 + \frac{L_{tot}}{L^*_{tot}}\right) + L^*\left[\frac{r_{tot}}{\beta} + K_d(1+\alpha) - L^*_{tot} - L_{tot}\right] - L^*_{tot}K_d = 0 \quad (7)$$

Knowing the total concentrations of labeled and unlabeled ligands and introducing the parameter values in eq. 7 leads to the computation of L^*. Then, L and b^*_{tot} can be successively computed by eqs. 6 and 5, respectively. If the experimental system fulfills the constraints of this model, then these different computational steps can be used (as shown in Table 1b) to fit a homologous displacement curve by nonlinear regression.

TABLE 1

Curve-fitting equations as introduced in the nonlinear regression module of the commercial program SigmaPlot

It is assumed that the independent and dependent variables are in columns 1 and 2 of the worksheet, respectively. If a weighted nonlinear regression is required, the expected experimental error is introduced in column 3.

A. Module for the fitting of a saturation curve

```
Parameters
  rt = 50,000; enter here a starting value for Rtot (cpm)
  kd = 1; enter here a starting value for Kd (nM)
  alpha = 0.5; enter here a starting value for α (no units)
Variables
  x = col(1); L*tot is in column 1 (in nM)
  y = col(2); b*tot is in column 2 (in cpm)
  w = 1/col(3)^2; weight = reciprocal of the square of the error
    given in column 3
Equations
  beta = 10,000; enter here the specific radioactivity (in cpm/nM)
  a = (1 + alpha)
  b = -(beta*(kd + x)*(1 + alpha) + rt + beta*x*alpha)
  c = beta*x*(alpha*beta*(kd + x) + rt)
  yth = (-b - sqrt(b*b - 4*a*c))/2/a; computation of predicted b*tot
  fit yth to y with weight w
```

B. Module for the fitting of a homologous displacement curve

```
Parameters
  rt = 50,000; enter here a starting value for Rtot (cpm)
  kd = 1; enter here a starting value for Kd (nM)
  alpha = 0.5; enter here a starting value for α (no units)
Variables
  x = col(1); Ltot is in column 1 (nM)
  y = col(2); b*tot is in column 2 (cpm)
  w = 1/col(3)^2; weight = reciprocal of the square of the error
    given in column 3
Equations
  conc = 1; enter here the radioligand concentration (nM)
  cpm = 10,000; enter here the corresponding radioactivity (cpm)
  a = (1 + alpha)*(1 + x/conc)
  b = rt*conc/cpm - conc - x + kd*(1 + alpha)
  c = -conc*kd
  ls = (-b + sqrt(b*b - 4*a*c))/2/a; computation of free ligand
    concentration
  l = ls*x/conc; computation of free unlabeled ligand concentration
  yth = rt*ls/(kd + ls + l) + alpha*ls*cpm/conc; computation of
    predicted b*tot
  fit yth to y with weight w
```

Results

General methods. The proposed methodology has been developed to deal with a binding system in which a significant fraction of added ligand is bound to specific and nonspecific sites. To assess the necessity to apply this methodology in this particular case, pseudo-experimental saturation and displacement curves have been generated and analyzed by computer fitting. For this purpose, the nonlinear regression routine in a commercially available, graphics software package (SigmaPlot for Windows, version 1.02; Jandel Corporation) has been used.

The first approach consisted of analyzing saturation and displacement curves obtained with a particular set of parameters, i.e., 5 and 1 nM for the total receptor concentration (R_{tot}) and the equilibrium dissociation constant (K_d), respectively, and 0.3 for α. For each point of the curves, a random error was introduced by generating a random number issued from a normally distributed population. This procedure was repeated to obtain 20 different pseudo-experimental curves, to show the usefulness of the proposed method. Then, a second approach was designed to define the range of situations where the use of the method should be recommended. For that purpose, theoretical curves obtained with different sets of parameters were analyzed by usual methods and the divergence between estimated and actual values of the parameters was examined.

Saturation curve. The typical pseudo-experimental saturation data presented in Fig. 1a were obtained in the absence or in the presence of an excess of unlabeled competitor. The latter situation simulated the experimental method commonly used to determine nonspecific binding. The total bind-

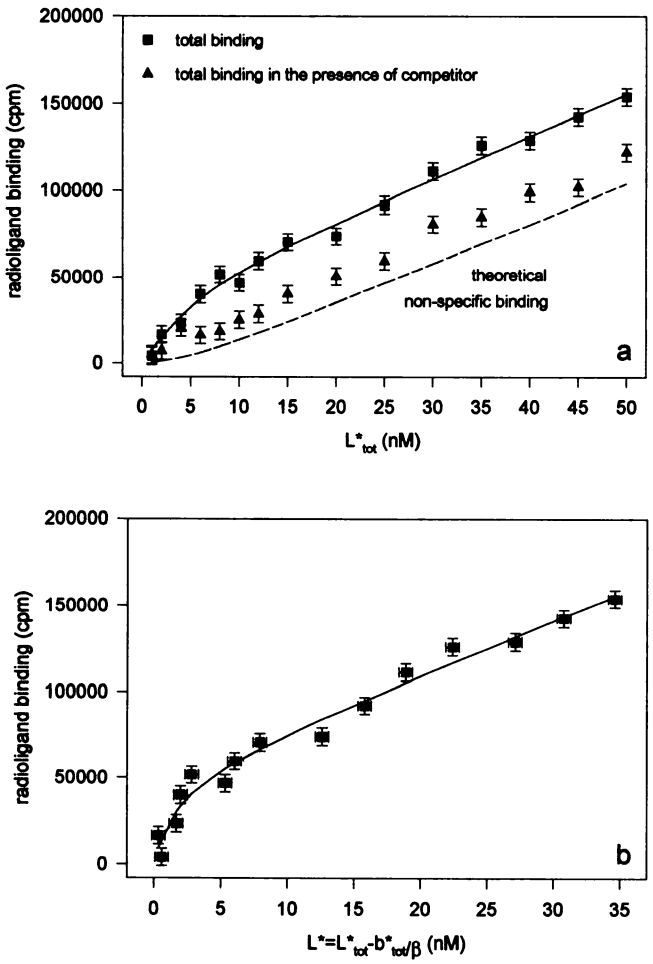

Fig. 1. Simulation and analysis of a saturation experiment. Pseudo-experimental data were generated by the model defined by eq. 2, using the following parameter values: R_{tot} = 5 nM, K_d = 1 nM, and α = 0.3. The specific radioactivity β is equal to 10,000 cpm/nM. The absolute error is equal to 5000 cpm. a, The data obtained in the absence or in the presence of an excess of competitor are plotted against the total radioligand concentration (mean ± standard deviation). *Solid line*, result of the curve fitting based on the procedure (method T) presented in Table 1a. *Dashed line*, theoretical nonspecific binding curve obtained in the absence of competitor. b, The same data are plotted against the free radioligand concentration, calculated as the difference between total and bound radioligand concentrations. *Solid line*, result of the curve fitting based on eq. 2 (method C).

ing data were fitted according to the proposed method T (T refers to "total") by using the computation module described in Table 1a. As expected, the estimates of the three parameters ($R_{tot,estimated}$ = 4.93 nM, $K_{d,estimated}$ = 1.42 nM, and $\alpha_{estimated}$ = 0.31) were compatible with the theoretical values used to generate the data.

Assuming that a significant difference between free and total ligand concentrations is observed, one could propose to plot the total binding, b^*_{tot}, against the calculated free ligand concentration, $L^* = L^*_{tot} - (b^*_{tot}/\beta)$ (Fig. 1b), and to fit this transformed curve on the basis of the "classical" eq. 2, which deals with free ligand concentrations (method C, with C referring to "calculated"). However, this theoretically correct procedure is reliable only if the experimental data are error-free. If this is not the case, the transformation introduces an error in the variable L^* (x-axis) and some dependence between the errors associated with the two variables, b^*_{tot} and L^*. As a consequence, the statistical constraint concerning the absence of error in the independent variable is violated, leading to biased estimates of the parameters (4). The analysis of the data plotted in Fig. 1b led to an apparently acceptable result ($R_{tot,estimated}$ = 5.19 nM, $K_{d,estimated}$ = 1.76 nM, and $\alpha_{estimated}$ = 0.30), but the reliability of the parameter estimates might be questioned (see below).

The untransformed saturation data (Fig. 1a) were also fitted by the classical eq. 2, i.e., ignoring the fact that the free ligand concentration was significantly lower than the total concentration, and thus the free concentration was approximated by the total concentration (method A, A referring to "approximated"). The result of the fitting ($R_{tot,estimated}$ = 5.28 nM, $K_{d,estimated}$ = 7.46 nM, and $\alpha_{estimated}$ = 0.22) demonstrated the inadequacy of the method in this situation, especially concerning the K_d estimate, which greatly deviated from the actual value.

The latter method, based on eq. 2, implicitly assumed that nonspecific binding was a linear function of ligand concentration, and this assumption seemed to be verified by the pseudo-experimental binding data obtained in the presence of an excess of unlabeled competitor (Fig. 1a). However, it appeared that this experimentally measured nonspecific binding deviated from the actual theoretically computed curve (Fig. 1a), which exhibited an upward curvature. This curvature is due to the fact that the nonspecific binding is proportional to the free ligand concentration, which is not proportional to the total ligand concentration. The discrepancy between experimental and theoretical nonspecific curves is explained as follows. The addition of the competitor leads to the displacement of the radioligand from the specific receptor sites, and thus the free radioligand concentration is higher in the presence than in the absence of competitor. Therefore, the residual binding obtained in the presence of the competitor does not reflect the amount of radioligand that is bound to nonspecific sites in the absence of the competitor, demonstrating that this experimental determination of nonspecific binding is misleading when a significant fraction of the radioligand can be sequestered by the specific receptors. Incidentally, it must be recalled that, in any case, the quantitative analysis of a saturation curve based on the fitting of total binding would be preferred to the fitting of specific binding calculated by subtracting experimentally measured nonspecific binding from total binding (5).

To show the possible presence of bias in the parameter estimates obtained by the three considered methods, the procedure used to generate the pseudo-experimental data shown in Fig. 1 was repeated 20 times. The fitting of the curves led to 20 different sets of parameter estimates. Fig. 2 shows the distributions of the ratios of estimated and actual values for R_{tot} and K_d. The proposed method T, in which the total ligand concentration was considered, gave distributions centered on 1, demonstrating, as expected, that the parameter estimators are unbiased. For method C, in which free ligand concentrations were calculated, both estimators appeared to be biased. It must be noted that, with this method, the bias was due only to the presence of error in the x-variable. Finally, for method A, in which free ligand concentrations were approximated by total concentrations, the K_d estimator was obviously biased, whereas the R_{tot} estimator seemed to be unbiased. It was thus interesting to investigate how the estimator bias depended on parameter values for

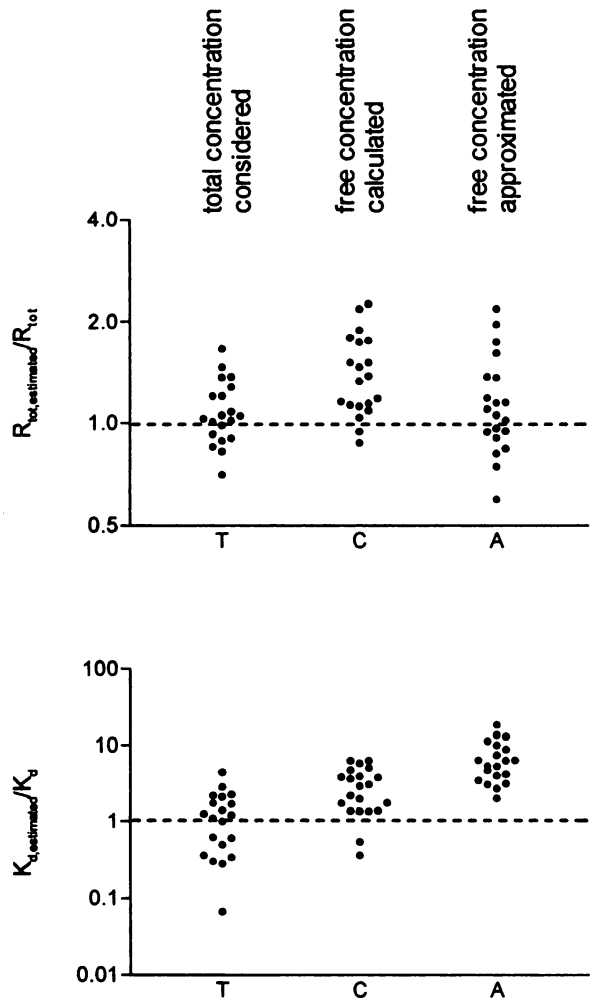

Fig. 2. Results of the fitting applied to 20 saturation curves obtained as in Fig. 1. R_{tot} and K_d estimates, relative to the actual parameter value, were obtained according to three methods, as follows: method T, proposed fitting method dealing with the total ligand concentration; method C, fitting method applied to the transformed data plotted as a function of the calculated free ligand concentration; method A, fitting method applied to the original data, assuming that the free ligand concentrations can be approximated by the total concentrations.

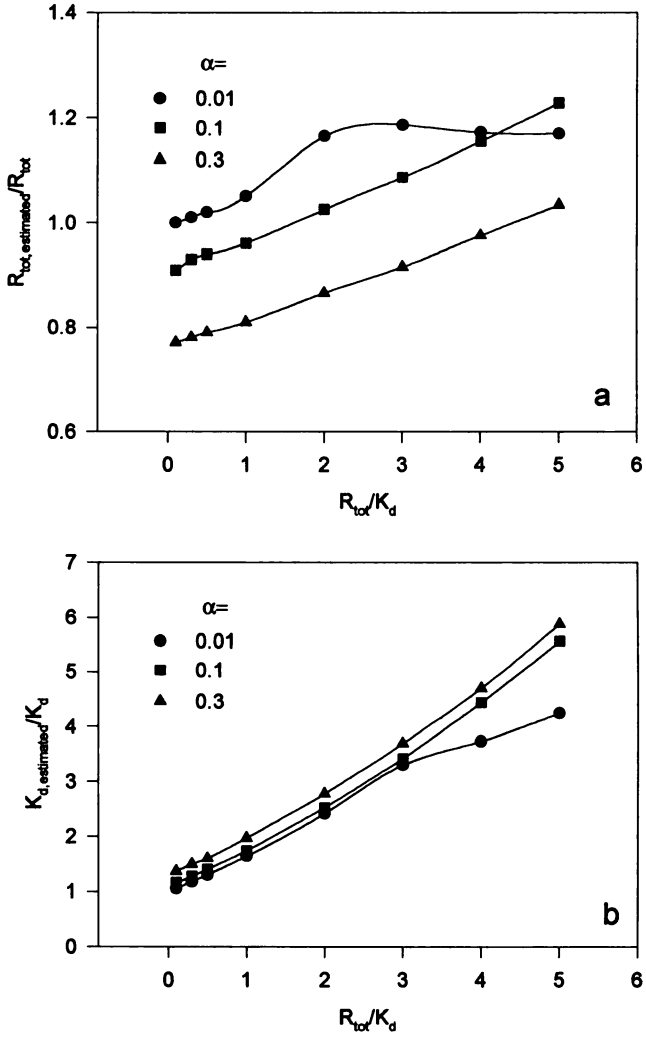

Fig. 3. Bias in R_{tot} and K_d estimates obtained in different situations with the fitting method that assumes that the free ligand concentrations can be approximated by the total concentrations (method A in Fig. 2). The fitting was applied to error-free saturation curves generated as in Fig. 1, but with different values of R_{tot} and α. K_d was always equal to 1 nM.

method A. Fig. 3 shows the results obtained by fitting error-free saturation curves generated in different situations. As expected, both estimators were unbiased only when R_{tot}/K_d and α were low. Indeed, only in this case were free and total ligand concentrations not significantly different. If α was increased, a significant fraction of ligand bound to nonspecific sites, leading to biased estimators; R_{tot} was underestimated, whereas K_d was overestimated. On the other hand, an increase of R_{tot} led to overestimated values of both parameters. However, in the considered situations, the error in the estimate of R_{tot} never exceeded 25%. Interestingly, in the presence of a significant fraction of nonspecifically bound ligand, there existed a range of R_{tot} values for which this parameter was accurately estimated. This was the case for the particular example treated in Figs. 1 and 2. The inadequacy of method A was more evident concerning the K_d estimates, which, in the considered situations, might exceed the actual value by a factor of 6.

Homologous displacement curve. The same general strategy was followed for the analysis of displacement curves. The pseudo-experimental data were obtained by assuming that the constant total concentration of radioligand was equal to 1 nM. Fig. 4 shows typical displacement data, which were fitted according to the proposed method T by using the computation module described in Table 1b. The parameter estimates were in good agreement with the theoretical values used to generate the data ($R_{tot,estimated}$ = 4.57 nM, $K_{d,estimated}$ = 1.06 nM, and $\alpha_{estimated}$ = 0.30). If the same data were fitted by eq. 5, i.e., wrongly supposing that free ligand concentrations could be approximated by total concentrations, the K_d estimate greatly deviated from the expected value ($R_{tot,estimated}$ = 5.71 nM, $K_{d,estimated}$ = 8.7 nM, and $\alpha_{estimated}$ = 0.22), suggesting the inadequacy of this approximated method A. This analysis applied to 20 different curves generated with the same parameter values led to the distributions of parameter estimates shown in Fig. 5. As expected, the proposed method T, dealing with total ligand concentrations, exhibited unbiased parameter estimators, whereas method A, which approximated free concentrations by total concentrations, led to biased overestimated parameter val-

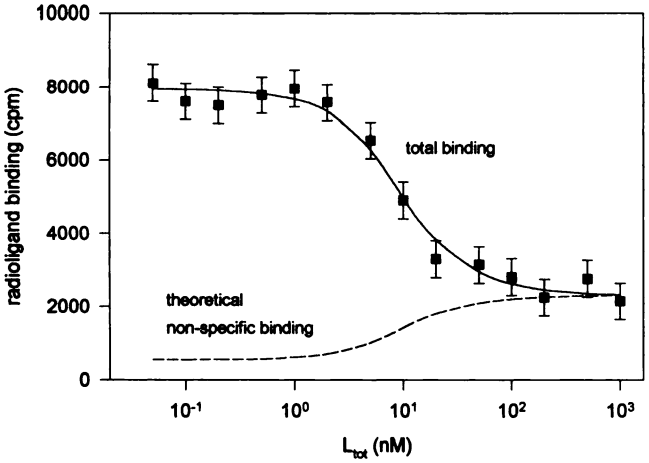

Fig. 4. Simulation and analysis of a homologous displacement experiment. Pseudo-experimental data were generated by the model defined by eq. 5, using the following parameter values: R_{tot} = 5 nM, K_d = 1 nM, and α = 0.3. The specific radioactivity β is equal to 10,000 cpm/nM. The constant total concentration of radioligand is equal to 1 nM. The absolute error is equal to 500 cpm. The data (mean ± standard deviation) are plotted against the total concentration of unlabeled ligand. *Solid line*, result of the curve fitting based on the procedure (method T) presented in Table 1b. *Dashed line*, theoretical nonspecific binding curve.

ues. As was the case for the saturation curves, the bias in the parameter estimates obtained with this method depended on the actual values of the parameters (Fig. 6). The general conclusion was that the bias in the K_d estimate was very sensitive to the receptor concentration and thus to the fraction of ligand bound to the specific sites, whereas R_{tot} might be overestimated or underestimated depending on the parameter values. It must be noted that the fitting of a displacement curve generated with R_{tot} = 0.3 nM, K_d = 1 nM, and α = 0.01 overestimated K_d by 25%, although the fraction of bound radioligand was only 15% in the absence of unlabeled ligand. Thus, it seemed that the proposed method T should be preferred even when the fraction of bound radioligand is low.

The correction of the Cheng-Prusoff equation proposed by Munson and Rodbard (2) as a tool to estimate binding affinity deals only with the specific binding component of the displacement curve. Moreover, it requires the determination of the total concentration of unlabeled ligand producing half-maximal displacement of the specific sites (ED_{50}). This can be achieved by fitting the data with, for instance, the logistic equation (6). To determine the specific binding component, it is tempting to subtract, from the total binding, the residual binding obtained at high concentrations of unlabeled ligand. It must be stressed that such an approach is misleading, essentially because it does not account for the fact that nonspecific binding of the radioligand increases with respect to unlabeled ligand concentration (Fig. 2). Indeed, when the unlabeled ligand concentration increases, more radioligand molecules are displaced, leading to an increase in free radioligand concentration and thus in nonspecific binding of the radioligand. It can be concluded that, when a significant fraction of added radioligand can be sequestered by the receptor, it is not possible to directly determine the nonspecific binding from a homologous displacement curve.

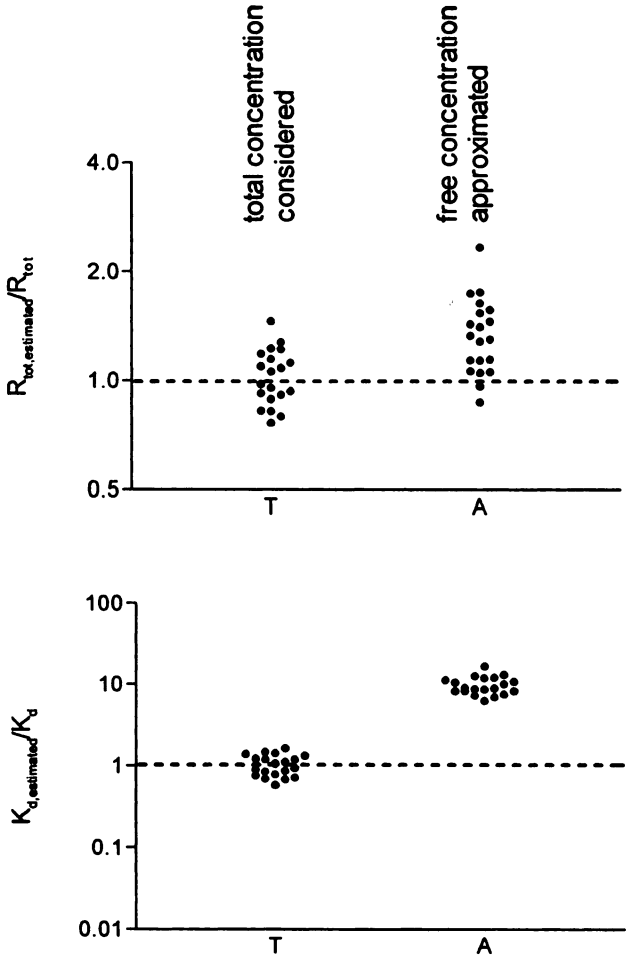

Fig. 5. Results of the fitting applied to 20 displacement curves obtained as in Fig. 4. R_{tot} and K_d estimates, relative to the actual parameter value, were obtained according to two methods, as follows: method T, proposed fitting method dealing with the total ligand concentration; method A, fitting method assuming that the free ligand concentrations can be approximated by the total concentrations.

Discussion

The quantitative interpretation of binding curves requires an adequate numerical treatment of the data. It has been repeatedly claimed that methods based on the linearization of binding curves may lead to bad estimates of binding parameters (3). Such methods, like Scatchard analysis, which were highly useful in the past, are now obsolete, compared with more sophisticated computer-aided approaches (7). Today, the most widely used technique is based on weighted nonlinear regression of the data and is implemented in several commercially available graphics packages. It must be stressed that this method requires that only the dependent variable, associated with the y-axis of the plot, presents experimental uncertainty, which is Gaussian in its distribution. In contrast, the independent variable, associated with the x-axis, must be virtually error free. If this constraint is not fulfilled, nonlinear regression is statistically incorrect because it deals with biased parameter estimates. In this case, another numerical method must be used, such as, for instance, the maximum-likelihood curve-fitting method (8). Unfortunately, such a method requires a special numerical

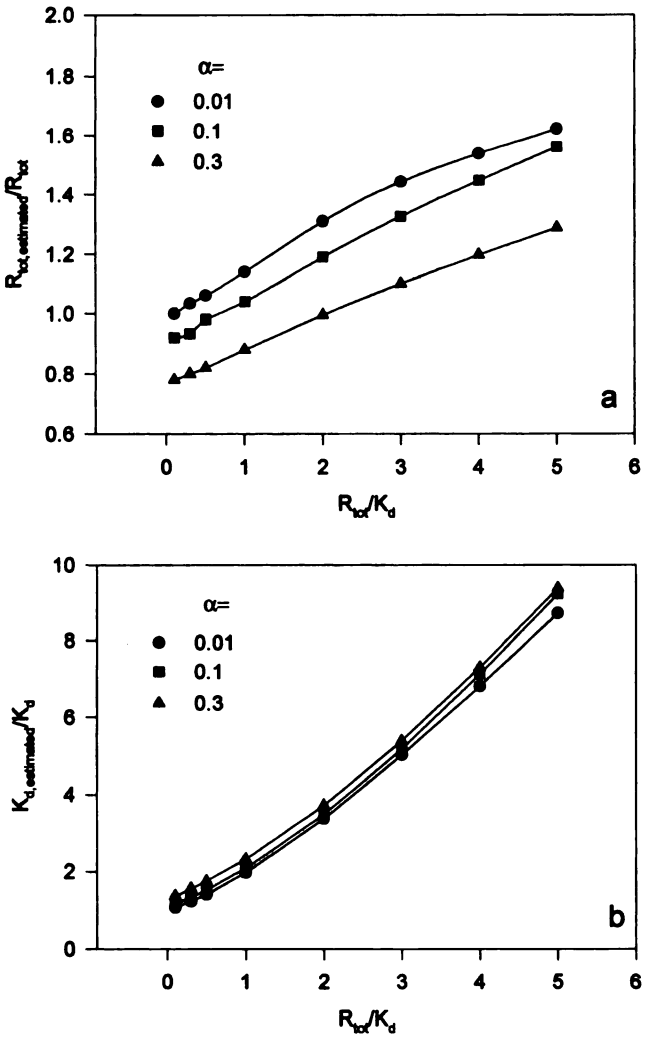

Fig. 6. Bias in R_{tot} and K_d estimates obtained in different situations by the fitting method that assumes that the free ligand concentrations can be approximated by the total concentrations (method A in Fig. 5). The fitting was applied to error-free displacement curves generated as in Fig. 4 but with different values of R_{tot} and α. K_d was always equal to 1 nM.

algorithm that is not currently implemented in commercial graphics packages.

Our approach is based on the implicit assumption that the experimental data meet the statistical constraints of the least-squares regression method. The curve fitting is applied to an untransformed plot of the data, in which x-axis and y-axis refer to the error-free independent variable and a measured dependent variable, respectively. In most experimental binding situations, the dependent variable corresponds to the amount of bound radioligand and the independent variable is defined as the total concentration of added ligand. When this total concentration is virtually equal to the free ligand concentration, well known equations describing ligand binding as functions of the free ligand concentration are useful for curve fitting. However, if the amount of bound ligand is not negligible, compared with the added amount, other equations dealing explicitly with the total ligand concentration as the independent variable must be considered. Such equations, defined in Table 1 for saturation experiments and homologous displacement experiments, have been shown to be useful because unbiased parameter estimates are provided by nonlinear regression.

Numerical simulations of both saturation and displacement experiments have demonstrated that nonspecific binding cannot be determined according to the usual empirical approach. For a saturation experiment, nonspecific binding is traditionally described by a straight line, the slope of which is identical to the asymptotic slope of the total binding curve. However, when a significant fraction of the ligand is in the bound form, nonspecific binding is not linear with respect to the total ligand concentration and exhibits an upward concavity. Moreover, it is demonstrated in Fig. 1a that the actual fraction of radioligand bound to the nonspecific sites cannot be measured by adding an excess of competitor to the different radioligand concentrations. Thus, the indirect determination of specific binding by subtraction of the experimentally observed nonspecific binding from the total binding is meaningless when a significant fraction of the added radioligand is bound to the receptor. In the case of competitive displacement curves, it is generally considered that the residual binding value obtained at high concentrations of unlabeled ligand reflects the constant amount of nonspecifically bound radioligand. However, it is demonstrated in Fig. 4 that this amount becomes smaller as the concentration of unlabeled ligand decreases. This is due to the fact that the free radioligand concentration decreases concomitantly, because of the increasing specific binding to the receptor. Again, the estimate of the specific binding component cannot be obtained by subtracting the asymptotic binding value from the total binding values obtained at the different concentrations of unlabeled ligand. In conclusion, the quantitative analysis must deal with the measured total binding and not with specific binding values indirectly obtained by an incorrect calculation.

A last word of caution concerns the interpretation of competitive displacement of a radioligand by a nonhomologous unlabeled ligand. In this case, the fraction of unlabeled ligand bound to the receptor and to nonspecific sites cannot be determined. Generally, it is assumed that total and free concentrations of the unlabeled ligand are not significantly different, but it is not possible to check the validity of this assumption. Therefore, the estimate of the equilibrium dissociation constant of the heterologous ligand may be questioned because of the possible and unverifiable existence of a significant partition between bound and free forms of this unlabeled ligand.

In conclusion, the analysis of binding curves obtained in the presence of high receptor concentrations demonstrates that specific binding cannot be estimated by subtracting nonspecific binding from total binding. Therefore, the equations describing total radioligand binding as a function of total added ligand concentration have been established. These equations, incorporated in a nonlinear regression routine, are absolutely required to estimate the maximal specific binding and the equilibrium dissociation constant when the receptor concentration is such that the free ligand concentration cannot be approximated by the total concentration.

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