The result of equilibrium-constant calculations strongly depends on the evaluation method used and on the type of experimental errors

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The determination of equilibrium constants is a widespread tool both to understand and to characterize protein–protein interactions. A variety of different methods, among them Scatchard analysis, is used to calculate these constants. Although more than 1000 articles dealing with equilibrium constants are published every year, the effects of experimental errors on the results are often disregarded when interpreting the data. In the present study we theoretically analysed the effect of various types of experimental errors on equilibrium constants derived by three different methods. A computer simulation clearly showed that

certain experimental errors, namely inaccurate background correction, inexact calibration, saturation effects, slow kinetics and simple scattering, can adversely affect the result. The analysis further revealed that, for a given type of error, the same data set can produce different results depending on the method used.

Key words: complex-formation rate constant, direct-calibration ELISA, kinetics, protein–protein interactions, Scatchard analysis.

INTRODUCTION

In order to quantify the various interactions between biological macromolecules and thus to comprehend functional and regulatory mechanisms, the determination of equilibrium dissociation constants is a common and essential tool. Since the determination of this constant is not restricted to a special type of protein, more than 1000 articles dealing with this constant are published every year.

The importance of equilibrium constants led, in the course of time, to the development of several different methods suitable for calculating these constants. Most methods are based on linearization procedures of the Law of Mass Action, among them the Scatchard analysis [1], the double-reciprocal plot described by Lineweaver and Burk [2], the Woolf plot [3] and the method described by Liliom et al. [4]. Another linearization procedure, the *F*-plot [5], takes into consideration, in addition to the Law of Mass Action, also the kinetics of equilibrium formation. All these methods glitter in principle with their simplicity and transparency. In the last two decades, the improvement of computer technology led to the development of non-linear curve-fitting algorithms and neuronal-network strategies [6–8]. Novel experimental methods for the determination of association and dissociation rate constants, such as surface plasmon resonance, gave rise to further computer-supported facilities that allow the determination of equilibrium dissociation constants [9]. However, all the latter techniques hold complicated and hidden obscure mathematical procedures that make it difficult to interpret the precision and reliability of the results.

In spite of the modern possibility of calculating dissociation constants using black-box computer programs, the determination of these constants by linearized plots seems to be more prevalent, probably owing to the transparency of the methods used. However, systematic errors may cause serious effects on the final result, and it has to be presumed that the different

linearization procedures respond differently to the same type of experimental error. Therefore, in the present study, we used computer simulations to generate a variety of data sets representing results with exactly defined systematical errors. These errors included inaccurate background correction, inexact calibration, the influence of saturation effects and deviations caused by slow kinetics. Additionally, we also investigated the effect of random scattering. Our analysis clearly shows that the value of the calculated equilibrium constant strongly depends on the evaluation method used and on the type of experimental errors. The results enable the user to take preventative measures in order to avoid misinterpretations and to obtain more accurate results.

EXPERIMENTAL

Generation of the basic data set

All calculations were done with Microsoft Excel 98 (Macintosh edition) using MacOS 8.1 on a machine with 604e processor. In order to simulate conditions that resemble a typical experimental environment for a binding curve, we created eight basic data points and a zero control. The data points result from initial ligand concentrations (L_0) of 0.35, 0.8, 1.4, 2.2, 4.0, 7.0, 11.0 and 16.0 nM. Assuming a dissociation constant (K_d) of 1 nM and an initial receptor concentration (R_0) of 2 nM, the theoretically expected (c_e) values for the amount of complex formed (RL_c) can be easily calculated as:

$$RL_c = \frac{L_0 + K_d + R_0 - \sqrt{L_0^2 + 2 \cdot (K_d - R_0) \cdot L_0 + (K_d + R_0)^2}}{2} \quad (1)$$

Effect of the kinetics

Since recording of a binding curve is typically performed by immobilizing a constant amount of receptor on a solid phase and

Abbreviations used: K_d , equilibrium dissociation constant; k_c , complex formation rate constant.

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varying the concentration of the soluble ligand, a first-order kinetic can be assumed [5,10]. Thus the amount of complex formed at time t can be described as:

$$RL_e = RL_{e(t \rightarrow \infty)} \cdot (1 - e^{-k_e t}) \quad (2)$$

The complex-formation rate constant, k_e , was defined as 0.001 s^{-1} and the incubation time was assumed to be 4 h. For these values, $RL_{e(t \rightarrow \infty)}$ is identical with $RL_{e(t=240 \text{ min})}$ until the fifth decimal place. Thus, under these conditions, the influence of other parameters is unaffected by the kinetics.

Generation of transfer plot data

The determination of equilibrium constants by the F -plot [5] requires an additional data set. Experimentally, the unbound ligand (L) is transferred after a defined short incubation period to another receptor-coated reaction vial and incubated again for the same time. The transfer is repeated n times (for details, see [5]). In the theoretical model, seven transfer series were simulated for six different incubation periods ($t = 6, 9, 13, 18, 24$ and 31 min). According to eqns (1) and (2), the signals of the transfer assay were calculated as (3):

$$RL_n = 0.5 \cdot \left[(L_{n-2} - RL_{n-1}) + K_d + R_0 - \sqrt{(L_{n-2} - RL_{n-1})^2 + 2 \cdot (K_d - R_0) \cdot (L_{n-2} - RL_{n-1}) + (K_d + R_0)^2} \right] \cdot (1 - e^{-k_e t}) \quad (3)$$

Generation of variable data sets

In order to determine the effect of systematical and random data recording errors on different methods for calculating equilibrium constants, each of the basic parameters (L_0, R_0, K_d, k_e) was varied independently, as indicated in the Figures.

Generation of systematical errors

Four types of systematical errors were simulated in the present study: (1) inaccurate background correction, (2) inexact calibration, (3) saturation and (4) too short incubation periods.

Inaccurate background correction leads to the addition (or subtraction) of a constant value C_B to (from) the measured signal. In order to compare independent series of signals, C_B is expressed as a fraction of the average expected signal of a complete data series.

Inexact calibration results from an incorrect conversion of the measured signal into a protein concentration. The inexact calibration results in the multiplication of the signal by a constant factor, C_C .

Saturation effects can arise, among others, from the limited time resolution of counters in a radioimmunoassay, from substrate scarcity or from absorbance values outside the range of the limited conditions of the Beer–Lambert Law in an immunosorbent assay. Typical for saturation effects is that high signals are significantly lowered, whereas low signals appear to be unaffected. The saturation effect is expressed by a constant, C_S , that represents the fraction of signal decrease at maximal saturation ($RL = R_0$). Before reaching maximal saturation, the actual signal reduction [$C_{S(RL)}$] is alleviated by S^{th} power of the ratio of the expected signal (RL_e) to the signal at maximal saturation (R_0), with S called the bending parameter:

$$C_{S(RL)} = C_S \cdot \left(\frac{RL_e}{R_0} \right)^S \quad (4)$$

This definition of the saturation effect implies that the deviation of the erroneous signal from the true signal diminishes at small values of RL_e . In fact, the deviation of RL_e decreases below a given limit ϵ [$\epsilon = (RL_e \text{ without saturation effect} - RL_e \text{ with saturation effects}) / RL_e \text{ without saturation effects}$] if $RL_e < \sqrt{\frac{\epsilon}{C_S}} \cdot R_0$. By the use of ϵ , the bending parameter, S , may also be expressed as:

$$\frac{RL_e}{R_0} = C_\epsilon = \left(\frac{\epsilon}{C_S} \right)^{\frac{1}{S}} \Leftrightarrow S = \frac{\log \epsilon - \log C_S}{\log C_\epsilon} \quad (5)$$

In other words, C_ϵ is the fraction of the saturation concentration (R_0) below which the deviation caused by the saturation effect is less than 1 % ($\epsilon = 0.01$). In the present study, unless otherwise stated, S was set to 1, i.e. if the signal decrease at maximal saturation is 5 % ($C_S = 0.05$), all values above 20 % ($C_\epsilon = 0.2$) of the maximal expected signal are affected by more than 1 % ($\epsilon = 0.01$).

The effects caused by short incubation times are described by eqn (2). Thus, in summary, all computer-simulated signals (RL_e) were calculated by eqn (6).

$$RL_e = RL_{e(t \rightarrow \infty)} \cdot (1 - e^{-k_e t}) \cdot (1 + C_C) \cdot \left[1 - C_S \cdot \left(\frac{RL_e}{R_0} \right)^S \right] + C_B \cdot \frac{\sum_1^p RL_p}{p} \quad (6)$$

In this equation, p represents the number of values in a complete data series.

Generation of statistical scattering

Statistical scattering was generated by adding to every data point (RL_e) a random value obeying a Gauss distribution. The amount of diversification (C_D) was defined as the relative range around the expected value RL_e in that 95.4 % (corresponding to $\pm 2 \text{ S.D.}$) of all random values will be found. Rare events of extreme values (0.06 % of all values) were excluded by introducing a cut-off at $1.7 \cdot C_D \cdot RL_e$ (i.e. the maximal deviation from the expected value RL_e was limited to $\pm 17 \%$ if $C_D = 10 \%$). The computer-simulated data sets that include statistical diversification (RL_d) were calculated as follows:

$$RL_d = RL_e \pm \lambda(\theta) \cdot \sigma$$

with $\sigma = C_D \cdot \frac{RL_e}{2}$ and $\theta(\lambda) = \frac{1}{\sqrt{2\pi}} \int_0^\lambda e^{-\frac{x^2}{2}} dx$ (7)

$\theta(\lambda)$ was set randomly to a value between 0 and 0.4997 (0.4997 represents the cut-off). In order to quantify the effect of scattering, each simulated experiment was performed either in triplicate (to simulate a typical laboratory situation) or as a sevenfold repetition (to obtain more precise results). In total, 12 experiments were performed for each different value of C_D .

Calculation of equilibrium constants

The calculation of equilibrium constants from all data sets was performed automatically according to the three linearization procedures described by Scatchard [1] ($K_d = -1/m$), Liliom et al. [4] ($K_d = 1/m$) and Fuchs et al. [5] [$K_d = R_0(1-m)/m$], where m represents the slope of the respective plot. The method of Liliom et al. [4] contains a quotient, i , which equals RL/R_0 ($R_0 = RL_{\max}$). For ideal data sets (i.e. $i = RL_e/R_0$), i is always less than 1. However, for real data sets or simulated data sets that represent real conditions, RL_d (eqn 7) may be slightly greater than R_0 . Since a quotient of $1/(1-i)$ is necessary for further calculation, $RL_d > R_0$ may result in negative dissociation constants and $RL_d \approx R_0$ in extreme incorrect values. Therefore, a cut-off for quotients $i > C_L$ was introduced. Unless otherwise stated, C_L was set to 0.975.

RESULTS

Principle determination of the equilibrium dissociation constant

In order to demonstrate the principle of our simulations, we first generated an ideal data set and a modified data set containing two extreme systematical errors. The simulated errors comprised an inexact calibration of 20% ($C_c = 0.2$) and a saturation effect of 30% ($C_s = 0.3$) with 1% deviation ($\epsilon = 0.01$) at a signal of 20% from the expected maximum ($C_e = 0.2$). The resulting binding curves that are essential for K_d determination by the three methods are shown in Figure 1(A). The assumed calibration error of +20% (i.e. recorded signals appear 20% greater than they actually are) leads to higher signals for the erroneous data set (broken line) at low ligand concentrations. At higher ligand concentrations, the saturation effect predominates more and more over the calibration error, resulting finally in lower values for the erroneous data set in comparison with the ideal data set. Using the two data sets shown in Figure 1(A), equilibrium dissociation constants were derived as described by Liliom et al. [4] (Figure 1B) and Scatchard [1] (Figure 1C). The dissociation constants derived from the ideal data set fit exactly with the expected value of 1.0000 nM. For the erroneous data set, in contrast, K_d was calculated to be 0.55 nM (Liliom) and 0.46 nM (Scatchard). Surprisingly, the correlation coefficient (r^2) of the Scatchard plot for the erroneous data set turned out to be 0.998, demonstrating that a perfect correlation coefficient is not a cogent clue to an accurate result.

The results of the F -plot (for details see [5]) are shown in Figure 1(D). A striking observation is that the slope of the regression curve in the F -plot is almost identical for the ideal data set and the erroneous data set. This may be attributed to the almost perfectly parallel slopes of the transfer plots that were used to create the data points in the F -plot (cf. continuous and broken lines in Figures 1E and 1F). The stunning similarity despite the extreme calibration and saturation errors (●) can be explained by two factors. First, inexact calibration is characterized by the multiplication of the expected value by a constant factor, thus affecting the absolute value of the signal but not the ratio between two values, leaving the slope unaffected. Secondly, the transfer plots are usually performed at short incubation times and thus far away from the maximal signal and thereby unaffected by saturation effects. As expected, the absolute slopes of the regression curves increase with an increasing transfer time, leading to the F -plot displayed in Figure 1(D).

Although the slopes of the F -plot are extremely similar for both data sets, the derived value of K_d for the erroneous data set was found to be 0.73 nM and is thus relatively inaccurate. The reason for this is that the equilibrium constant was calculated as:

$$K_d = R_0(1-m)/m$$

where m is the slope in the F -plot, and the apparent R_0 was lowered by saturation effects (Figure 1A). Nevertheless, for this extreme example, the F -plot led to the best result in comparison with the other two methods. For the ideal data set, K_d was calculated to be 1.0098 nM. Thus, in contrast with the methods of Liliom et al. and Scatchard, the F -plot produces small deviations even with ideal data. This can be traced back to an approximation that is made by the derivation of the F -plot [5]. However, this less than 1% inaccuracy is very small in comparison with the deviations resulting in all three methods from slight systematic errors or scattering (see below).

Effect of basic parameters on K_d determination

In order to quantify the effect of isolated systematic errors on the calculated dissociation constant, each of the described parameters

Figure 1 Binding experiment, K_d determination and transfer assay

(A) Ligand variation for a typical binding experiment; (B) Liliom plot derived from the data in (A); (C) Scatchard plot derived from the data in (A); (D) F -plot derived from the data in (E) and (F); (E) transfer plot for 6 min incubation periods; (F) a series of transfer plots for different incubation periods (6, 9, 13, 18, 24 and 31 min) as are required for composing the F -plot (D) by the method of Fuchs et al. [5]). Each plot shows the curves for an ideal data set (○—○) and a data set affected by extreme systematical errors (●—●). These errors comprise 20% incorrect calibration ($C_c = 0.2$) and 30% saturation effect ($C_s = 0.3$). The saturation effect reaches 1% deviation from the expected value at a signal of 20% from the expected maximum ($C_e = 0.2$). In the F -plot, the difference between the two data sets is very subtle, so that the erroneous-data-set and the ideal-data-set results are superimposed.

Figure 2 Variation of basic parameters

(A) Variation of the ligand concentration (L_0) in the transfer assay; (B) variation of the concentration of coated receptor (R_0) with constant ratio R_0/L_0 ; (C) variation of the equilibrium dissociation constant (K_d). Each data point represents the result of an F -plot ($\circ$), of a plot according to Lilliom ($\square$) or of a Scatchard plot ($\diamond$). Arrows point to the value that is used for all other experiments, i.e. when other parameters are varied. In (C) the relative error is given instead of the calculated dissociation constant, since the dissociation constant was varied. However, as K_d was set to 1 in all other experiments, the relative error is equivalent to the calculated dissociation constant in the other panels.

was independently varied. For each single data point in Figures 2–4, a complete data set (binding curve, Scatchard plot, Lilliom plot, kinetics, transfer assay and F -plot) was generated and the K_d calculated. All results are qualitatively summarized in Table 1. Since the Lilliom and Scatchard methods do not require a transfer plot, the variation of the initial ligand concentration (L_0) in the transfer assay does not influence the K_d determination (Figure 2A). The K_d determined by F -plots deviates by more than 1% at L_0 concentrations greater than 0.05 nM (i.e. for ratios of $R_0/L_0 < 40$). This reflects the approximation that the F -plot is only valid if $L_0 \ll R_0$. A value of $L_0 = R_0/40$ was chosen for all further experiments, since on the one hand it is small enough to get an inaccuracy that was less than 1% for ideal data sets and on the other hand large enough to be clearly detected in a real

Table 1 Qualitative summary of all results

Variable	Sign	Conditions	Scatchard		Lilliom		F -plot	
			Apparent K_d^*	Effect intensity	Apparent K_d^*	Effect intensity	Apparent K_d^*	Effect intensity
Ligand	L_0	+	Unaffected	—	Unaffected	—	Increasing	High
Receptor	R_0	+	Unaffected	—	Unaffected	—	Decreasing to optimum	Moderate
	R_0	+	Unaffected	—	Unaffected	—	Relatively maximum	Slight
Dissociation constant	K_d	+	Unaffected	—	Unaffected	—	Relatively maximum	Slight
	K_d	+	Unaffected	—	Unaffected	—	Constant but inaccurate	Minute
Rate constant	k_c	+	Decreasing to optimum	Extremely high	Decreasing to optimum	Extremely high	Decreasing to optimum	Moderate
	k_c	+	Constant but inaccurate	Dependent on $k_c \times t_b$	Constant but inaccurate	Dependent on $k_c \times t_b$	Decreasing to optimum	Slight
	k_c	+	Constant but inaccurate	Dependent on $k_c \times t_b$	Constant but inaccurate	Dependent on $k_c \times t_b$	Constant but inaccurate	Dependent on $k_c \times t_b$ and $k_c \times t_i$
Calibration	C_C	+	Decreasing	High	Decreasing	High	Decreasing	Slight
	C_C	—	Increasing	High	Increasing	High	Increasing	Slight
Background correction	C_B	+	Decreasing	Extremely high	Decreasing	High	Increasing	Extremely high (moderate†)
	C_B	—	Increasing	Extremely high	Increasing	High	Decreasing	Extremely high (moderate†)
Saturation	C_S	+	Decreasing	Moderate	Decreasing	Moderate	Decreasing	Moderate
Bending	C_e	+	Relatively minimum	Moderate	Relatively minimum	Moderate	Relatively minimum	Moderate
Scattering	C_0	±	Distributed	Moderate	Increasing	Extremely high	Distributed	Slight

* For increasing absolute values of the variable.

† If only the first four values of the transfer assay were used.

Figure 3 Kinetic effects, calibration errors and background correction

(A) Effect of the incubation time on the calculated equilibrium dissociation constant at a given rate constant of $k_c = 0.001 \text{ s}^{-1}$; (B) effect of calibration errors (C_C); (C) effect of background correction errors (C_B). Data points were derived by F -plots ($\circ$), Liliom plots ($\square$) and Scatchard plots ($\diamond$). $\odot$ in (C) represent F -plots derived from transfer assays in which only the first four values ($1 \leq n \leq 4$) were used for all further calculations.

transfer experiment [5]. A variation of the immobilized receptor concentration (R_0) at a constant R_0/L_0 ratio of 40 showed that the calculated K_d is only slightly affected by R_0 in the F -plot (Figure 2B) with an error maximum of about 1% at 2 nM R_0 (i.e. at an R_0/K_d ratio of 2). This error solely depends on the R_0/K_d ratio, since a K_d variation with constant R_0/K_d and R_0/L_0 ratios resulted in identical ratios of expected and calculated dissociation constants for all K_d values considered (Table 1). A K_d variation with constant values for R_0 and L_0 showed a small error maximum of about 1% at 1 nM (Figure 2C). Thus the standard values for R_0 and K_d used for all further experiments represent the maximal error possible for the F -plot when applied to ideal data sets. The calculated dissociation constant is theoretically not affected by R_0 and K_d when using the Liliom or Scatchard plot (Figure 2). However, if the ratio of R_0/K_d is low, the signal obtained in the binding curve of a real experiment can become too small for an accurate K_d calculation, since effects caused by incorrect back-

ground correction or scattering can completely diminish the theoretical accuracy (see below).

Effect of kinetics and systematic errors on K_d determination

The complex-formation rate constant, k_c , can dramatically affect the calculated K_d . Figure 3(A) shows the effect of a variation of the incubation time (common duration of experiments) in the binding experiment (t_B). Assuming $k_c = 0.001 \text{ s}^{-1}$, the calculated K_d strongly increased for incubation periods shorter than 70 min when using either the Liliom or Scatchard plots (note the different scale in the y -axis in comparison with other Figures!). At $k_c = 10^{-4} \text{ s}^{-1}$, this increase amounts to 40% (Liliom) and 45% (Scatchard), even if the incubation period is extended to 240 min (results not shown). In contrast, the F -plot is much less affected by the variation of the incubation time. In order to obtain errors less than 1% at $k_c = 0.001 \text{ s}^{-1}$, incubation times of at least 6.6, 14.1 and 14.5 h are required in the F -plot, the Liliom plot and the Scatchard plot respectively.

If the product $k_c \times t_B$ is kept constant, the F -plot still depends slightly on the kinetics, whereas the apparent K_d determined by the Scatchard or Liliom plot becomes constant, but inaccurate (Table 1). We could show that this behaviour can be traced back to the incubation time t_T in the transfer assay. If the product of $k_c \times t_T$ is kept constant in addition to the product of $k_c \times t_B$, the apparent K_d becomes thus constant in the F -plot, even if k_c is varied (Table 1).

Calibration is always error-prone for many reasons, including the inaccurate determination of protein concentrations or specific radioactivity. A calibration error clearly affects the calculated K_d in the Scatchard and in the Liliom plot in a similar manner (Figure 3B), i.e. a 6% calibration error results in about 10% error in K_d . In contrast, the K_d determined by the F -plot is almost unaffected by a calibration error. This favourable behaviour is attributed to the transfer plot, which serves as an internal calibration [5].

The effects of inaccurate background corrections are immense (Figure 3C). A background error of only 3% leads to errors in K_d determination of 19% (F -plot), -12% (Liliom) and -28% (Scatchard). The discontinuous function for the F -plots in the range of negative background correction errors is due to undefined values in the transfer assay; whenever a background corrected signal decreases to below 0, the logarithm is undefined and the data point will be ignored for regression analysis, leading to discontinuities in the plotted function. The observation that the calculated K_d becomes more accurate after passing a point of discontinuity led us to propose to use only the first four transfer numbers ($1 \leq n \leq 4$) in each transfer plot for regression. In this case ($\odot$ in Figure 3C), the effects of background correction errors in the F -plot are notably diminished (e.g. from 19.2 to 4.5% at 3% error) and clearly smaller than in the Scatchard and Liliom analyses.

Since saturation effects can be caused by a variety of parameters, the impact on the recorded signal can only be expressed by an empirical formula. There are two key parameters describing a saturation effect: the maximal signal decrease (C_s) and the fraction of the maximal signal at which significant saturation occurs (C_e). When varying C_s (Figure 4A), the apparent K_d decreases with increasing saturation effect in all plot types. In comparison with the calibration error, the consequence of saturation effects is less momentous. It seems that the Liliom plot is the least saturation-affected method, whereas the F -plot is the most affected. However, a variation of C_e at constant saturation ($C_s = 10\%$ as indicated by the arrow in Figure 4A) shows that the saturation-dependent effect is also dependent on

Figure 4 Influence of saturation effects

Influence of saturation effects on the calculated equilibrium dissociation constant (**A**), when the maximal saturation effect (C_S) was varied ($S = 1$, $C_e = 0.01 \cdot C_S^{-1}$) and (**B**) when the recorded signal at which the saturation effect becomes significant (C_e) was varied. In the latter case, the maximal saturation effect was kept constant [$C_S = 0.1$, $S = -(\log C_e)^{-1}$]. Data points were derived by *F*-plots ($\circ$), Liliom plots ($\square$) and Scatchard plots ($\diamond$). The arrows indicate those values that are used as the constant in the other panel ($C_S = 0.1$, $S = 1$, $C_e = 0.1$).

C_e (Figure 4B). The resulting deviations of the derived K_d from its true value show a maximum of approx. 10% at C_e of about 0.3–0.4 in the case of the Liliom and Scatchard plots. The Scatchard plot appears to be most sensitive to this type of error (for $0.15 < C_e < 0.85$), whereas the *F*-plot seems to be most resistant and displays its maximum error at much smaller values of C_e . In addition, the deviation of K_d does not depend in the *F*-plot as much on C_e as in the other two methods. In summary, saturation effects are less important than calibration or background effects and affect all three described methods to a similar extent.

Effect of scattering on K_d determination

In addition to the systematic errors, random scattering is a striking problem in the evaluation of recorded signals. In contrast with the systematic errors, statistical scattering cannot be reduced by systematic variation of the basic parameters, but can be partially overcome by multiple independent repeats of the same experiment. In order to simulate scattering effects, an ideal data set was superimposed by individual offsets for each value. The offsets were randomly generated in a way that the entirety of all offsets are Gaussianly distributed. For every ideal data set, seven scattered data sets were generated to simulate multiple repeats and called an 'experiment' in the present paper. For each condition, 12 experiments were generated to simulate possible differences from laboratory to laboratory.

When simulating $\pm 20\%$ distribution ($C_D = 0.2$, S.D. = $RL_e/10$), the errors of the average apparent K_d (central thick

Figure 5 Effect of statistical scattering

Effect of statistical scattering (C_D) on the equilibrium dissociation constant calculated with the respective linearization procedures (*F*-plot, Scatchard and Liliom). The scattering was analysed for six different intensities (2, 4, 7, 10, 15 and 20%; for definition, see the Experimental section). For each intensity, 12 independent experiments with seven complete data sets for each experiment were generated and analysed. The thick lines (—) indicate the maximal, the average, and the minimal dissociation constant calculated for all 84 data sets for each intensity. The dash-dotted lines (— · —) represent the average S.D. for the seven data sets in all experiments. The thin lines (—) indicate the maximal and the minimal dissociation constant calculated from the 12 experiments (as the average of seven data sets). The broken lines (---) represent the S.D. for the 12 experiments.

continuous lines in Figure 5) of all experiments were $+3\%$ (*F*-plot), $+5\%$ (Scatchard) and $+87\%$ (Liliom). The S.D. of the different experiments comprised intervals of -1 to $+7\%$ (*F*-plot), -2 to 12% (Scatchard) and $+53$ to $+120\%$ (Liliom) of the expected value (broken lines). This analysis shows that the *F*-plot and the Scatchard analysis produce acceptable results, but have a slight tendency to exhibit a higher K_d . In contrast, the Liliom plot produced unusable results at this scatter intensity. For the Liliom plot, the highest just acceptable scatter intensity was $\pm 10\%$, which results in an S.D. interval of -5 to $+16\%$ for the different experiments and an average interval of -25 to $+36\%$ for the seven data sets of each experiment (— · — lines).

At a scatter intensity of 20 %, the average S.D. intervals for the seven data sets were -6 to 12 % (F -plot) and -14 to $+27$ % (Scatchard). This result clearly indicated that a single recording of an experimental binding curve is unacceptable in the scientific context.

The same data sets were also created for experiments done in triplicate instead of a sevenfold repeat (results not shown). These data exhibited basically an identical behaviour. As expected, the S.D. between different experiments was slightly elevated, whereas the maximal observed error was smaller (because less experiments reduce the likelihood of the appearance of extreme values). However, when data sets were created in triplicate, for scattering intensities of 7 – 20 %, the expected value in the Scatchard analysis was outside the S.D. interval in 54 % of the results, feigning a more accurate determination than it really is.

A further analysis of the Liliom plots revealed that, in 11 of 12 cases ($C_D = 15$ %), the results were 28 % more accurate when applying the geometrical mean instead of the arithmetic mean. In addition, the Liliom method contains a quotient, $1/(1-i)$, with $i = RL/R_0$, that can cause negative dissociation constants or extremely inaccurate values (see the Experimental section). A detailed analysis (not shown) revealed that the S.D. decreases if reading points with quotients $i > C_L = 0.975$ were ignored. Therefore C_L was set to 0.975 in all experiments presented above.

DISCUSSION

Comparison of the different methods

Scatchard analysis is a widespread method for the determination of equilibrium constants, and a variety of critical aspects have been already discussed. Most of the literature has dealt with the problem of receptor–ligand stoichiometry and the existence of binding sites of different affinity [11–14]. These theoretical considerations were performed with error-free simulated ideal data sets. In addition, other workers applied their theoretical considerations to an experimental data set [15,16].

In contrast, the present study assumes a $1:1$ stoichiometry and only one subset of molecules for each type of binding partner, thus resulting in a single binding constant. The focus lay on the effect of different kinds of error in data acquisition on the determination of K_d . The required data sets can in principle be gained experimentally as well as by simulation. The latter method, however, allows one to distinguish clearly the different kinds of errors and to generate a large number of data sets. Using experimental data sets, Nekhai et al. [17] previously showed that the choice of the initial ligand and receptor concentrations can systematically effect the shape of the Scatchard plot. They attributed this effect to inaccuracies in the determination of free ligand, but did not correlate this discrepancy to a specific kind of error.

In order to find methods that are extremely robust against experimental errors, a variety of different plots have been developed. Nevertheless, using several experimental data sets, a comparison of a non-linear plot developed by Klotz [18] with the Scatchard plot did not reveal any particular advantage for either method [18,19]. The effect of outliers systematically inserted into an experimental data set on three different linearization procedures (Scatchard plot [1], Woolf plot [3] and Lineweaver–Burk plot [2]) was analysed by Keightley and Cressie [20], who showed [21] that the best results were achieved using the Woolf plot and an uncommon regression analysis.

In the present study, we have systematically examined a variety of errors, including inaccurate background correction, inexact calibration, the influence of saturation and effects caused by slow kinetics and simple scattering. The calculation of K_d was

performed by three different methods: Scatchard analysis [1], Liliom analysis [4] and the method of direct calibration (F -plot) [5]. These methods were chosen because the Scatchard plot is the most common analysis, the Liliom plot does not require absolute concentrations and is therefore suitable for K_d determination from an ELISA, and the F -plot is the only plot that includes an internal calibration, but requires additional data and cannot be derived by linearization of the Michaelis–Menten relationship.

A comparison of the Scatchard and Liliom methods showed that these methods produced very similar results for most types of errors analysed. Two exceptions were observed: First, the Liliom plot was more precise with insufficient background correction and, secondly, the Scatchard plot produced a substantially better K_d in scattering experiments. When the number of repeated data acquisition was low (3 in this study), the true K_d was, however, outside the S.D. in more than 50 % of all determinations.

The crucial point in the F -plot is the ratio of R_0 to the initial concentration of L_0 in the transfer assay. On the basis of the present data we recommend using an R_0/L_0 ratio of at least 40 . In comparison with the Scatchard and Liliom analyses, the results of the F -plot are more precise. The built-in calibration of the transfer assay minimizes calibration errors, and its kinetic approach diminishes problems caused by low rate constants.

For all three methods, background correction is a major problem. If the average absorbance in a binding curve is 0.5 , an overcompensation of the background of only 0.015 (corresponding to -3 %) in a Scatchard analysis leads to an apparent K_d that is 34 % greater than the actual value. It is noteworthy that it is not the absolute value of the background signal that is decisive, but the deviation of the recorded average background signal from the ‘real’ background signal. Since the signals in the transfer assay are often low for higher transfer numbers, background errors have a stronger effect on these values. Therefore, for the F -plot analysis, the background problems can be reduced by utilizing only the first four or five values of each transfer plot (Figure 3C and Table 1).

Scattering caused an average S.D. of 8 % (F -plot), 48 % (Liliom) and 20 % (Scatchard) when seven data sets were generated per experiment and C_D was set to 20 %. Concerning random scattering, Woosley and Muldoon [22] had also studied this effect on different methods of evaluation. They generated 100 simulated data sets with a normal distribution about the mean of the expected value and analysed the data by Scatchard plot, Lineweaver–Burk plot and a direct linear plot according to Eisenthal and Cornish-Bowden [23]. They found the direct linear plot to be the best, followed by Scatchard plot and a rather inaccurate Lineweaver–Burk plot. The unreliability of the Lineweaver–Burk plot was also shown by Keightley and Cressie [20]. When we compared our S.D. data with those obtained by Woosley and Muldoon, using the Scatchard analysis, similar results were observed. Since the definition of random data generation was slightly different in both studies, a quantitative comparison was not possible.

Biological applications

The present study reveals important aspects concerning the determination of equilibrium constants and will be helpful for the experimental design and the subsequent evaluations that are necessary for accurate, reliable and reproducible results. The pros and cons of different methods are qualitatively summarized in Table 1. The present study revealed that the accuracy of the determination of equilibrium constants should not be overrated.

Assuming a background error of only 1 %, a calibration error of 3 % and a saturation effect of also 3 %, the resulting systematical error can reach, depending on the sign of the errors, up to –13 % (*F*-plot), –10 % (Liliom), or –17 % (Scatchard). Experimentally derived values of K_d are often listed with two or even three decimal points, thus feigning an accurate result. In order to avoid this misperception and to make it easier to compare equilibrium binding constants of different reactions that may easily vary by several orders of magnitude, we propose to list the negative decadic logarithm pK_d , as in the case with the dissociation constants of acids and bases (pK_a and pK_b).

When planning an experiment, one should have in mind that the most critical point for all methods is the correct background determination. Therefore, the experimental conditions should be chosen in a way that the background *variation* is as low as possible and the zero control should be performed at least in quadruplicate. If noticeable calibration errors (caused e.g. by protein determination or the signal detector) are observed or scattering effects are very high, the *F*-plot should be the method of choice, unless an R_0/L_0 ratio of at least 40 cannot be attained. When larger scattering is unavoidable, the user should take into consideration during data interpretation that all three methods analysed lead to an erroneous increase of the apparent K_d . This effect is extremely large when using the Liliom plot. It is also important to note that this deviation of K_d cannot be significantly reduced by increasing the number of measurements.

In order to avoid systematical errors caused by slow kinetics, the time-dependent binding of the ligand should be recorded prior to the saturation binding experiment. For the saturation binding curve, the product of incubation time and complex-formation rate should be at least 4 when using Scatchard analysis. If the necessary incubation time is experimentally unfeasible, the *F*-plot is preferred. For the Scatchard analysis, direct labelling of the ligand is required. If the label is suspected to alter the affinity of the ligand (H. Fuchs and R. Gessner, unpublished work), either the Liliom plot (or *F*-plot) should be used or the considerations of Hollemans and Bertina [24], who analysed the effect of heterogeneity in binding affinity of labelled and unlabelled ligand on Scatchard plots, should be taken into account.

In summary, the *F*-plot produces in most cases the most accurate results, but requires a more exhaustive experimental procedure and thus more time to perform. On the other hand it saves time (as does the method of Liliom) by avoiding the need to first label the ligand.

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