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Estimation of Ligand Binding Parameters by Simultaneous Fitting of Association and Dissociation Data: A Monte Carlo Simulation Study

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SUMMARY

A new procedure for analysis of ligand binding kinetics was evaluated by Monte Carlo simulations. In this, all association and dissociation data were fitted simultaneously to a set of nonlinear equations. This should have several advantages over more conventional methods; data are better used in a single fitting procedure in which the degrees of freedom are maximized and the error term is spread over more observations; all relevant parameters (B_{max}, k_1, and k_{-1}) are obtained directly; values obtained from measurements are not treated as errorless; and it yields a single residual term that can be used for statistical comparison among binding models and/or experiments. We have compared this approach with the common practice of analyzing the association and dissociation phases separately, either by nonlinear regression or by linear regression after suitable transformations. With respect to both the precision and accuracy of parameter estimates, the simultaneous procedure was superior to the other two methods. The properties of the simultaneous procedure were further investigated, concerning both parameter estimation and the probability of reliably detecting a second binding site. For the latter, the relative density of receptor subtypes and the dissociation rate constants were found to be of major importance, whereas association rate constants and ligand concentration were of minor importance in this respect. The probability of resolving two sites by kinetic or equilibrium data under similar conditions with the aid of a single labeled ligand was examined. When the selectivity of the ligand was low, the resolution was found to be more probable when based on kinetic, rather than equilibrium, data. This was true at higher selectivities as well, provided kinetic data were obtained at two different ligand concentrations.

Kinetic analysis of radioligand binding is often performed for a few specific purposes, namely, to determine appropriate incubation times for equilibrium binding studies, to demonstrate the reversibility of ligand-receptor interaction, and to confirm the estimates of binding affinities obtained from such experiments. Thus, kinetic studies are often regarded as subordinate to equilibrium binding studies. This may partly be due to the relative inefficiency of the procedures commonly used to analyze binding kinetics. Such data are usually evaluated by LR on transformed data (1), although the use of nonlinear least squares curve fitting (NLR) has increased in recent years (2–7). In both these procedures, the association and dissociation phases are evaluated separately rather than simultaneously. This makes it necessary to use either the total receptor concentration (B_{max}), obtained from equilibrium binding studies, or the dissociation rate constant (k_{-1}), from the dissociation curve, in the calculation of the association rate constant (k_1). Errors in these B_{max} or k_{-1} estimates will unduly influence the resulting estimate of k_1 and thus also the kinetically determined binding affinity (K_d). Furthermore, if B_{max} from equilibrium binding experiments is used, the K_d obtained from the kinetic studies will not be truly independent. Separate analysis of the association and dissociation curves will yield two residual terms, which impedes statistical comparison between ligand-binding models. Linear regression of transformed data involves additional disadvantages; the equilibrium binding level (B_{eq}) has to be estimated and this estimate is treated as an indefectible value in the subsequent analysis; the linearization results in an error transformation that is not considered in the linear regression; and heterogeneous binding curves resulting from multiple binding sites are not easily evaluated.

In the search for a more efficient kinetic analysis, we have developed a novel procedure, namely SNLR of association and dissociation data (8), in which the above-mentioned drawbacks of traditional analysis are avoided. In the present study we have compared the three fitting procedures (LR, NLR, and SNLR) with respect to estimation properties, when fitting one-site binding data. We have further characterized factors influencing parameter estimation by SNLR.

The binding of a radioligand to several receptor populations

ABBREVIATIONS: LR, linear regression of transformed data; NLR, separate nonlinear regression of association and dissociation data; SNLR, simultaneous nonlinear regression of association and dissociation data; CV, coefficient of variation; L_T, total ligand concentration.

can usually be characterized at equilibrium through the different affinities of the interactions. Every selectivity of a ligand results from differences in the association and/or dissociation processes and will be evident in its binding kinetics. Furthermore, ligands that are nonselective at equilibrium have shown large differences in their kinetics at receptor subtypes (3, 9, 10). Kinetically selective ligands will thus encompass all traditionally selective ligands as well as others. This implies that, theoretically, receptor subtypes can be resolved by a single ligand in more binding systems by kinetic than by equilibrium studies. In practice, however, several factors influence the possibility of recognizing receptor subtypes. In this study, some conditions that may influence the resolution of two binding sites by SNLR were evaluated, namely, differences in the rates of association and dissociation, relative binding densities, receptor occupancy, scatter in data, and number of data points. In addition, we compared the probability of detecting two binding sites through kinetic modeling with their discrimination by equilibrium studies. Throughout, this model comparison was restricted to one or two independent binding site populations. For both parts of this study, we used Monte Carlo simulations (11), which have proven to be a powerful tool in the validation of other analytical procedures (12–14).

Materials and Methods

Hard- and software. Data were generated with the aid of the simulation computer program ACSL, using a second-order Runge-Kutta algorithm (15). Theoretical models were fitted to the simulated kinetic data by the nonlinear least-squares curve fitting program PCNONLIN (16), using the Nelder-Mead simplex algorithm (17). Both the generation and analysis of data were performed on IBM personal computers.

Simulations of binding to one site. Ligand-receptor interactions according to Eqs. 1 and 2 (the number of binding site populations, n, is equal to one) were simulated and a normally distributed random noise was added to the calculated values of concentration of bound ligand (B).

Association:

$$dB/dt = \sum_{i=1}^{n} \left[k_i \cdot (B_{\max,i} - B_i) \cdot \left(L_T - \sum_{i=1}^{n} B_i \right) - k_{-i} \cdot B_i \right] \quad (1)$$

Dissociation:

$$dB/dt = \sum_{i=1}^{n} [-k_{-i} \cdot B_i] \quad (2)$$

The noise function [variance (B) = (CV · $(B$ + 0.05 · $B_{eq}))^2$] was chosen so as to give an error essentially proportional to the concentration of bound ligand, but with a minor component mimicking the subtraction of a nonspecific binding amounting to 5% of the specific binding at equilibrium.

Different plausible experimental conditions were mimicked by varying the scatter, B_{max}, L_T, and the number of data points. For each combination of parameters and experimental conditions, 30 data sets were generated. Each data set consisted of 14 "observations," sampled at 10, 30, 45, 60, 75, 90, 98, and 99% of B_{eq} in the association phase and 90, 75, 60, 45, 30, and 10% of B_{eq} in the dissociation phase, and every increase in the number of data was accomplished by multiple sampling at these levels. Equilibrium was assumed to have been attained before the initiation of dissociation. The default binding parameters used were as follows: B_{max} = 10^{-12} concentration, K_d = 10^{-9} concentration, k_1 = 10^7 concentration^{-1} time^{-1}, and k_{-1} = 0.01 time^{-1} (arbitrary units).

Simulations of binding to two sites. The association and dissociation of a ligand to two binding sites were simulated according to Eqs. 1 and 2 (two sites, n = 2). The default procedures with respect to scatter, number of simulations, and number and spacing of data points were essentially the same as described for the one-site model. Unless otherwise stated, the total concentration of binding sites was 10^{-12} concentration units, and the densities of the two sites were equal. The kinetic rate constants of site 1 were the same as those described above for the one-site model, and those of site 2 were as stated in the text. The chosen ligand concentrations normally gave an equilibrium occupancy of 50%, either for both sites or for the site with the lowest affinity.

LR. The binding data from the dissociation phase were evaluated by linear regression of the logarithmic transforms:

$$\ln B = \ln B_{eq} - k_{-1} \cdot t \quad (3)$$

The association of ligand was transformed according to the equation:

$$\ln[B_{eq}/(B_{eq} - B)] = k_1 \cdot t \cdot L_T \cdot B_{max}/B_{eq} \quad (4)$$

This equation assumes pseudo-first order conditions (i.e., $L_T \gg B_{eq}$) (1), which was a reasonable assumption because at most 0.1% of the added ligand was bound in the data sets evaluated by this method. The calculation of k_1 requires an estimate of B_{eq}. This was obtained as the mean value of bound ligand for the samples at 98% and 99% of B_{eq}. In a few data sets, the value obtained at 90% of B_{eq} exceeded this estimate, in which case B_{eq} was obtained as the mean of the three highest concentrations of B (i.e., the 90, 98, and 99% levels) in order to avoid logarithmic transforms of negative values. The slope of the association plot was determined by linear regression and, in the calculation of k_1 from this slope, the true value of B_{max} was inserted unless otherwise stated.

NLR. The analysis according to NLR was performed in steps. First, k_{-1} was estimated by fitting dissociation data to Eq. 5. This k_{-1} estimate for each data set was then introduced as a constant in Eq. 6 (one site, n = 1), which was used for the estimation of B_{max} and k_1 from association data.

$$B = B_0 \cdot e^{-k_{-1} \cdot t} \quad (5)$$

$$B = \sum_{i=1}^{n} B_{\max,i} \cdot (1 - e^{-(k_{-i} + k_i \cdot L_T) \cdot to}/(1 + k_{-i}/(k_i \cdot L_T)) \quad (6)$$

In Eq. 6, it is assumed that $L_T \gg B_{eq}$ and that equilibrium is attained before initiation of dissociation, which was the case in the data sets analyzed by NLR. The same weighting procedure was used for NLR as described below for SNLR.

SNLR. Eqs. 1 and 2 were fitted simultaneously to the data from the association and dissociation phases, respectively. When applicable ($L_T \gg B_{eq}$), these equations were replaced by Eqs. 6 and 7. The variables $B_{max,i}$, k_i, and k_{-i} were thus estimated from each data set in a single fit.

$$B = \sum_{i=1}^{n} B_{\max,i} \cdot e^{-k_{-i} \cdot t}/(1 + k_{-i}/(k_i \cdot L_T)) \quad (7)$$

The reciprocal of the error function used to generate data was used for weighting in the nonlinear regression. An iterative reweighting procedure (described in the PCNONLIN manual) was used to calculate the individual weights, because weighting according to the predicted, rather than the observed, concentrations has slightly superior estimation properties (18, 19).

Statistics. As a measure of accuracy, the geometric mean was calculated from the 30 individual parameter estimates. The geometric range, calculated as the difference in the asymmetric 95% confidence interval limits, normalized to the mean estimate, is given as a measure of precision. The parameters reported were normalized by calculating the ratio between the estimates and the true parameter values characterizing site 1.

To determine whether data were better explained by two than by

one receptor population, discrimination between the rival models was accomplished by the use of a partial F test, according to Eq. 8 (20):

$$F \text{ value} = [(WSS_1 - WSS_2)/(df_1 - df_2)]/(WSS_2/df_2) \quad (8)$$

where WSS_1 and WSS_2 are the sums of the weighted squares of residuals with models 1 and 2, and df_1 and df_2 are the corresponding degrees of freedom. An improvement of the fit by use of the two-site model was considered significant at $p < 0.05$.

Results

Comparison of kinetic estimation procedures. In contrast to LR, the precision and accuracy of NLR and SNLR were correlated to the receptor occupancy. At ligand concentrations equal to or higher than K_d, both nonlinear fitting procedures were superior with respect to both accuracy and precision, whereas at low ligand concentration the opposite was true (Table 1). The above findings held true whether the arithmetic or geometric mean was used as a measure of accuracy. However, the geometric mean was used throughout, because the parameter estimates were approximately log-normally distributed. The low precision of the nonlinear fitting procedures in determining B_{max} and k_1 at low occupancies was accompanied by an increase in the correlation between these two parameters, in which an overestimation of B_{max} paralleled an underestimation of k_1. This uncertainty in k_1 resulted in poor estimates of K_d. As in LR, an equilibrium binding estimate of B_{max} may be introduced in the nonlinear fitting procedures. Through this, the independence of kinetic estimates are lost but, when the true value of B_{max} was inserted, it resulted in approximately 5- to 10-fold higher precision in the parameter estimates with nonlinear fitting than with LR. When a normal random error with a standard deviation of 25% was added to the fixed B_{max} value, the inferiority of LR was still evident (not shown).

The precision of parameter estimates was higher for SNLR than NLR, irrespective of whether B_{max} was estimated as a variable or introduced as a constant. Under optimal experimental conditions, the two nonlinear methods performed similarly whereas, under less optimal conditions, SNLR was clearly superior. Therefore, we proceeded with a further characterization of the properties of SNLR only.

Factors affecting SNLR estimates. Because the precision and the accuracy of the estimates declined with reduced receptor occupancy, three strategies to improve the estimates from experiments run at low ligand concentrations were tried; firstly, the number of samples were doubled; secondly, the ligand concentration was doubled; and thirdly, the number of samples was doubled and the ligand concentration was increased by 50% in half the samples and reduced by the same amount in the other half. The applied designs all involve doubling of the amount of ligand used in the original experiment. It was found that the latter design resulted in the largest increase in both the precision (Fig. 1) and accuracy (not shown) of the parameter estimates.

In a number of simulations with medium scatter, conditions were chosen that would make the concentration bound not negligible in relation to the total ligand concentration. These data sets, with 20, 40, and 60% of added ligand bound and with equilibrium receptor occupancies of 29, 38, and 44%, respectively, were fitted with SNLR according to the full second-order model. The uncertainties of the B_{max} estimates were 0.20, 0.15, and 0.12 (normalized geometric range), respectively, which are approximately as expected from the level of occupancy (compare Fig. 1). The accuracy of the B_{max} estimates and the accuracy and precision of the other parameter estimates were also as expected from the occupation level.

Kinetic binding data usually reflect the binding to specific and nonspecific binding sites. If the amount of nonspecifically bound ligand can be considered constant throughout the studied time interval, the estimation of this binding can easily be included in the data analysis by adding an extra term to the fitted equations. We found that this yielded slightly increased precision, in comparison with manual subtraction of the nonsaturable binding (not shown).

The association and dissociation rate constants (and the ligand concentration) determine the shape of kinetic binding curves. Therefore, two receptor subtypes differing in either one or both of this pair of constants may be resolved in kinetic binding experiments. If the relative rate of association (k_2/k_1) and the relative rate of dissociation (k_{-2}/k_{-1}) of two binding sites are equal, these sites will behave as a single homogeneous site at equilibrium, whereas in kinetic experiments they may be resolved. The probability of resolving the two sites depends on their relative kinetic rates and on their relative binding densities (Table 2). The most favorable conditions for differ-

TABLE 1
Parameter estimates from SNLR, NLR, and linearization procedures of simulated one-site binding curves

Parameters	Receptor occupancy at equilibrium	Geometric mean ± range								
		SNLR			NLR			LR		
		CV = 2%	CV = 4%	CV = 8%	CV = 2%	CV = 4%	CV = 8%	CV = 2%	CV = 4%	CV = 8%
	%	% of true value								
k_1	9.1	90 ± 28	69 ± 82	38 ± 147	86 ± 35	50 ± 156	18 ± 279	109 ± 12	106 ± 14	102 ± 27
	50	99 ± 3.5	98 ± 7.3	96 ± 15	99 ± 3.5	98 ± 7.6	96 ± 16	104 ± 7.4	105 ± 16	94 ± 24
	91	100 ± 1.7	99 ± 3.4	99 ± 6.3	100 ± 2.1	99 ± 4.2	99 ± 7.5	104 ± 7.8	111 ± 21	104 ± 26
k_{-1}	9.1	100 ± 0.9	101 ± 1.6	101 ± 3.4	100 ± 1.0	100 ± 1.7	101 ± 4.0	100 ± 1.1	101 ± 1.8	101 ± 5.5
	50	100 ± 0.8	101 ± 1.5	102 ± 3.1	101 ± 1.0	101 ± 2.0	103 ± 4.2	101 ± 1.0	101 ± 1.9	103 ± 5.6
	91	100 ± 0.9	101 ± 1.9	101 ± 3.5	100 ± 1.2	101 ± 2.0	101 ± 4.0	100 ± 1.1	101 ± 2.3	101 ± 5.5
B_{max}	9.1	111 ± 28	146 ± 82	268 ± 143	116 ± 34	199 ± 155	559 ± 275	—[a]	—	—
	50	101 ± 2.7	102 ± 5.4	105 ± 11	101 ± 2.5	101 ± 5.8	105 ± 12	—	—	—
	91	100 ± 0.8	100 ± 1.8	101 ± 3.4	100 ± 1.3	100 ± 2.4	104 ± 10	—	—	—
K_d	9.1	112 ± 29	146 ± 84	268 ± 148	116 ± 37	200 ± 166	557 ± 308	92 ± 13	94 ± 15	100 ± 27
	50	101 ± 4.0	102 ± 8.0	107 ± 17	101 ± 3.6	102 ± 8.4	106 ± 17	97 ± 8.0	96 ± 17	109 ± 22
	91	101 ± 2.3	100 ± 4.8	103 ± 8.8	101 ± 2.5	100 ± 4.4	102 ± 8.9	97 ± 8.0	91 ± 20	98 ± 24

[a] Estimates are not obtained with this procedure.

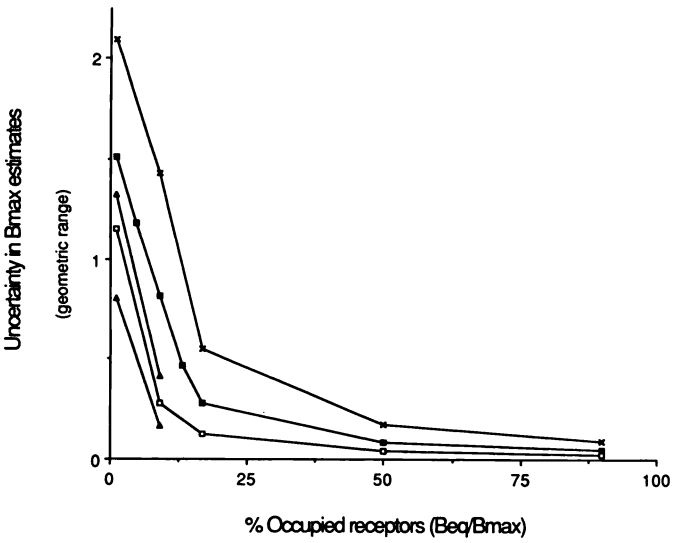

Fig. 1. Relationship between receptor occupation and precision of B_{max} estimates by the simultaneous method. The *points* represent the geometric range of the 30 B_{max} estimates from simulated experiments. The results are from data sets with a total of 14 observations and a CV of 2% (□). 4% (■), and 8% (×). and data sets with 28 observations and a CV of 4%. The latter were simulated as two separate experiments, each with 14 observations, with either the same (△) or different (▲) ligand concentrations, fitted together. The ratio of the different ligand concentrations was 3, and shown are the geometric range in relation to occupancy at the mean of the two concentrations.

entiating two sites are high relative kinetic rates and equal binding densities. Subtypes with binding densities of the same order ($B_{max,\ site\ 1}$ is 25–75% of total B_{max}) could be determined if the relative rates of the binding processes were at least 3 to 5, and relative rates of 5 to 10 were recognizable when the minor binding component accounted for 10–25%. It was found that, the higher the relative density of a receptor subtype, the better determination of binding parameters. Because the dissociation process was not followed beyond the time at which 90% of the initial ligand-receptor complex was dissociated, the slow binding component (k_{-1}) was poorly estimated.

To test whether the differentiation between two sites is mainly determined by the relative rate of association or the relative rate of dissociation, each of these was set at unity while the other was varied (Fig. 2). Receptor subtypes with a relative rate of association equal to unity or equal to the relative rate of dissociation were resolved to a similar degree. Ligand-site interactions differing only in their rate of association were identified to a significantly lesser extent.

Because we found that the level of occupancy influenced the precision of parameter estimates, potentially similar effects on model discrimination were studied. When two binding sites differed only in their association rate constants, a high positive correlation between receptor occupancy and discrimination power was found, whereas no correlation could be found under other conditions (Fig. 3). The influence of the number of observations on the probability of identifying a complex model was also studied. Model discrimination improved exponentially with increasing number of observations, irrespective of the scatter in the data (Fig. 4). A more efficient way of increasing the resolution than through the mere addition of data points was to rerun the experiment at different ligand concentrations.

To examine the sensitivity of the model discrimination with respect to the approximation of ligand excess, data sets with a 5-fold difference in the rates of association and dissociation were simulated with receptor concentrations equal to 10 and 20% of the ligand concentration. When, thus, the fraction of added ligand bound was increased from below 0.1% to approximately 5% and 10%, a slight decrease in the F values was observed.

Comparison of model discrimination by kinetic and by equilibrium binding data. Because two binding sites may be resolved by both kinetic and equilibrium studies, the relative merits of these two techniques were compared on similar premises. For this purpose, Monte Carlo simulations of equilibrium binding experiments with nonlinear least-squares curve fitting

TABLE 2

Influence of relative receptor densities and relative kinetic rates on model resolution and parameter estimation of two binding sites with equal affinity

Relative rates (k_2/k_1)[a]	Relative density of site 1	F values (mean ± SD)	Improved two-site fits[b]	Parameter estimates (geometric mean ± range)[c]					
				k_1	k_2	k_{-1}	k_{-2}	$B_{max,\ 1}$	$B_{max,\ 2}$
	%		%	% of true values					
2	25	1.2 ± 1.2	3	85 ± 62	101 ± 26	105 ± 24	106 ± 16	116 ± 66	91 ± 27
	50	2.2 ± 2.3	13	86 ± 50	83 ± 34	94 ± 21	104 ± 19	96 ± 51	112 ± 29
	75	1.3 ± 1.5	7	83 ± 59	79 ± 53	99 ± 10	102 ± 38	95 ± 27	132 ± 46
3	10	1.3 ± 1.5	7	95 ± 104	92 ± 13	78 ± 108	105 ± 13	55 ± 288	101 ± 16
	25	4.5 ± 2.7	47	91 ± 61	94 ± 24	89 ± 47	108 ± 15	112 ± 60	94 ± 22
	50	10 ± 8.0	83	85 ± 73	96 ± 23	84 ± 61	114 ± 22	99 ± 52	96 ± 30
	75	4.8 ± 4.0	50	64 ± 34	96 ± 24	91 ± 9	79 ± 27	96 ± 26	138 ± 32
	90	1.9 ± 2.6	13	82 ± 26	60 ± 160	96 ± 7	99 ± 70	118 ± 21	178 ± 84
5	10	3.5 ± 2.6	33	63 ± 126	94 ± 8	19 ± 342	101 ± 7	101 ± 73	102 ± 8
	25	23 ± 15	100	109 ± 65	87 ± 28	89 ± 47	96 ± 39	102 ± 52	99 ± 35
	50	33 ± 19	100	81 ± 31	98 ± 16	99 ± 10	104 ± 15	110 ± 20	106 ± 16
	75	13 ± 6.1	97	88 ± 24	84 ± 42	99 ± 5	98 ± 35	105 ± 13	123 ± 39
	90	5.0 ± 5.4	30	95 ± 22	105 ± 85	99 ± 4	91 ± 63	100 ± 12	112 ± 80
10	10	12 ± 8.7	93	108 ± 83	105 ± 12	57 ± 245	105 ± 10	146 ± 64	93 ± 13
	50	53 ± 25	100	87 ± 38	95 ± 17	99 ± 6	100 ± 14	109 ± 22	105 ± 16
	90	9.7 ± 4.4	90	90 ± 17	78 ± 56	101 ± 3	120 ± 56	107 ± 9	136 ± 41

[a] Because the two sites have equal K_d are they characterized by their relative rates, i.e., k_2/k_1 (which is equal to k_{-2}/k_{-1}).
[b] $F > 4.08$ corresponds to $p < 0.05$, which was considered significant.
[c] Mean and range for all 30 data sets.

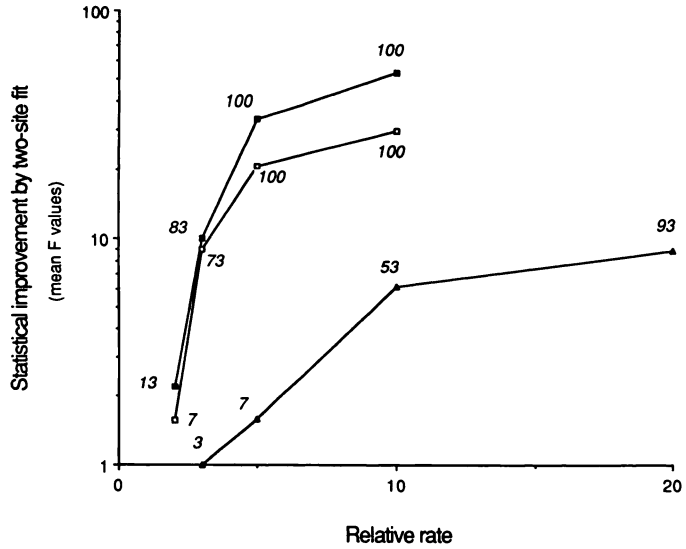

Fig. 2. The relationship between the quotient of the association and/or dissociation rate constants and resolution of two binding sites. Kinetic binding curves with a CV of 4% were generated for a two-site model with a difference in either the association (▲) rate constant, the dissociation (□) rate constant, or both (■). In the latter case the two receptors had equal K_d values. The *abscissa* gives the ratio of k_2/k_1 and/or k_{-2}/k_{-1}. An F value > 4.07 corresponds to a p value < 0.05 and was considered statistically significant (F values corresponding to $p = 0.01$ and $p = 0.001$ are 7.59 and 15.8, respectively). The *number in italics* at each *point* represents the percentage of data sets from which two sites were successfully detected.

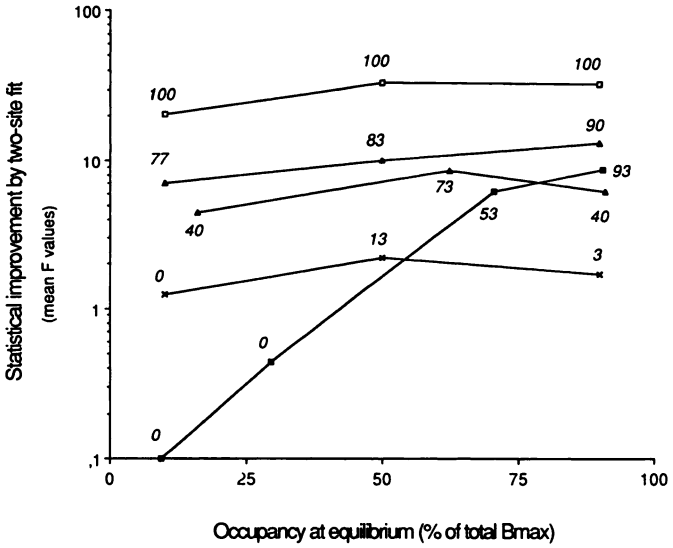

Fig. 3. The influence of equilibrium occupancy on model discrimination at a 10-fold difference in the association (■) or 3-fold difference in the dissociation (▲) rate constants between the receptor subtypes. Experiments were also simulated with a 2- (×), 3- (△), or 5-fold (□) difference in both association and dissociation rate constants. *CV*, significance level; and *numbers in italics* as in Fig. 2.

were taken from Bürgisser (Table 2 in Ref. 12). The same conditions with respect to scatter [variance in bound ligand $(B) = (3\% \cdot B)^2$], number of observations ($n = 20$), K_d ratios, and total amount of radioligand were then used when simulating kinetic data. The selectivity of a ligand toward two sites at equilibrium is determined by the ratio of their K_d values. Such a ratio can, however, reflect an infinite number of combinations between the rates of reaction and, thus, an n-fold selectivity will prevail as long as k_{-2}/k_2 is n times higher than k_{-1}/k_1. At a

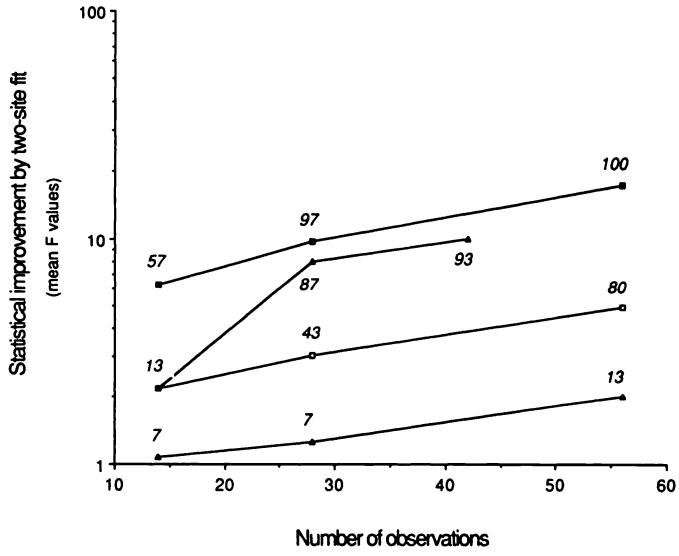

Fig. 4. Effect of scatter and number of observations on the detection of two binding sites. Data were simulated at a single receptor occupancy of 50% and a CV of 2% (■), 4% (□), or 8% (△). ▲ denotes the resolution when data sets run at receptor occupancies of 50%, 10%, and 90%, respectively, with a CV of 4% were sequentially fitted together. Significance level and *numbers in italics* are as in Fig. 2. F values for 14, 28, and 54 observations at $p = 0.05$ are 4.07, 3.05, and 2.80, and corresponding values at $p < 0.001$ are 15.8, 7.80, and 6.38, respectively.

5-fold K_d ratio, the resolution power was higher for the kinetic than the equilibrium binding experiments at all relative rates of the two reactions, except when the dissociation rate constants were similar (Fig. 5A). As the selectivity of a ligand has often been ascribed to differences in the dissociation time from the receptor subtypes, further comparisons were made under the assumption of similar association rate constants. Equilibrium binding experiments were superior in resolving the binding of ligands with high selectivity, whereas kinetic experiments were more advantageous for less selective ligands (Fig. 5B), irrespective of the relative binding densities of the two subtypes (Fig. 5C). Simultaneous analysis of two kinetic experiments run at different ligand concentrations was superior to both equilibrium binding experiments and kinetic experiments run at a single ligand concentration.

An example of kinetic and equilibrium fitting of binding data for a ligand interacting with two sites, at a 5-fold selectivity, is given in Fig. 6. Although both procedures do identify the two-site model, the kinetic procedure yields more accurate parameter estimates.

Discussion

Nonlinear fitting of untransformed kinetic binding data has, *a priori*, several advantages over linear regression of transformed data. The estimates of the kinetic binding parameters are independent of B_{max} estimates from equilibrium binding studies, and there is no need either to attain or to determine the equilibrium binding level, or to treat this estimate as an indefectible value. The use of untransformed data makes it possible to weight data according to their known or estimated variance. The use of SNLR renders further advantages over both LR and NLR. All the relevant parameters (B_{max}, k_1, and k_{-1}) are determined simultaneously, and their interdependence will be revealed by the correlation matrix. As the total number

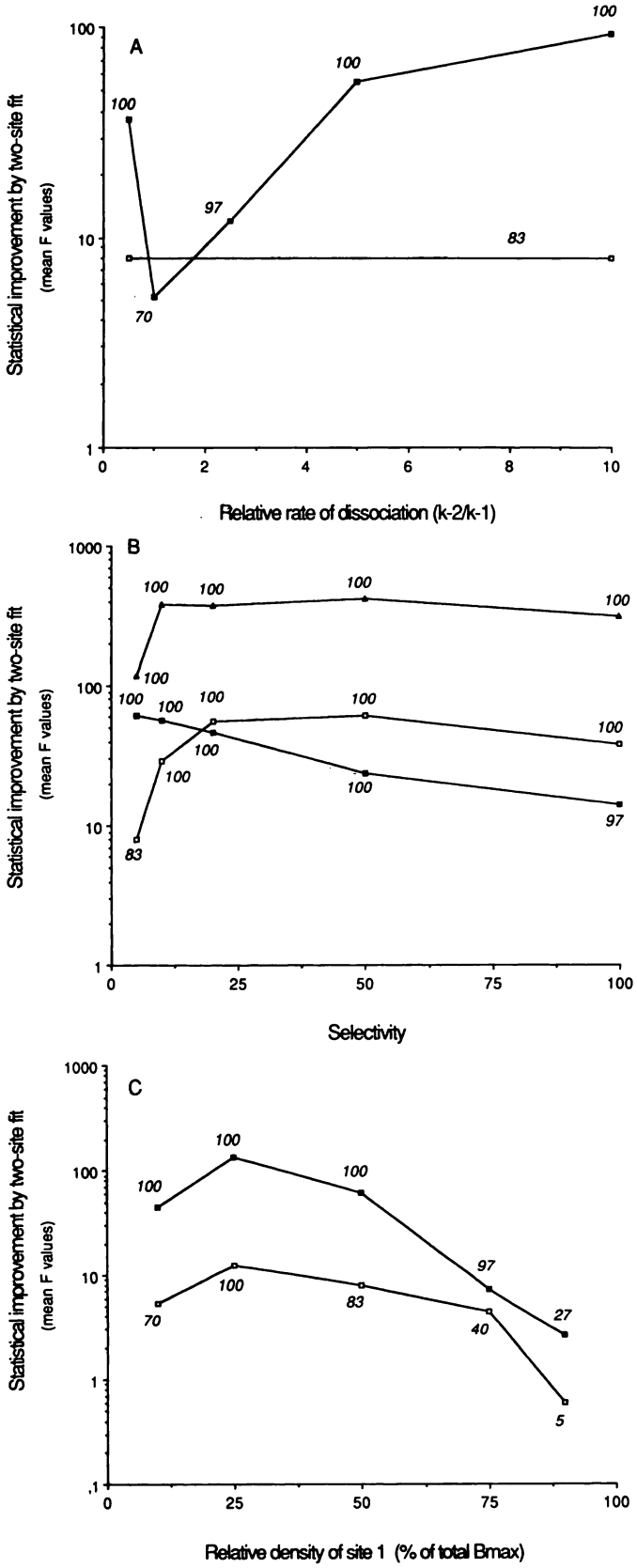

Fig. 5. Resolution of two existing binding sites, from Monte Carlo simulations of kinetic (■) and equilibrium (□) binding to two sites. The same conditions with respect to K_d ratio, receptor concentrations, scatter, number of observations, and total amount of ligand were used in corresponding experiments with the two techniques. The *numbers in italics* give the success rate of detecting two sites ($p < 0.05$), given as percentage. The F values corresponding to p values of 0.05, 0.01, and 0.001 are 3.34, 5.56, and 9.73 for the kinetic, and 3.63, 6.23, and 11.0 for the equilibrium binding experiments, respectively. A, The influence of different ratios between k_2 and k_1 (equal to k_{-2}/k_{-1}) on kinetic resolution. The ligand shows a 5-fold selectivity towards the two sites and the two sites have equal binding densities. Resolution of two sites from equilibrium binding data is not dependent on k_2/k_1, but the single estimate is given as a *straight line* for comparison. B, Resolution of two sites at different selectivities. The ligand selectivity was due to differences in the dissociation rate constant, and the binding densities of the two sites were equal. ▲ denotes the resolution when kinetic data were obtained at two different ligand concentrations. The total amount of ligand, the total number of observations, and other conditions are as in the other experiments. C, Resolution of two sites at different relative binding densities when the 5-fold selectivity of the ligand is determined solely by differences in the dissociation rate constant.

of parameters is smaller, the error term will be spread over more observations. The data analysis gives a single residual term, which in principle enables model discrimination to be performed statistically with respect to all kinetic data, in a manner similar to methods used in analysis of equilibrium data (21).

A given fitting procedure is of little value, irrespective of other advantages, without sufficient accuracy and precision in the estimation of parameters. We have evaluated SNLR in this respect and compared it with the generally used NLR and LR. At medium and high levels of receptor occupancy, the nonlinear fitting procedures were superior to the linearization procedure in every respect. There was, however, a decline in both the accuracy and precision of the nonlinear fitting procedures with decreasing equilibrium occupancy. Thus, LR was superior in estimating the kinetic rate of constants at low occupancy. This superiority is based on the assumption of a known B_{max} value, which is introduced as a constant in the fitting process. When data were analyzed by the nonlinear procedures under the same assumption, the resulting estimates were highly superior to those obtained by LR. However, because several of the advantages of nonlinear fitting are lost with this procedure, we have investigated other strategies to increase the precision for SNLR. The inability to determine k_1 and B_{max} correctly at low ligand concentrations ($L_T \cdot k_1 \ll k_{-1}$) can be predicted from Eq. 6, which then approximates to:

$$B = B_{max} \cdot k_1 \cdot L_T \cdot (1 - e^{-k_{-1} \cdot t})/k_{-1} \quad (9)$$

This equation clearly shows the interdependence between k_1 and B_{max}, and the difficulty in determining the two parameters accurately in such circumstances is obvious. The use of a low radioligand concentration in kinetic binding studies is not uncommon in experimental practice. In such cases, diluting the radioligand with unlabeled ligand and running the experiments at the former levels of radioactivity will suffice to increase the performance of the simultaneous fitting procedure. The most favorable way to increase precision and accuracy, in the event of a limited amount of ligand, is to run the kinetic experiments at different ligand concentrations and then fit all the data together.

The treatment of nonsaturable binding poses a problem in binding experiments. If the nonsaturable binding can be considered constant over time, it may be estimated by inclusion of an extra parameter in the kinetic model of SNLR. The benefits of adopting this procedure are similar to those obtained when

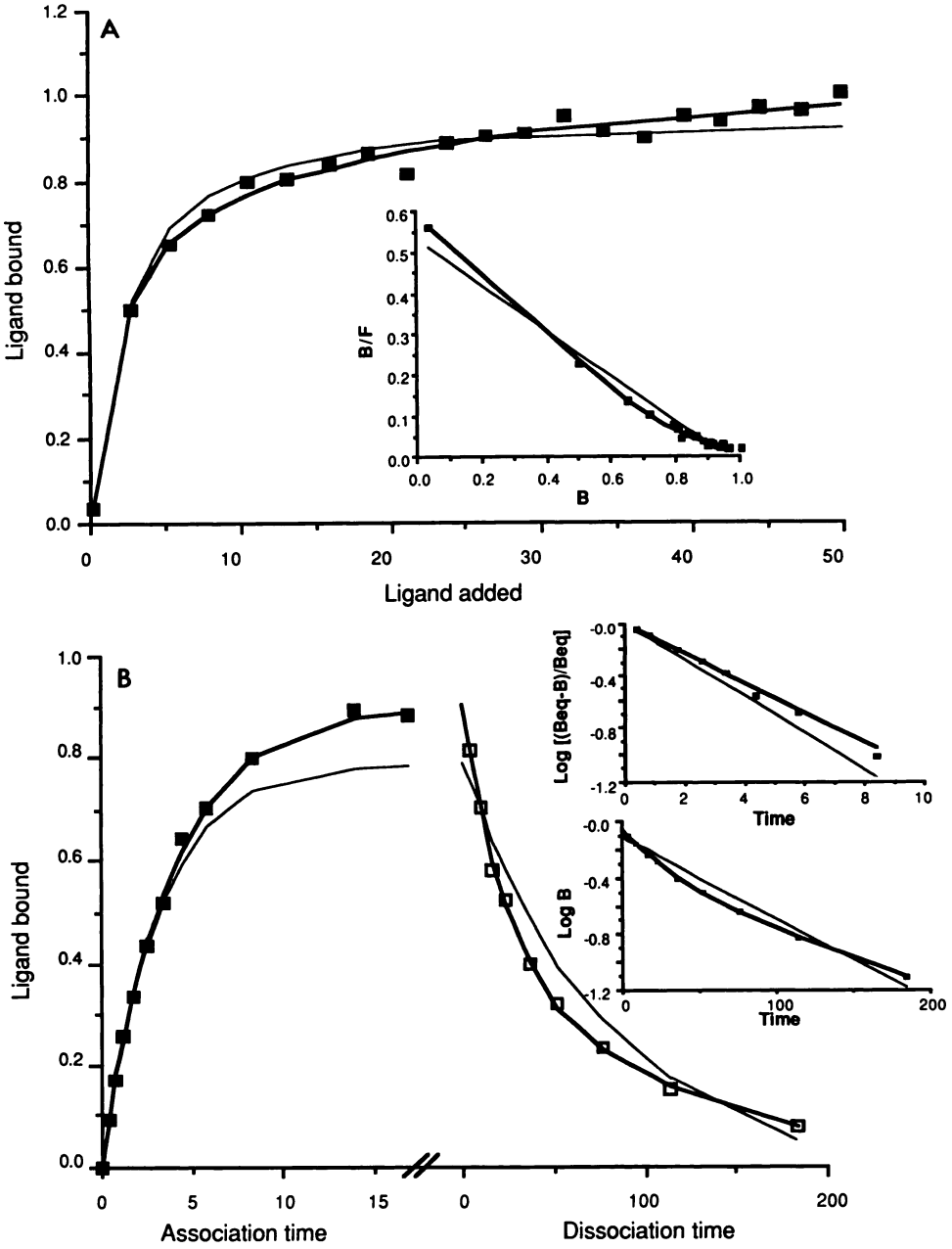

Fig. 6. Examples of ligand binding to a mixture of two receptor subtypes of equal density and a 5-fold difference in affinity, caused by differences in rate of dissociation. Shown are the best fit of one- (——) and two-(——) site models to data containing a relative error of 3%. A, Equilibrium binding data were generated by Feldmans equation, error was added to the true values, and the resulting data were fitted in LIGAND, according to the method of Bürgisser (12). Significantly better fit was obtained with the two-site model ($F = 22.8$, $p < 0.001$). Parameters for the two-site fit were (estimate ± standard error as percent of the true values) $K_{d,1} = 133 \pm 12$; $K_{d,2} = 690 \pm 636$; $B_{max,1} = 157 \pm 12$; $B_{max,2} = 70.4 \pm 15.6$. *Inset*, Scatchard plot of the same data. B, Time-course of ligand binding. Association (■) and dissociation (□) were fitted simultaneously, yielding a significantly better fit for the two-site model ($F = 178$, $p < 0.001$). Parameter estimates for the two-site fit were (estimate ± standard error as percent of true values) $k_1 = 86.9 \pm 24.5$; $k_{-1} = 94.3 \pm 4.6$; $k_2 = 104 \pm 23$; $k_{-2} = 92.8 \pm 10$; $B_{max,1} = 92.6 \pm 7.2$; $B_{max,2} = 108 \pm 6.4$, $K_{d,1} = 108 \pm 32$, $K_{d,2} = 88.8 \pm 18$. *Upper inset*, logarithmic transformation of the association data to show fraction of complex remaining to be formed; *lower inset*, logarithmic plot of the dissociation data.

fitting nonspecific binding to equilibrium binding data (21), namely, that the information regarding the nonsaturable level contained in the total binding data is taken into account and the determination of the nonsaturable binding is not treated as precise.

From the considerations above it is clear that LR is suboptimal and should be replaced by nonlinear regression techniques. In the choice of nonlinear technique, we advocate SNLR. This choice is based on several factors as follows: the superior estimation properties, the statistical advantages, and the relative simplicity of SNLR. We have, with experience using several computer programs, found that SNLR is easier to handle because it requires half the number of both model and data files and transfer of data from the dissociation fitting to the association fitting is avoided. One may assume that the relative merits of SNLR data will increase with increasing complexity of the underlying ligand-receptor interaction, as when two binding sites are possible. Then, both fitting of data to one and two sites, respectively, and statistical comparison become necessary. Two-site fitting by LR is, in general, impossible. Such fitting can be performed by NLR (5), but this procedure involves pairing of the right association and dissociation rate constants. The model discrimination with NLR is made more complex by summation of residuals and uncertainty relating to the interpretation of degrees of freedom, as some parameters are used twice.

The relative merits of model resolution by SNLR may best be assessed in relation to those of resolution based on equilibrium binding data. Different receptor subtypes are unlikely to be detected in equilibrium binding studies with ligands showing less than 5- to 7-fold selectivity (12, 13, 22). Major differences in kinetics have been found for ligands exhibiting low selectivity in the binding to opiate, γ-aminobutyric acid, serotonin, dopamine, histamine, and α-adrenergic receptor subtypes (3, 9,

10). The binding kinetics can thus be utilized to detect receptor subtypes in systems in which equilibrium binding studies will fail. This inability of equilibrium binding experiments to resolve separate sites with a ligand showing similar affinities is well known and several alternative equilibrium (23–25) and kinetic (26, 27) techniques have been developed. But, whereas each of these techniques demands previous information of the binding sites in question and access to selective ligands, we have recently shown this is not needed when SNLR analysis is used (8). Another situation in which equilibrium studies may give unreliable results is when a ligand has low specific activity. Although this could lead to an improperly defined binding isotherm in the low concentration range at equilibrium, model discrimination by kinetic data is usually improved by the use of a high ligand concentration.

Most available ligands are, however, moderately selective. Our studies indicate that, whereas both equilibrium and kinetic binding data obtained with such ligands will probably pick up a second binding site, properly designed kinetic experiments have far superior resolving power. Apart from the higher resolving power of the kinetic procedure, the results shown in Fig. 6 indicate that this procedure is likely to yield more accurate parameter estimates than equilibrium studies, but we have not pursued this issue further. Because kinetic data is usually collected in any event, attention to experimental design and efficient data analysis may reveal erroneous models or serve as an independent verification of parameters obtained through equilibrium binding.

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