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Number of Receptor Sites From Scatchard and Klotz Graphs: Complementary Approaches

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Estimates of number of receptor sites and evaluation of the complexity of the binding process require collection of a spectrum of binding measurements and selection of a theoretical model to fit the experimental data. The appropriateness of the measurements and of the model can be visually judged on graphic displays of the model-data fitting curves in Scatchard and semilogarithmic coordinates. This approach is helpful for detecting the two types of errors most frequently found in reports of binding studies: (1) underestimating the number of binding sites, and (2) failure to recognize the complexity of the binding process. While the former is readily recognizable on semilogarithmic but not on Scatchard plots of the model fitting the data, the latter might not be apparent on either plot. Collection of extensive measurements over a wide range of ligand concentrations with graphic display of the model-data fitting curves in Scatchard and semilogarithmic coordinates should be used to recognize and prevent both errors.

Key words: binding interactions, binding models, graphical display

Ligand-receptor binding studies can be evaluated graphically and analyzed mathematically. Unfortunately, the distinction between graphical evaluation and mathematical analysis by model fitting of binding data is unclear in many reports. In addition, the basis on which some investigators select one mathematical approach over another to analyze binding data is frequently vague despite the fact that such a selection might be crucial to the analysis. In recent years, the Scatchard plot has emerged as perhaps the preferred graphic method [1]. Under optimal experimental conditions and in the presence of a single class of non-interacting sites, binding studies generate linear Scatchard plots. Such plots are assumed to provide reliable estimates of receptor density and affinity, derived from the intercept on the abscissa

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and the slope of the line, respectively. These estimates reflect the binding isotherm, notwithstanding a certain degree of bias introduced by a host of experimental factors which adversely affect the accuracy of the data and their interpretation. On the other hand, biologic systems, particularly those involving live cells, are much more complex and more often than not generate curvilinear Scatchard plots. Difficulties in interpreting such Scatchard plots are compounded by doubts regarding the exact molecular mechanism underlying the binding process and the selection of the most appropriate mathematical model for analysis. Limitations in receptor methodology and analysis preclude unambiguous demonstration of the molecular mechanism underlying such binding data. Thus, interpretation of curvilinear Scatchard plots and mathematical resolution of data that generate such plots, remain controversial [2,3]. Moreover, in a recent publication, Klotz questioned the reliability of predictions of receptor binding capacity calculated from Scatchard plots. Using examples taken from the literature, he found such values to markedly underestimate total binding capacity derived from semilogarithmic plots (bound versus log of free ligand), which he views as the more valid approach [4]. However, the informational content of a set of binding data plotted in different coordinate systems is theoretically the same. Thus, discrepancies in conclusions drawn from graphs of models fitted to the same binding data reflect more selection of inappropriate models to fit the data than an intrinsic value of these plots. Indeed, the major utility of graphic displays of models fitted to binding data is to judge visually the "goodness of fit" of each model proposed as a possible solution for the data and to identify the best fitting model [5]. Model selection is a most important step in binding analysis, as it is possible to fit a model to a set of data for which the model assumptions are invalid or have no biologic implications.

Thus, the recent controversy regarding Scatchard versus semilogarithmic (Klotz) plots [4–9] must be examined in light of the real cause for the reported discrepancies: that the known portion of the curve derives from a spectrum of binding measurements often insufficient to permit its extrapolation to a reliable total receptor capacity. In such cases, the true extent of the unknown portion of the curve is not readily apparent in the Scatchard plot, possibly leading to erroneous estimates of the equilibrium binding parameters. In contrast, the semilog plot provides a clear indication as to the extent of extrapolation in relation to the data. The extent of extrapolation is inversely proportional to the known portion of the curve. Thus, selection of a narrow range of ligand concentrations can lead to an underestimation of total binding capacity directly proportional to the fraction of unoccupied receptors. Such underestimation will occur even in circumstances where graphic interpretation and statistical analysis of the data appear satisfactory. In addition, the use of narrow ranges of ligand concentrations may preclude recognition of the curvilinearity of Scatchard plots which is characteristic of complex binding systems [6,10]. Thus, the range and magnitude of ligand concentrations selected must be sufficient to minimize errors in estimates of binding parameters and reveal the degree of complexity of the binding interactions. An example is provided by our work with the leukoagglutinin(LPHA) lymphocyte-binding system [10–14]; a model that closely mimics the insulin-IM9 system in its binding interactions including the generation of curvilinear Scatchard plots.

MATERIALS AND METHODS

Cells

Blood lymphocytes from healthy volunteers and patients with chronic lymphatic leukemia were isolated by ficoll-hypaque gradient centifugation as previously described [10].

LPHA Purification and Iodination

The LPHA was prepared from PHA-P (Difco Laboratories, Detroit, MI) by column chromatography and was iodinated with reductant-free ^{125}I, specific activity 17Ci/mM (ICN Life Sciences Group, Irvine, CA) by the chloramine-T method. Details of the LPHA purification and iodination procedures have been published elsewhere [12].

Binding Measurements

Aliquots of 10^6 lymphocytes suspended in 0.1ml of ME medium containing 0.1% albumin were incubated in duplicate plastic culture tubes presoaked with 0.5% albumin in phosphate-buffered saline, pH 7.2 with ^{125}I-LPHA of constant specific activity in concentrations ranging from 1×10^{-9} M to 2×10^{-5} M in 0.4 ml of phosphate-buffered saline containing 0.1% albumin. After reaching equilibrium, the reaction was abruptly terminated by addition of 10 ml of phosphate-buffered saline-albumin at 4°C. Unbound ligand was removed by washing three times with ME medium-albumin at 4°C. Cells were collected on 0.5% albumin-presoaked 0.45μ filters (Millipore Corp., Bedford, MA), and cell-bound radioactivity was measured in a Beckman/Gamma 4000 Counter (Beckman Instruments, Inc., Irvine, CA).

Data Analysis

Data are presented as averages of replicate experiments as indicated in the legends. Within each experiment, each datapoint, assessed in duplicate, exhibits a standard deviation generally less than 5%. Data were analyzed by curve-fitting using the least-square "ligand" program [15] adapted to Apple Soft BASIC for the Apple II Plus microcomputer and confirmed by the "ligand" program on DEC-10 computer (courtesy of J.E. Fletcher, NIH). Fitted parameters are graphically displayed in both Scatchard and semilogarithmic coordinates.

RESULTS

The spectrum of LPHA concentrations used in our binding studies is based on the biological effect of this ligand upon normal lymphocytes [10] and is thus functionally relevant. However, because of the demonstrable occupancy-dependent activation threshhold [14], and the fact that most receptors are redundant [10,14], the range of ligand concentrations selected is greater than that necessary to elicit the full range of functional responses [10]. Our binding data can be fitted by the generalized Scatchard model [15], which identifies a two-class binding site model as "best fitting" (2 sites vs 1 $p < .01$; 3 vs 2 sites $p > .05$): a small first component class (21 [SEM 3] ng/10^6 cells) of high-affinity receptors and a second component class of low-affinity sites corresponding to 707 (SEM 14) ng/10^6 cells, for a total of 728 ng/10^6 cells. These results are graphically displayed in both Scatchard and semilogarithmic coordinate systems as shown in Fig 1a and 1b, respectively. Another example of data fitted by the Scatchard model is shown in Figure 2, which displays Scatchard and sigmoid plots of LPHA binding to malignant lymphocytes (chronic lymphatic leukemia cells). A two-class model generates estimates of 2 ng and 120 ng/10^6 cells for the first and second components, respectively.

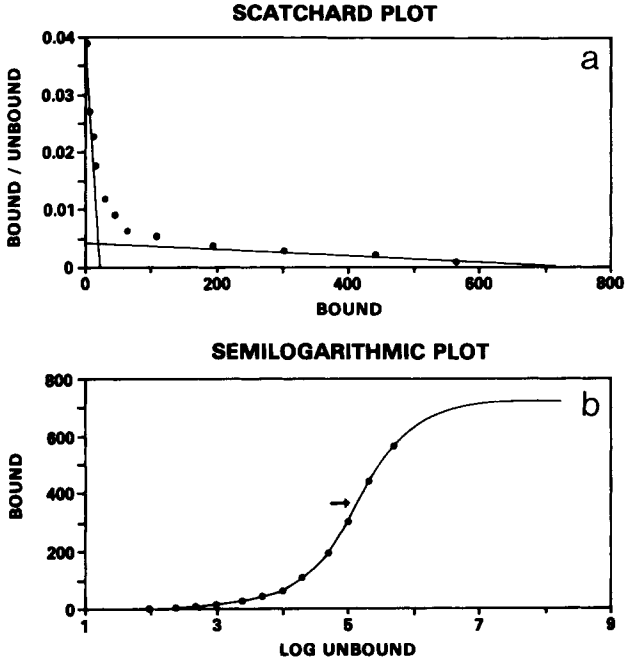

Fig. 1. Scatchard analysis of receptor-specific binding of LPHA to normal human lymphocytes (n = 26). A two-site model (solid line) fitted to the data (●) is graphically displayed in Scatchard (panel a) and semilogarithmic coordinates (panel b). The inflection point is marked by the arrow on the semilog plot.

DISCUSSION

Estimates of the maximal binding capacity are arrived at by extrapolation of the fitted model through an unmeasured and thus unknown portion of the binding curve. Thus, the accuracy of the estimates are critically dependent on the data in the known portion of the curve, which in turn reflects the range of ligand concentrations used. The appropriateness of ligand concentrations selected and their impact on estimates of equilibrium constants can better be judged from the sigmoid plot. As shown in Figures 1b and 2b, experimental datapoints are available over more than half of the S (50% receptor saturation) with several values above and below the inflection point. Binding data achieving less than 50% receptor occupancy should be viewed as an inadequate basis for deriving estimates of total receptor sites. This is easily demonstrated by using the narrow range of ligand concentrations that generates the first 6 datapoints of our database. Scatchard model analysis of this data-set identifies one single class of binding sites with a capacity of 57 (SEM 9) ng/10^6 cells. While the graphic display of the fitted model in Scatchard coordinates (Fig. 3a) indicates a reasonably good model fit to this partial data-set, display in semilogarithmic coordinates (Fig. 3c) clearly demonstrates that the curve has not reached the inflection point and cannot be extrapolated to a plateau value reflecting the maximum binding capacity. Thus, the value of 57 ng/10^6 cells derived from the Scatchard analysis and supported by the Scatchard plot is incorrect.

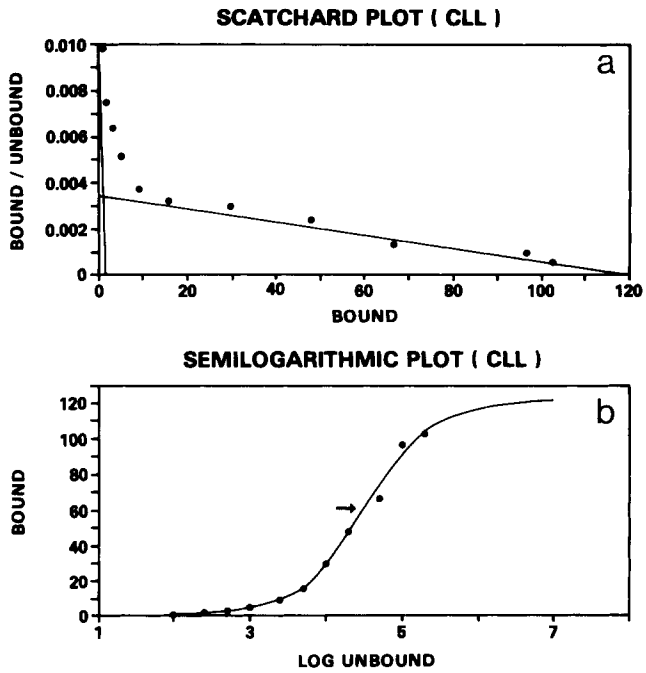

Fig. 2. Scatchard analysis of receptor-specific binding of LPHA to neoplastic (chronic lymphatic leukemia) lymphocytes (n = 6). A two-site model (solid line) fitted to the data (●) is graphically displayed in Scatchard (panel a) and semilogarithmic coordinates (panel b). The inflection point is marked by the arrow on the semilog plot.

On the other hand, data surpassing the 50% saturation point are not necessarily associated with reliable estimates. This is exemplified in Table I, which presents Scatchard model analysis of our data after sequential deletion of the last 0, 1, 2, and 3 datapoints to progressively reduce the database available to detemine the extrapolated portion of the curve. It is clear that the wider the spectrum of ligand concentrations utilized, the greater the predicted number of sites occupied by the ligand (from 194 ng to 564 ng/10^6 cells). This, in turn, results in increased total receptor capacity estimates (from 513 ng to 707 ng/10^6 cells) and in a progressively shortened extrapolated portion of the curve (from 62% to 20% of the total curve). The impact of each additional datapoint on estimates of capacity is shown by improvements in the mean square and in relative precision (SEM/estimate) of segmental estimates, thus providing a measure of validity proportional to the distance of extrapolation. In contrast, the suggestion of using relative receptor occupancy to judge the distance of extrapolation [4] and, therefore, the accuracy of capacity estimates is misleading for it is based on the very capacity (an uncertain value) it is expected to validate. In general, the longer the extrapolation, the greater the underestimation of true capacity and, reciprocally, the greater the overestimation of true occupancy. However, capacity estimates tend to be more unstable as binding approaches 50% occupancy (Table I). Therefore, no inferences should be made regarding extrapolations of experimental data which have not substantially surpassed the 50% saturation point.

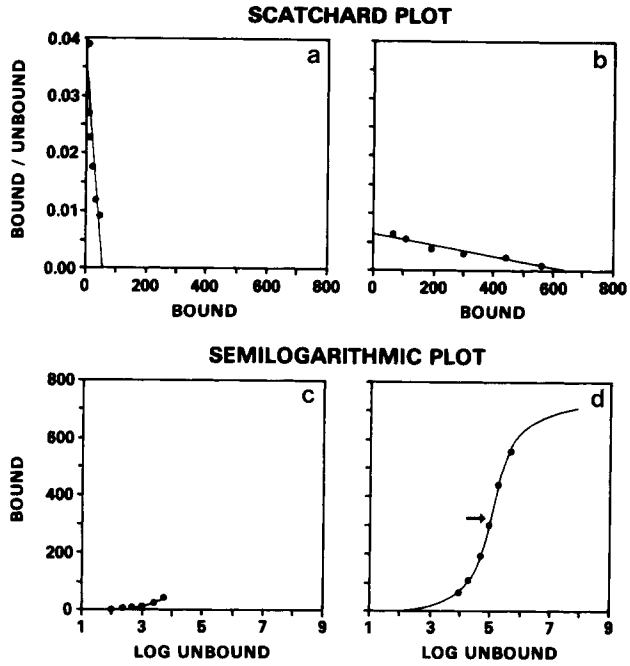

Fig. 3. The data-base shown in Figure 1 was divided into two subsets of data including the first and last six datapoints (shown as left and right panels, respectively). A single-site model (solid line) best fit to each data subset (●) is graphically displayed in Scatchard and semilogarithmic coordinates. The inflection point is marked by the arrow on the semilog plot.

TABLE I. Estimates of Binding Capacity Derived From Scatchard Model Analysis Effect of Reducing the Extrapolated Region of the Curve*

Segment analyzed		Scatchard analysis			
Seg[a]	Max. bd[b]	Capacity[c]	Occup[d]	Prec (%)[e]	Mean sq
A	194 (14)	513 (65)	.38 .27	13	97
B	303 (28)	737 (59)	.41 .43	8	86
C	443 (34)	830 (26)	.53 .63	3	80
D	564 (22)	707 (14)	.80 .80	2	71

*For sake of clarity and simplicity, this table and its discussion in the text present only data pertinent to the large class of low affinity sites.

[a]Segments A, B, C, and D, sequentially exclude the last 3, 2, 1 and 0 data-points (right most data-points shown on Figure 1).

[b]Maximum amount of bound ligand (SEM) measured for each segment, expressed in ng/10^6 cells.

[c]Total binding capacity (SEM) expressed in ng/10^6 cells calculated by Scatchard model analysis of each segment of the curve.

[d]Occupancy: Left column, relative receptor occupancy calculated as the ratio of maximal amount of bound ligand to binding capacity, estimated from each segment; right column, true receptor occupancy calculated as ratio of maximum amount of bound ligand for each segment to total binding capacity estimated from the entire curve (Segment D).

[e]Relative precision calculated as the ratio of standard error divided by capacity estimated from each segment.

A good model fit as judged by statistical analysis [5,6] and by visual display of the fitted model in both Scatchard and semilogarithmic coordinate systems [4,5] is adequate support for estimates of total receptor capacity. However, results of such analyses may not reflect the true nature of the binding isotherm. Consider the set of data including the last six datapoints from our database. Scatchard model analysis of this data-set identifies one single class of binding sites with a capacity of 653 (SEM 51) ng/10^6 cells. Graphic display of the fitted model in both Scatchard and semilogarithmic coordinate systems (Fig. 3b and 3d, respectively) provides convincing visual evidence for one single class of binding sites with the stated total receptor capacity. Yet, as shown above, analysis of our entire database demonstrated a somewhat greater receptor capacity and, more importantly, more complex binding interactions.

From the above examples, it is clear that binding data based on narrow ranges of ligand concentrations can lead to two types of errors depending on which end of the ligand concentrations range is missing: (1) underestimating both total receptor capacity and the complexity of the binding process (high end of concentration range missing); and (2) failure to recognize the complexity of the binding process inspite of a good mathematical and visual fit (low end of concentration range missing). The first type of error is more likely to occur using serial dilutions of a single concentration of radioligand as practiced in binding displacement studies [1,16–18]. The second type of error is more likely to occur when binding assays are conducted using increasing concentrations of a radioligand of constant specific activity [13]. While neither type of error is identifiable by the Scatchard plot, the semilogarithmic plot will easily reveal type one errors. However, type two errors remain unexposed by either the Scatchard or semilogarithmic plots. To avoid such errors, the investigator must select a range of ligand concentrations as wide as possible or practicable. Thus, binding data compatible with a single class of binding sites might be viewed with suspicion unless derived from extensive measurements over a very wide range of ligand concentrations. Because of these limitations, the adequacy of model fit should be visually judged by both Scatchard and semilogarithmic plots. The inflection point of the semilogarithmic plot should also be used as a guide for investigators to select ligand concentrations sufficient to achieve well over 50% receptor saturation. This reduces the unknown portion of the curve and makes estimates of total binding capacity less uncertain. The display of results of the analysis in Scatchard coordinates might, from its appearance, suggest complex binding sites or negative coopertivity [5,16–18] not revealed by the semilogarithmic plot. Equilibrium binding constants derived from such a methodical mathematical and graphic approach to the analysis of binding data are broadly valid, regardless of the binding isotherm, and are thus applicable to one or more classes of homogeneous non-interacting binding sites and to sites exhibiting positive or negative cooperativity, or both.

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