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COMMENTARY

THE CURVILINEAR SCATCHARD PLOT

EXPERIMENTAL ARTIFACT OR RECEPTOR HETEROGENEITY?

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For almost every ligand–receptor system studied, some investigators report findings inconsistent with the predictions of the Law of Mass Action for the simplest model of a reversible, bimolecular interaction between the ligand and a single set of identical, independent receptors. The Scatchard plot [1] of the ratio of bound ligand to free ligand against the amount (or concentration) of bound ligand has a curvilinear appearance, instead of the predicted straight-line relationship. The most common pattern is for the Scatchard plot to appear progressively less steep as it approaches the abscissa axis (upwardly concave); this is interpreted as evidence of some non-uniformity in the interaction between ligand and receptor, such as the presence of a heterogeneous population of receptors. This concept of functional receptor heterogeneity is widely accepted in the receptor field; much work is now focused on its likely nature and biological relevance. The term "functional heterogeneity" is used here in its broadest sense to encompass differences in behavior between individual receptors (due, for example, to the influence of neighboring molecules) as well as any actual molecular differences between receptors.

Despite the numerous reports of curvilinear Scatchard plots, many questions about the explanation for the curvature remain unanswered. One reason for this is that, although some investigators appear to find clear evidence of a curvilinear plot, with the same receptor systems other investigators often obtain a linear plot. Thus, for essentially every receptor system, the case for receptor heterogeneity is best regarded as unproven.

Theoretical analyses have indicated that a number of experimental artifacts may explain the curvilinear nature of the Scatchard plot without the need to invoke receptor heterogeneity [2–6]. The artifactual explanations outnumber the real models of heterogeneity. In practical receptor studies, however, the potential artifacts are usually dismissed, often in a quite cursory manner, in favor of the more exciting concept of receptor heterogeneity. This concept is of particular interest because of its probable relationship to the manner in which the receptor functions in transducing the external stimulus into a signal from the effector; current evidence suggests that the functional heterogeneity of many receptors (if it exists) may be related to the association reactions between the receptor and a guanine nucleotide-binding protein that is then coupled to the appropriate effector [see Ref. 7 for review].

The principal purpose of this review is to remind receptor practitioners of the existence of the various artifacts, and to illustrate their potential contribution to the observed binding pattern in actual receptor studies. It is an attempt to bridge the gap between the theoreticians who understand the artifacts [see Refs. 3 and 4 for more complete theoretical analyses], but are rarely concerned with real receptor-binding data, and the practitioners who generally underestimate the potential impact of the artifacts. Now seems a particularly apposite time to reconsider the potential contribution of these artifacts as the focus of receptor research has turned to the nature, rather than the mere existence, of receptor heterogeneity. The search for this nature could be sorely hindered by even a small distortion of the true binding pattern. For some, perhaps many, receptors these artifacts may even explain completely the observed curvature of the Scatchard plot.

The principal artifacts are listed in Table 1, together with their usual effect on the Scatchard plot. Essentially all receptor studies are affected by some or all of these artifacts. The magnitude of their impact on the Scatchard plot, however, differs greatly from one study to another. Each artifact will be discussed in the following section. The emphasis will be on artifactual sources of upward concavity of the Scatchard plot because this is the pattern usually seen in practice; this is not meant to imply, however, that the artifactual sources of downward concavity do not also arise.

Estimation of non-specific binding

The current experimental definition of non-specific binding, that is binding in the presence of an excess of unlabeled ligand, may be the principal artifactual source of curvilinear Scatchard plots. Although this definition is used almost universally, it clearly has some major inadequacies [5]. First, it is not a definition of non-specific binding, but rather one of non-saturable binding. These two terms are not synonymous since the components of non-specific binding (i.e. that not related to the receptors under study) do tend to saturate as the ligand concentration is increased [5, 8], albeit usually at higher concentrations than saturate the true receptors. The choice of an appropriate excess of unlabeled ligand thus becomes important.

Table 1. Experimental artifacts in receptor-binding studies and their impact on the pattern of the Scatchard plot

Nature of artifact	Effect on Scatchard plot	Comments
Imprecise estimate of non-specific binding	Upward curvature (usually)	Very common Effect may be severe
Poor procedure for data analysis	Exaggerates other artifacts	Importance underrated
Affinity difference between labeled and unlabeled ligands	Upward curvature (usually)	Common with iodinated ligands
Impure labeled ligand	Upward or downward curvature	Common problem
Effect of membrane microenvironment	Upward or downward curvature	May be important
Contamination of bound by free	Upward curvature	Similar to non-specific binding
Incomplete recovery of bound fraction	Downward curvature	Usually minor effect
Irreversible binding and internalization	Downward curvature	Common with whole cells
Ligand degradation	Downward curvature (usually)	May not be common
Receptor degradation	Downward curvature	Often minor effect
Non-equilibrium binding conditions	Downward curvature	To reduce degradation

Some studies that I performed with ^{125}I-labeled thyrotrophin illustrate this point [9, 10]. One component of non-specific binding, that to the receptor-assay tube, was reduced substantially (by 54% on average, compared with binding of radioligand alone) on addition of the unlabeled thyrotrophin excess (3×10^{-7} M) usually used to define non-specific binding in studies of this receptor. The reduction was somewhat ameliorated by the use of a 10-fold smaller excess just sufficient to saturate the receptors. Saturability of the non-specific binding to receptor-bearing biological materials (target cells, plasma-membrane extracts, or solubilized proteins) clearly cannot be assessed as readily as that to the assay tube (or filter, if that is used to collect the receptor-bound ligand). However, studies with non-target tissues and with ligands not directed towards the receptor have demonstrated that this component of non-specific binding is also saturable.

The consequence on the Scatchard plot of underestimating non-specific binding, through partial saturation, is illustrated in Fig. 1. Even in the case of a good receptor study where non-specific binding appears unimportant (e.g. 10% of total binding), its underestimation can result in noticeable artifactual curvature of the Scatchard plot (Fig. 1b). Not surprisingly, the resulting curvature is much more pronounced in other receptor studies where non-specific binding is greater (e.g. 50% of total binding) [10]. The degree of saturability of non-specific binding undoubtedly depends on the ligand used, and on the source of receptors. It certainly occurs, however, with many ligands [see Refs. 5 and 8 for further discussion], and is a much more widespread source of error than usually recognized. The best way to minimize its impact is to select an assay protocol with a high ratio of specific to non-specific binding and to be careful in choosing an appropriate definition of non-specific binding. The impact is particularly great and difficult to obviate in studies of low affinity receptors, for which saturation of receptors and non-specific sites may occur at similar ligand doses.

There is little value in criticizing the current definition of non-specific binding unless a better definition can be proposed. Ideally, non-specific binding should be measured at ligand concentrations relevant for receptor binding (e.g. at the K_d). From an experimental standpoint, it thus seems better to define non-specific binding as the sum of two separate components: the first represents binding to non-biological materials (assay tube, filtration apparatus) and can be measured accurately without addition of excess unlabeled ligand; the second represents binding to sites other than receptors on the biological materials (cells, membranes, protein extracts) and can only be measured approximately (for example, using a limited excess of unlabeled ligand). This is a significant improvement over the usual experimental definition whenever the first component contributes substantially to the overall non-specific binding. It is also worth pointing out here that it is generally a misconception to base the unlabeled excess on the concentration of labeled ligand used, for example to use a 100-fold or 1000-fold excess. Such an approach is only valid when the labeled ligand is itself used at a saturating concentration. Under other circumstances, the excess should instead be chosen so as to saturate the receptors, and thus based on the K_d for the ligand–receptor interaction. A limited excess, for example $100 \times K_d$, which will normally block about 99% of the receptors, is preferred over a larger excess because it is less likely to cause significant saturation of the non-specific sites [13]. Even with this improved experimental definition of non-specific binding, it is best to regard the resulting value only as a guide to its true extent, and to use a curve-fitting approach in which a non-specific binding parameter is fit to the data along with the parameters to characterize the receptors (see below).

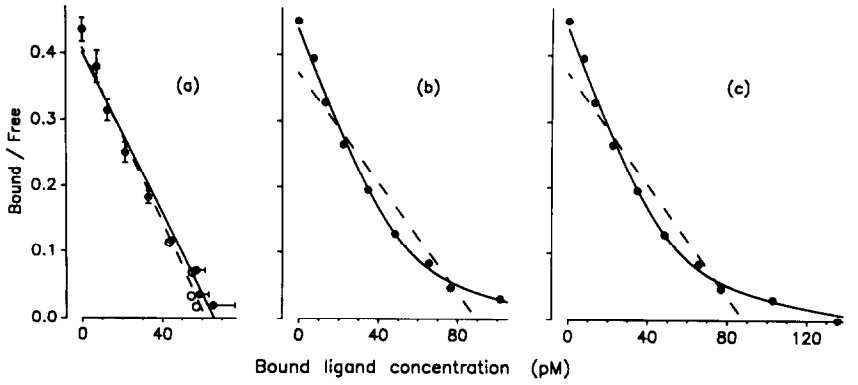

Fig. 1. Effect of underestimation of non-specific binding on the Scatchard plot. The data shown are representative of binding studies with thyrotrophin; they are one set from a series of twenty reconstructions in which random errors (based on the experimental error pattern) were reapplied to an idealized Law of Mass Action model for binding to a single set of receptor sites ($K_a = 7 \times 10^9$ l/mol, capacity = 60 pM) together with an ideal (not saturable) non-specific binding component [10, 11]. Means of duplicate "measurements" are shown throughout in the form of a Scatchard plot. (a) Data (closed circles) corrected for an unbiased estimate of non-specific binding (although this does include a random error component). The error bars shown are SD based on data from thirteen experimental studies. The solid line shows the optimal one binding site fit obtained by the LIGAND program [12] with the "experimental" non-specific binding regarded as an exact (error-free) value; the binding capacity is estimated as 66 pM. The hatched line is the optimal fit regarding non-specific binding as a fitted parameter, and the open circles represent the corresponding data points. In this case, the binding capacity is estimated as 62 pM, and the use of a two binding site model does not produce a significantly better fit to the data ($P > 0.1$, partial F-test). (b) Data corrected for a non-specific binding estimate that includes a bias towards underestimation typical of that seen in actual experiments. The solid curve shows the optimal two binding site fit obtained by LIGAND with the "experimental" non-specific binding regarded as exact. The high and low affinity sites have capacities of 50 and 102 pM respectively. This fit is much better than the optimal one binding site fit (hatched line) under these circumstances ($P < 0.005$). (c) Data of (b) reanalyzed by the LIGAND program with the "experimental" non-specific binding treated as an additional data point at a 100-fold excess dose. The optimal two binding site fit (solid curve; binding capacities 50 pM and 106 pM) is very similar to that of the preceding case, and is again much better than the optimal one binding site fit (hatched line; $P < 0.005$).

Procedure for data analysis

The graphical Scatchard plot remains a useful illustrative tool for receptor-binding studies. It no longer seems appropriate, however, to use it either for quantitative evaluation of the binding parameters (affinity and capacity) or for establishing the presence of functional receptor heterogeneity. The Scatchard plot suffers from two serious flaws in such uses. First, its shape is critically dependent on an accurate correction for non-specific binding and such a correction is not usually a realistic demand (see above). Second, it grossly distorts the measured binding data, particularly in the vicinity of the two axes, such that a seemingly small measurement error can result in a badly misplaced data point. It is important to realize that linear regression analysis cannot overcome these defects in the Scatchard plot, even if severe weighting is applied to compensate for the gross distortion of measurement errors. The variables plotted on the ordinate and abscissa (bound-to-free ratio and amount bound) are clearly interdependent (both being governed by the concentration bound), thereby invalidating a fundamental assumption of regression analysis that there be an independent and a dependent variable. Furthermore, neither of the two possible regression lines ("y" on "x", or "x" on "y") is appropriate because the errors are predominantly in the direction of the ordinate near the ordinate axis and in the direction of the abscissa near the abscissa axis (Fig. 1a) [see Ref. 14 also].

Many alternative graphical representations of receptor-binding data have been considered. The binding isotherm, showing the concentration (or amount) bound plotted against the total (or free) ligand concentration (Fig. 2a), has the advantage that it is the most direct representation of the data. The linear scale on the abscissa, however, compresses the most important information on specific binding into a small region near the origin. This difficulty is overcome by using a logarithmic scale for the abscissa (Fig. 2b), as recommended by Klotz [15, 16]. The latter plot would be a useful, direct representation were it not for the influence of non-specific binding, whose contribution to the overall binding curve is the least apparent in this plot. A progressive rise in the concentration of bound ligand can be caused by non-specific binding as well as by increasing occupancy of a saturable receptor (Fig. 2b). The error pattern is also markedly non-uniform [14], just as with the Scatchard plot. In contrast, the displacement plot of the percentage of ligand bound against the logarithm of the total ligand concentration (Fig. 2c) provides the clearest indication of non-specific binding (as a non-zero lower limit for the binding percentage at high ligand concentrations). None of these alternative plots, however,

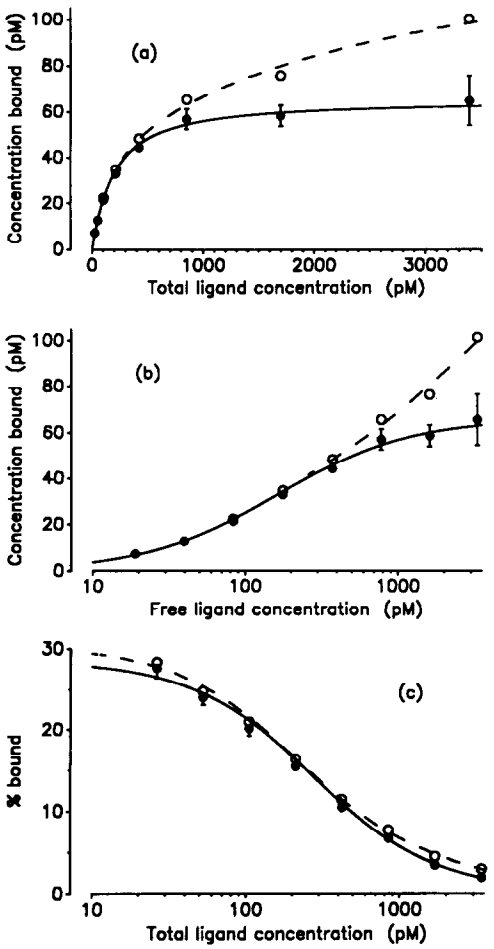

Fig. 2. Alternative graphical representations of receptor-binding data: (a) binding isotherm; (b) Klotz plot; (c) displacement plot. The simulated one binding site data of Fig. 1 are replotted in terms of different coordinate systems. The data are shown corrected for either an unbiased (closed circles) or a biased (open circles) estimate of non-specific binding (corresponding, respectively, to the data represented by the closed circles of panels a and b of Fig. 1). Error bars are shown for the former case. The solid line in each plot represents the optimal one binding site fit to the unbiased data, and the hatched line the optimal two binding site fit to the biased data (for which the one binding site model provides an inadequate fit).

provides information on binding heterogeneity (if it exists) in as clear and succinct a manner as the Scatchard plot. Moreover, these plots are all distorted as much as the Scatchard plot (though perhaps in a less obvious manner) by the various experimental artifacts discussed subsequently in this review.

Thus, the analytical drawbacks of the Scatchard plot should not be considered a reason for choosing a different graphical representation. Instead, they are best overcome by applying curvilinear regression methods to analyze the raw (untransformed) binding data. The total ligand concentration can be regarded essentially as an error-free independent variable, and curvilinear regression can be applied to the relationship between this and the bound radioligand concentration (dependent variable). This forms the basis of the approach used by the LIGAND computer program originally developed by Munson and Rodbard [12, 17]. This and other similar programs have since been adapted for many computer systems (including Apple II and IBM-PC, as well as minicomputers and mainframes). Copies of several such programs are available from the Biomedical Computing Technology Information Center (Vanderbilt Medical Center, Nashville, TN). When used correctly with appropriate weighting of the data (usually on the basis of equal percentage errors), such programs yield reliable estimates of the binding parameters. In view of this, the application of linear regression to transformed (e.g. Scatchard) data should no longer be tolerated as an acceptable approach.

Another advantage of computerized curvilinear regression is that it allows flexibility in the definition of non-specific binding. Three distinct strategies are possible. The first is to treat the experimental measurement of non-specific binding as a precise figure to be used (preferably by the program) to correct all measurements of total (specific + non-specific) binding. Such an approach has little merit, except that an average value is used for correction instead of a separate non-specific binding estimate at each dose. The second strategy is to regard the non-specific binding measurement as an additional data point (at a very high ligand concentration) to be included with the remaining data; the program is then made to fit (optimize) a non-specific binding parameter as well as the specific binding characteristics. This is the approach recommended by the authors of the LIGAND program [12]. In principle, it is clearly preferable to the first strategy. In practice, however, the non-specific data point is so remote from the other points (typically differing in total ligand concentration by a factor of 100 from its nearest neighbor) that, even with the usual weighting pattern, it has a disproportionate influence on the curve-fitting process. Its influence remains so great that any saturability of the non-specific binding component still results in a distorted apparent specific binding pattern suggestive of receptor heterogeneity (Fig. 1c), a pattern which is indistinguishable from that obtained by direct subtraction of experimental non-specific binding (Fig. 1b).

The problem of saturability of non-specific binding is overcome by the third strategy, in which the non-specific data point is omitted entirely from the analysis, yet a non-specific binding parameter is again included in the fitting process. Binding measurements are made over a limited range of ligand concentrations appropriate to the receptors, for example from $0.1 \times K_d$ (for the highest affinity sites, if multiple sites are present) up to $10 \times K_d$ (for the lowest affinity sites). The data for total (specific + non-specific) binding are then fit to a model comprising the specific binding site, or sites, together with a non-specific binding component. The non-specific binding estimate is thus based only on ligand concentrations relevant for the receptor-binding interaction. This latter strategy does have the disadvantage that the

non-specific binding parameter may be too loosely defined, resulting in greater uncertainty in the estimates of the specific binding characteristics. The use of this third strategy, however, greatly reduces the likelihood of finding heterogeneity where none exists (Fig. 1a); it provides a much more rigorous test for the existence of functional receptor heterogeneity and should thus be the preferred procedure.

Computerized data analysis offers other significant advantages in the study of receptor heterogeneity, particularly in that the binding patterns for several ligands can be analyzed simultaneously [see Refs. 17 and 18 for examples]. Its principal limitation at present is that the programs available are only suitable for examining some of the possible models of heterogeneity (e.g. multiple independent receptor model and cooperativity model), not others (e.g. interconvertible affinity model).

Affinity difference between labeled and unlabeled ligands

One of the fears of the earliest investigators involved in receptor-binding studies was that a radioiodinated ligand might behave differently from the corresponding native ligand in its receptor interactions. This was a real fear because the radioiodine introduced into the ligand was a "foreign" atom not present in the native ligand. However, despite reports of the alteration of receptor-binding properties of several ligands upon radioiodination [2, 9, 19, 20], this possibility has largely been ignored by more recent investigators. One reason may be the difficulty of comparing the receptor-binding properties of the labeled and unlabeled ligands in a reliable manner [see Ref. 21 for further discussion]; the methods usually used can probably only detect gross differences in behavior.

The theoretical consequences of a difference in ligand affinity were examined by Taylor [2]. For receptor studies using a tracer concentration of labeled ligand competed by increasing concentrations of unlabeled ligand, he showed that the Scatchard plot would appear upwardly concave if the labeled ligand were of higher affinity than the native ligand and downwardly concave if it were of lower affinity. In practice, since the incubation conditions for a receptor assay are usually chosen so as to maximize binding of the labeled ligand, the pattern of upward concavity may be expected to predominate whenever radiolabeling influences the binding properties of a ligand.

The following case is a useful illustration. Studies of the thyrotrophin receptor revealed three different patterns of Scatchard plot (linear, upwardly concave, downwardly concave) depending on the degree (and method) of radioiodination and the incubation conditions used for the binding reaction (Fig. 3). With one form of ^{125}I-labeled thyrotrophin (low specific radioactivity, prepared by the Bolton–Hunter method [22]), the Scatchard plot was always linear regardless of the incubation conditions [10, 11]. With another form (high specific radioactivity, prepared by the chloramine-T method [23]), however, the Scatchard plot was upwardly concave under incubation conditions chosen to increase radioligand binding, but downwardly concave under the optimal incubation conditions for the first tracer [10]. These data can be explained by the analysis of Taylor in the context of a simple homogeneous receptor model: the Bolton–Hunter radioligand appears equipotent with the native ligand [9, 10], hence its linear Scatchard plots; the other radioligand, due to "distortion" from excessive radioiodination [9], is less potent under normal incubation conditions (pH 7.5), but under incubation conditions (pH 6.5) chosen to favor its binding and disfavor binding of the native ligand it becomes more potent than its native counterpart.

These observations suggest that a useful strategy to investigate the potential for anomalous behavior of other radioligands may be to perform similar comparative studies with different radiolabeling methods. If all the radioligands behave in an equivalent manner, confidence that this behavior truly reflects that of the native ligand may be justifiable. An alternative strategy for validating the behavior of a radioligand is to compare its biological potency before and after radiolabeling [19, 24], preferably using a bioassay that is closely akin to the chosen receptor assay. This alternative has the advantages of being simpler and needing only a single form of radioligand. It is appropriate, however, only for those radioligands in which a large proportion of the ligand molecules have been radiolabeled (preferably at the same site), as the bioassay will respond to unlabeled as well as labeled ligand molecules. Moreover, it may not yield meaningful information when the radioligand has been purified extensively after radiolabeling (as the radioligand may then be in a more pure form than the native ligand), or when its specific radioactivity is imprecisely defined [21].

Impure labeled ligand

Many of the radioligands used for receptor-binding studies may not be entirely pure, whether prepared commercially or by the individual investigator [21]. In some cases, the degree of purity can be quite low, perhaps no more than 30% [24–27]. This is a particular problem with the complex glycoprotein hormones, but can also be a problem with some much simpler, yet labile, compounds [21]. Repurification on a regular basis, either by standard biochemical techniques or by receptor adsorption [11, 24, 28], should overcome this problem.

The importance of measuring purity in a manner relevant to the receptor-binding study has been discussed in detail in a previous report [21]. It is useful to determine the receptor-reactivity ("bindability") of the radioligand from measurements of its binding to an increasingly large excess of receptors [11, 21]. The use of an impure radioligand will normally result in a Scatchard plot showing a downwardly concave pattern [3, 21]. This is always the case in the absence of a correction for the receptor-reactivity of the radioligand. When an impurity itself possesses significant receptor-binding activity, however, an overcompensation can occur upon correction for the receptor-reactivity, leading to an upwardly concave Scatchard plot [21].

Influence of the membrane microenvironment

The membrane microenvironment of the receptor

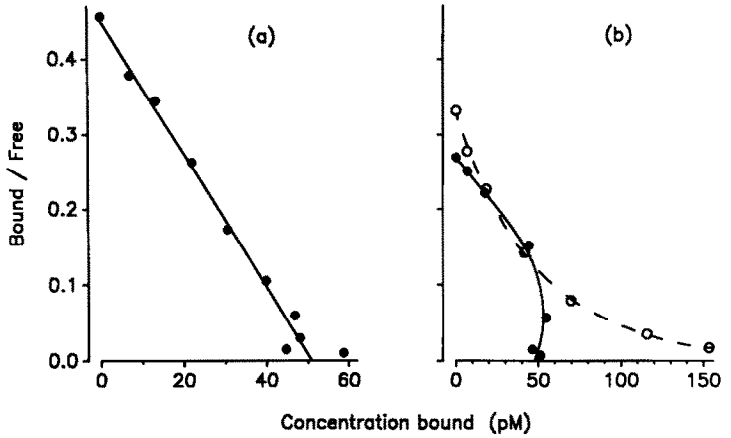

Fig. 3. Effect of radioiodination of thyrotrophin on the Scatchard plot for binding to receptors on thyroid membranes. All binding data were obtained using a tracer concentration of labeled ligand competed by increasing concentrations of unlabeled ligand [10, 11]. (a) Thyrotrophin was radioiodinated to low specific radioactivity by the Bolton–Hunter method [22], and binding was assayed at pH 7.5 under conditions optimal for its binding. (b) Radioiodination was to high specific radioactivity by the chloramine-T method [23]; binding was assayed either at pH 6.5 (open circles, hatched line) or at pH 7.5 (closed circles, solid line). Binding at pH 7.5 was measured using twice the concentration of thyroid membranes so as to achieve a similar maximal binding level.

can influence the ligand–receptor binding pattern in several ways. First, it may play a part in supporting the interactions between membrane proteins that are responsible for functional receptor heterogeneity (for example, interactions between receptor and guanine nucleotide-binding protein); in this respect, the membrane can be regarded as an indispensable part of the mechanism of action of the receptor. Second, binding to the membrane represents a major component of non-specific binding; the membrane is thus a potential source of artifactual curvature of the Scatchard plot (as discussed above). Finally, the membrane can alter the interaction between ligand and receptor by perturbing the local concentrations of the reactants. Such perturbations are regarded here as an artifactual source of curvature of the Scatchard plot, although the alternative view that they represent an intrinsic part of receptor function is also tenable.

In applying the Law of Mass Action to the analysis of ligand–receptor binding data, the effective concentration of each reactant is assumed to correspond to its average concentration in the complete reaction mixture. This should be a realistic assumption in studies of solubilized receptors, but it becomes questionable when the receptors are membrane-bound. Not only are membrane-bound receptors themselves non-uniformly distributed in the mixture, gradients of free ligand concentration may also arise. Charged ligands become attracted towards an oppositely charged membrane so that the local concentration in the vicinity of the receptor exceeds the average concentration [6, 29]. For a typical biological membrane (of surface potential −40 mV), a 5-fold concentration gradient is expected per unit positive charge on the ligand [6]. Lipophilic ligands tend to be further concentrated in the membrane bilayer, and amphiphilic ligands may be brought into a particular orientation in the membrane favorable (or unfavorable) for interaction with the receptor [6, 30].

These processes all have a qualitative effect on the apparent affinity of the ligand–receptor interaction. Because they usually tend to be saturable, they can also affect the shape of the Scatchard plot: for a positively charged ligand interacting with a receptor on the plasma membrane, for example, significant upward concavity of the Scatchard plot may arise [6, 29]. The contribution made by the membrane microenvironment to the pattern of the Scatchard plot will vary substantially from one ligand–receptor system to another, depending on the nature of both the ligand (e.g. its charge and amphiphilicity) and the membrane (e.g. its surface potential) and on the intrinsic affinity of the ligand–receptor interaction. This contribution appears unimportant for receptors of high intrinsic affinity ($K_a \approx 10^{10}$ l/mol), but may be very significant for low affinity ones.

Contamination of bound fraction by free radioligand

The inclusion of some free radioligand in the bound fraction (e.g. by trapping in a cell or membrane pellet) causes artifactual upward curvature of the Scatchard plot [3]. Such contamination of the bound fraction is usually regarded as part of the non-specific binding component. A reliable estimate of non-specific binding (see above) will thus effectively remove the influence of this artifact. Strictly, a slight error in the estimate of binding affinity arises with this approach [see Ref. 3], but the correction is qualitatively correct and the magnitude of the quantitative error is very small. An effective way to reduce the trapping of radioligand in the pellet is to centrifuge the cells or membranes through a layer of silicone oil [18, 31] or another inert material. Other useful techniques include dilution of the reaction mixture prior to separation and rinsing of the pellet

afterwards, although both these can result in significant dissociation of bound radioligand (particularly in the case of low affinity receptors).

Incomplete recovery of bound fraction

Inadequate separation of receptor-bound from free ligand during centrifugation or filtration causes downward curvature of the Scatchard plot [3]. Loss of bound ligand due to dissociation from the receptors during the separation step has the same result [3]. In each case, the effect is equivalent to that seen with an impure labeled ligand (see above); an appropriate measurement of receptor-reactivity should thus take these effects into account and allow for their correction.

Irreversible binding of ligand and internalization of ligand–receptor complex

A major difficulty with receptor-binding studies on intact cells is the tendency for the ligand to become irreversibly bound to the receptor and subsequently be internalized. Both these processes cause a distortion of the binding pattern, generally resulting in downward curvature of the Scatchard plot [4]. Such effects are minimized by assaying binding at a reduced temperature. This change, however, will also slow down (and may even prevent) intermolecular reactions that are an intrinsic part of receptor function.

Ligand degradation

Some receptor preparations contain enzymes that may degrade the ligand while a receptor-binding study is being performed. If the free ligand concentration is defined in the normal manner for receptor studies (as the difference between the total concentration added and the bound concentration), such degradation of the ligand causes an artifactual downward curvature of the Scatchard plot [4]. If the true concentration of intact free ligand at the end of the binding reaction is used instead to construct the Scatchard plot, the opposite pattern of curvature arises. For this, however, an assessment of the purity of the free ligand after incubation is necessary (not just a measurement of radioactivity in the free fraction).

Although many investigators have reported degradation of various ligands during receptor-binding studies, their evidence has usually been based on a reduction in the binding activity of the residual free ligand [32]. Such a reduction, however, is only definitive evidence of ligand degradation when the original ligand is known to be pure (100% receptor-reactive); otherwise, a reduction in binding activity is to be expected simply as a result of the selective removal of the receptor-reactive ligand [18].

Receptor degradation

Degradative enzymes can damage the receptor during incubation just as they can damage the ligand. This artifact again causes downward curvature of the Scatchard plot [4]. Degradation of both ligand and receptor can be reduced by assaying binding at a reduced temperature, by using the minimal incubation time that ensures equilibrium binding, or by the inclusion of proteolytic enzyme inhibitors in the reaction mixture.

Non-equilibrium binding conditions

Binding reactions are sometimes terminated prematurely in an attempt to minimize the extent of degradation of ligand and receptor. Measurement of ligand binding before equilibration, however, again results in a downwardly concave Scatchard plot [4]. In this context, because the binding reaction is slowest at the lowest ligand concentration, it is important to realize that the time for equilibration should be determined at the lowest ligand concentration to be used.

Conclusions

In view of all these artifactual sources of distortion of the pattern of a Scatchard plot, it is unrealistic to interpret the finding of an upwardly concave Scatchard plot as immediate, definitive evidence of functional receptor heterogeneity. It is surely necessary first to rule out at least the principal artifactual sources of upward curvature of the plot. This requires the use of a pure labeled ligand that is equipotent in receptor-binding activity with the unlabeled ligand (equal potency only being essential for "labeled + unlabeled" studies), a careful assessment of nonspecific binding, and an appropriate computerized analysis of the binding data. Very few receptor studies performed to date adequately meet these requirements, so conclusions about even the existence of receptor heterogeneity appear premature for most ligand–receptor systems. Neither should the potential influence of the membrane microenvironment of the receptor on its binding pattern be disregarded, although removal of the receptor from this microenvironment by solubilization may not be the appropriate way to eliminate this influence (as it could also disrupt receptor–receptor or receptor–effector interactions responsible for the functional heterogeneity). Nor should the potential artifactual sources of downward curvature of the Scatchard plot be ignored.

Only when very careful consideration is paid to all these potential artifacts can there be any real hope that receptor-binding studies will contribute to unraveling the likely nature of functional receptor heterogeneity (even if this does exist). Also likely to prove necessary are corroborative studies involving different approaches, such as reconstitution studies to demonstrate the disappearance of heterogeneity in the absence of a particular membrane component or genetic studies to prove the existence of more than one receptor subtype.

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