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Binding of Hormones to Receptors. An Alternative Explanation of Nonlinear Scatchard Plots†

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ABSTRACT: Binding of ^{125}I-labeled hormones to receptors can usually be inhibited by addition of unlabeled hormone. In analyzing such binding-inhibition data, it is commonly assumed that both labeled and unlabeled hormones are bound with equal affinity. When this assumption is made incorrectly, an artifactually nonlinear Scatchard plot results. Equations to describe these nonlinear Scatchard plots are derived. These results are discussed with regard to previously published observations of nonlinear Scatchard plots for binding of insulin to its receptor.

Interactions of polypeptide hormones with membrane receptors are the subject of intense research interest (Roth, 1973). One approach to the investigation of these interactions has been to study the binding of radioactively labeled hormone analogs to receptors. Such binding data are routinely displayed graphically by plotting the ratio of bound to free hormone as a function of the concentration of hormone bound—i.e., a Scatchard plot (Scatchard, 1949). In the case of certain hormones—insulin (Hammond et al., 1972; House, 1971; Freychet et al., 1972; Kahn et al., 1974) among others (Lefkowitz et al., 1970; Bockaert et al., 1972; Shlatz and Marinetti, 1972)—nonlinear Scatchard plots have been reported. Many hypothetical models have been proposed to account for these nonlinearities: cooperative binding interactions (House, 1971; De Meyts et al., 1973), polymerization of hormone (House, 1971; Nichol et al., 1969), heterogeneity of receptors (Hammond et al., 1972; Freychet et al., 1972; Kahn et al., 1974; Klotz and Hunston, 1971), or heterogeneity of labeled hormone. It is the purpose of this communication to propose another possible explanation of apparent nonlinearity in the Scatchard plot. In addition, the plausibility of this model will be evaluated insofar as it applies to the binding of one hormone, insulin, to its receptor.

In a typical binding study, a low concentration of ^{125}I-labeled hormone is allowed to bind to receptors. Varied concentrations of unlabeled hormone are added and inhibition of ^{125}I binding is observed. The data are analyzed with the aid of a Scatchard plot—assuming that iodinated and na-

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tive hormone are bound by the receptor with equal avidity (e.g., see Kahn et al., 1974). If the assumption of identical behavior of iodinated and uniodinated hormone molecules is correct, then the calculations are valid. Problems arise if this assumption is incorrect. I questioned this assumption and derived equations to describe the consequences of differential binding. An incorrect assumption of equal binding gives rise to *artifactually* nonlinear Scatchard plots. In the case where iodinated hormone is bound less tightly than native hormone, Scatchard plots are concave downward. Tighter binding of iodinated hormone than of native hormone leads to plots that are concave upward.

Results

The simplest model for binding of a hormone (H) to its receptor (R) is described by

$$\mathrm{RH} \rightleftarrows \mathrm{R} + \mathrm{H}$$
$$K = (\mathrm{R})(\mathrm{H})/(\mathrm{RH})$$

When the concentration of RH is plotted as a function of the concentration of H, an hyperbola is obtained. It is possible to transform variables such that a linear relation is obtained:

$$B = (\mathrm{RH}) = S - K(\mathrm{RH})/(\mathrm{H}) = S - K(B/F) \qquad (1)$$

where $B = (\mathrm{RH})$, $F = (\mathrm{H})$, and S = total concentration of all forms of the receptor. In the commonly employed Scatchard plot, B is plotted on the abscissa and B/F is plotted on the ordinate. Many investigators have employed radioactively labeled hormone derivatives—frequently, iodinated hormones—to serve as tracers which facilitate measurement of the concentrations of the hormone in the bound and free forms (reviewed by Roth, 1973). Assume for the purpose of this derivation that the labeled hormone (H*) binds to the same receptor as the unlabeled hormone, but with a dissociation constant of k. Further assume that binding of H and H* are mutually exclusive. Then

$$B = S/[1 + K(1 + f/k)/F] \qquad (2)$$
$$b = S/[1 + k(1 + F/K)/f] \qquad (3)$$

where b and f represent bound and free forms of the labeled hormone (H*). It is possible to calculate an expression for the total concentration of all forms of hormone bound to receptor (i.e., $b + B$) in terms of easily measurable quantities (i.e., b and f). First, it is useful to solve eq 3 for F:

$$F = K(Sf - bf - bk)/bk \qquad (4)$$

Then

$$(b + B) = b + S/[1 + K(1 + f/k)/F]$$
$$= b + S/[1 + bk(1 + f/k)/(Sf - bf - bk)]$$
$$= S - k(b/f)$$

or

$$(b/f) = S/k - (b + B)/k \qquad (5)$$

If one could plot b/f vs. $(b + B)$, it would be a simple matter to estimate the values of S and k from the slope and intercepts of the line. Unfortunately, the investigator may not have sufficient data to construct such a graph. The concentrations of bound (b) and free (f) ^{125}I-labeled hormone are directly measurable. However, it is not always possible to calculate $(b + B)$ knowing only b, $I_0 = B + F$ and $i_0 = b + f$. However, it is possible to calculate $(b + B)$ if H and H* are bound with equal affinity (i.e., $k = K$). According to this assumption, bound and free hormones have equal specific radioactivities. Therefore

$$(b + B) = b(1 + I_0/i_0)$$

One may define a variable β as follows

$$\beta = b(1 + I_0/i_0) \qquad (6)$$

Now, for the case where $k = K$, it turns out that $\beta = b + B$. Nevertheless, even for $k \neq K$, it is possible to calculate a value for β according to eq 6. However, if $k \neq K$, then $\beta \neq b + B$. The remainder of this section will be devoted to deriving equations to describe the shape of the curves of plots of b/f vs. β. Except in the case of $k = K$, the plot of b/f vs. β will be nonlinear. It is to be emphasized that this nonlinearity arises from an *incorrect assumption* in the processing of the experimental data.

In order to investigate the implications of assuming $k \neq K$, it is helpful to express β as a function of the variables b and f. To accomplish this, it is necessary to eliminate I_0 from eq 6.

$$\beta = b[1 + (F + B)/i_0]$$
$$= b\{1 + (F + S/[1 + K(1 + f/k)/F])/i_0\}$$

Substitution of eq 4 yields

$$\beta = [(Sf - bk)(fK + bk) + f^2b(k - K)]/fi_0k \qquad (7)$$

I shall derive approximations of eq 7 for three cases: $k = K$, $k \ll K$, and $k \gg$ K. In addition, I shall derive linear equations which approximate the Scatchard plots of eq 7 over two regions: first, in the presence of such an excess of unlabeled hormone that $b \ll f$; and second, in the region where $\beta \simeq 0$. In the latter region ($\beta \simeq 0$), in the interest of brevity, I shall derive the linear approximations only under the assumption that $i_0 \ll k$. In all of these derivations, it is assumed that the ^{125}I-labeled hormone concentration is constant and that the unlabeled hormone concentration (I_0) is varied in order to generate the data for a plot of b/f vs. β.

Case 1: $k = K$. Figure 1 shows a plot of eq 7 for $k = K = 10$ and $S = 10$. The plot is linear and independent of the value of i_0. Assuming $k = K$, one can derive eq 8 from eq 7:

$$\beta = (Sf - bK)(f + b)/fi_0 = S - K(b/f)$$
$$b/f = S/K - \beta/K \qquad (8)$$

Equation 8 is the equation of the familiar Scatchard plot (2) with association constant of $1/K$ and a maximal binding capacity of S. As stated above, for $k = K$ the assumption that $\beta = b + B$ is correct.

Case 2: $k \ll K$. Figure 2 shows a plot of eq 7 for $k = 10$, $K = 100$, and $S = 10$. The curve is concave upward and varies according to the value of i_0. The curvature is more marked for small values of i_0. Assuming $k \ll K$, eq 9 can be derived:

$$\beta \simeq [(Sf - bk)(fK + bk) - f^2bK]/fi_0k \qquad (9)$$

For $b \ll f$, $fK + bk \simeq fK$. Thus

$$\beta \simeq [(Sf)(fK) - (bk)(fK) - f^2bK]/fi_0k$$

Substitute $f = i_0 - b$ and assume higher order powers of b (i.e., b^2, b^3, etc.) are negligibly small:

$$\beta \simeq [SK(i_0 - b)^2 - bki_0K - i_0^2bK]/fi_0k$$
$$\simeq [SK(i_0^2 - 2bi_0) - bkKi_0 - i_0^2bK]/fi_0k$$
$$= KS/k - (b/f)(K/k)(2S + k + i_0)$$
$$b/f \simeq S/(2S + k + i_0) - \beta k/K(2S + k + i_0) \qquad (10)$$

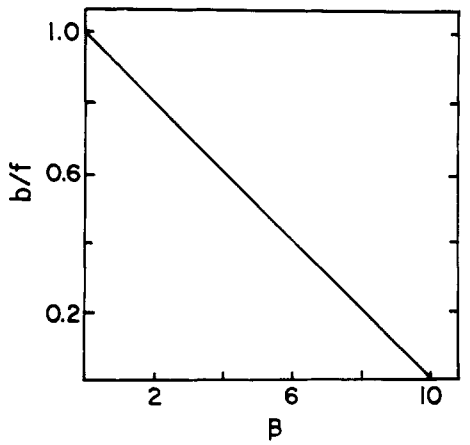

FIGURE 1: Linear Scatchard plot for case 1. This is a Scatchard plot of eq 7 for $k = K = 10$, $S = 10$, and i_0 may have any value.

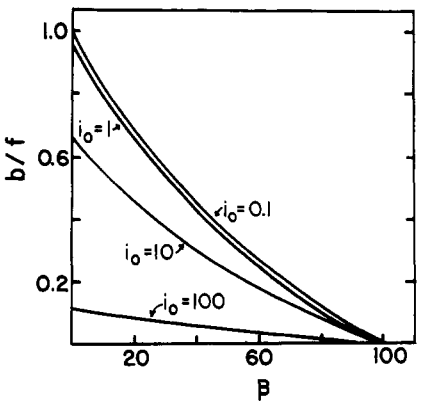

FIGURE 2: Upward concave Scatchard plot for case 2. This is a Scatchard plot of eq 7 for $k = 10$, $K = 100$, $S = 10$, and $i_0 = 0.1$, 1, 10, or 100.

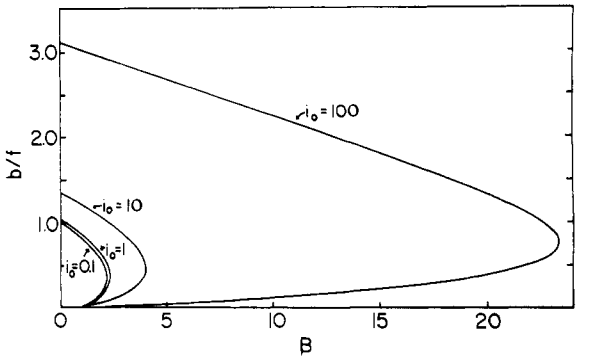

FIGURE 3: Downward concave Scatchard plot for case 3. This is a Scatchard plot of eq 7 for $k = 10$, $K = 1$, $S = 10$, and $i_0 = 0.1$, 1, 10, or 100.

For the part of the Scatchard plot corresponding to $b \ll f$, one would estimate an association constant of $k/K(2S + k + i_0)$ and a binding capacity of KS/k.

For $\beta = 0$, one can solve eq 9 to obtain the value of b/f which corresponds to the intercept on the ordinate. First, eq 9 is approximated by

$$\beta \simeq K(Sf - bk - fb)/i_0k = 0 \quad (11)$$

Substituting $f = i_0 - b$, one obtains

$$b^2 - b(S + k + i_0) + i_0S = 0 \quad (12)$$

$$b = \{S + k + i_0 - [(S + k + i_0)^2 - 4i_0S]^{1/2}\}/2 \quad (13)$$

For sufficiently small values of i_0, one can assume $i_0S \ll (S + k + i_0)^2$. Therefore, one can use the first two terms of the Taylor series expansion to approximate $(x + \delta)^{1/2} \simeq x^{1/2} + \delta/2x^{1/2}$ (where $\delta \ll x$) and thereby to derive eq 14 from eq 13:

$$b \simeq Si_0/(S + k + i_0) \simeq Si_0/(S + k) \quad (14)$$

Equation 11 allows one to solve for the ratio of b/f in terms of b:

$$Sf - bk - fb = 0$$
$$b/f = (S - b)/k \quad (15)$$

Solving eq 14 and 15 simultaneously, one obtains

$$b/f \simeq S[1 - i_0/(S + k + i_0)]/k$$
$$\simeq S(S + k)/k(S + k + i_0) \simeq S/k$$

Thus for $\beta \simeq 0$ and $i_0 \ll S + k$, $b/f = S/k$. The slope of the region of the curve near $\beta = 0$ can be obtained by evaluating $d(b/f)/d\beta$ at $\beta = 0$. Differentiating eq 11, one obtains

$$d\beta/df \simeq (K/i_0k)(S + k - b + f)$$
$$= (K/i_0k)(S + k + i_0 - 2b)$$
$$\simeq (K/i_0k)(S + k)$$
$$d(b/f)/df = -i_0/f^2$$
$$d\beta/d(b/f) \simeq -[k/(S + k)]^2(K/k)(S + k) \text{ for } \beta = 0$$
$$d\beta/d(b/f) \simeq -Kk/(S + k) \text{ for } \beta = 0$$

Thus in the region of $\beta = 0$, the Scatchard plot is approximated by

$$b/f = S/k - \beta(S + k)/Kk \quad (16)$$

In the neighborhood of $\beta = 0$, the apparent association constant is $(S + k)/Kk$ and the apparent maximal binding capacity is $KS/(S + k)$.

Case 3: $k \gg K$. Figure 3 shows a plot of eq 7 for $k = 10$, $K = 1$, and $S = 10$. The curve is concave downward and attains a maximal value of β at an intermediate value of b/f in the range between zero and one. Assuming $k \gg K$, one can derive eq 17 from eq 7:

$$\beta \simeq [(Sf - bk)(fK/k + b) + f^2b]/fi_0 \quad (17)$$

For $b \ll f$, further approximations are possible:

$$\beta \simeq [(Sf)(fK) + (Sf)(bk) - (bk)(fK) + f^2bk]/fi_0k$$
$$\simeq [SK(i_0 - b)^2 + Si_0bk - bi_0kK + i_0^2bk]/fi_0k$$
$$\simeq (SKi_0^2 - 2SKi_0b + Si_0bk - bi_0kK + i_0^2bk)/fi_0k$$
$$\simeq KS/k + (b/f)[S + i_0 - K(1 + 2S/k)]$$

$$b/f \simeq KS/k[S + i_0 - K(1 + 2S/k] + \beta/[S + i_0 - K(1 + 2S/k)] \quad (18)$$

For the part of the Scatchard plot corresponding to $b \ll f$, one would estimate an association constant of $-1/[S + i_0 - K(1 + 2S/k)]$ and a binding capacity of KS/k. Note that the apparent association constant is positive only for $i_0 < K - S + 2SK/k$. Figure 4 shows graphs of eq 7 for $k = 10$, $K = 1$, $S = 1$, and $i_0 = 0.04$, 0.2, and 1. In the region of $b \ll f$, the apparent dissociation constant is positive for $i_0 < 0.2$ and negative for $i_0 > 0.2$. For $i_0 = 0.2$, the plot is vertical (i.e., the slope is infinite) in the regions of $b \ll f$.

One can solve eq 19 to obtain the value of b/f at $\beta = 0$:

$$\beta \simeq [(Sf - bk)(b)(fK/bk + 1) + f^2b]/fi_0 = 0 \quad (19)$$

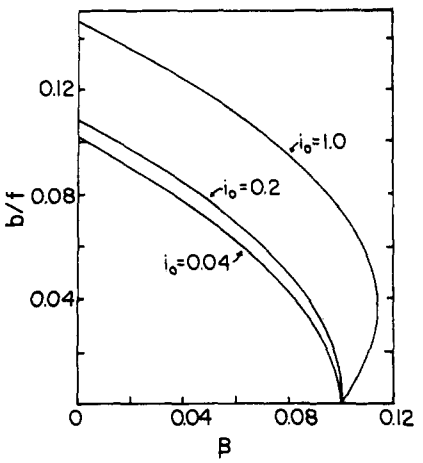

FIGURE 4: Effects of i_0 on the sign of the slope of the Scatchard plot for case 3. This is a Scatchard plot of eq 7 for $k = 10$, $K = 1$, $S = 1$, and $i_0 = 0.04$, 0.2, or 1.

If $K/k \ll b/f$, then eq 19 reduces to

$$\beta \simeq (b/f)(Sf - bk + f^2)/i_0 = 0 \quad (20)$$

For $b/f \neq 0$, $Sf - bk + f^2 = 0$, Therefore

$$Sf - (i_0 - f)k + f^2 = 0$$
$$f^2 + f(S + k) - ki_0 = 0$$
$$f = \{-(S + k) + [(S + k)^2 + 4ki_0]^{1/2}\}/2 \quad (21)$$

For $ki_0 \ll (S + k)^2/4$, one can derive eq 22 in a manner analogous to the derivation of eq 14:

$$f \simeq i_0k/(S + k) \quad (22)$$

$$b/f \simeq (i_0 - f)/f \simeq$$
$$i_0[1 - k/(S + k)]/[i_0k/(S + k)]$$
$$b/f \simeq S/k \quad (23)$$

The slope of the Scatchard plot may be derived from eq 17 in the region of $\beta = 0$ by evaluating the derivative $d\beta/d(b/f)$. Differentiation of eq 20 yields

$$d\beta/df \simeq \{-S + b^2k/f^2 + 2bk/f + b - f\}/i_0$$
$$= \{k(b/f)^2 + 2k(b/f) + (i_0 - S) - 2f\}/i_0$$
$$d\beta/d(b/f) = -(f/i_0)^2\{k(b/f)^2 + 2k(b/f) + (i_0 - S) - 2f\} \quad (24)$$

Assuming $b/f = S/k$ and $f = i_0k/(S + k)$ as demonstrated above, one can evaluate $d\beta/d(b/f)$ at $\beta = 0$: $d\beta/d(b/f) = -kS/(S + k)$ for $\beta = 0$ and $i_0 \ll k_0$.

Therefore, the linear approximation to the Scatchard plot in the region of $\beta = 0$ is given by

$$b/f = S/k - \beta(S + k)/kS \quad (25)$$

Equation 25 implies an apparent association constant of $(S + k)/kS$ and an apparent maximal binding capacity of $S^2/(S + k)$.

Discussion

Table I presents a summary of the principal results derived above. It should be emphasized that hyperbolic binding kinetics were assumed in these derivations. Therefore, according to this model, the Scatchard plot for the binding of hormone to receptor should be linear. Nevertheless, the use of two different hormone species—e.g., iodinated plus native hormone—without allowing for differential binding introduced artifactual nonlinearity into the Scatchard plot. Moreover, for $k \neq K$, the slopes and intercepts of linear approximations to the curves are related only indirectly to the physical parameters (i.e., binding affinity and capacity) of the receptor (Table I). Of course, for the case where $k = K$, the Scatchard plot remains linear because both hormonal species behave identically. Furthermore, only when $k = K$ is the Scatchard plot independent of the total concentration of tracer (i_0) which is employed (Figures 1–3). This suggests an approach to test the hypothesis that $k = K$. If two different concentrations of i_0 (e.g., $i_0 = k/10$ and $i_0 = 10k$) are employed in binding displacement studies, observation of different Scatchard plots for the two values of i_0 would indicate $k \neq K$. In some favorable circumstances, the data obtained from these studies might be displayed in a Dixon plot (Dixon, 1953). Here, the reciprocal ($1/b$) of the bound labeled hormone concentration is plotted vs. the free concentration (F) of unlabeled hormone. The Dixon plots for various values of i_0 would be expected to intersect at $F = -K$. This approach does not assume the equality of $k = K$. However, the difficulty in employing this plot resides in determining F. However, for $I_0 \gg S$, then $F \simeq I_0$.

The motivation for these derivations has been to provide a mathematical analysis of the type of binding displacement experiments which are common in the study of binding of hormones to receptors (Hammond et al., 1972; Freychet et al., 1972; Kahn et al., 1974; Shlatz and Marinetti, 1972; Cuatrecasas, 1971a,b; Cuatrecasas et al., 1971; Kono and Barham, 1971; Crofford, 1968; Gammeltoft and Gliemann, 1973). Such derivations seem not to have been reported. However, similar problems have been investigated by others. Vassent (1974) has studied "tracer behavior . . . under the assumption that it has exactly the same binding properties as the corresponding unlabeled part of the ligand." Feldman (1972) has treated the kinetics of *unlabeled* hormone binding in the presence of a fixed amount of labeled hormone whereas I discussed the kinetics of binding of *labeled* hormone. Since it is the labeled hormone which is

Table I: Estimation of Kinetic Parameters from Scatchard Plot.[a]

Case	Parameter	Site I[b]	Site II	Ratio (I:II)
1 ($k = K$)	Affinity	$1/K$	$1/K$	1
	Capacity	S	S	1
2 ($k << K$)	Affinity	$(S + k)/Kk$	$k/K(2S + k + i_0)$	$(S + k)(2S + k + i_0)/k^2$
	Capacity	$KS/(S + k)$	KS/k	$k/(S + k)$
3 ($k >> K$)	Affinity	$(S + k)/kS$	$1/[K(1 + 2S/k) - S - i_0]$	$[(S + k)[K(1 + 2S/k) - S - i_0]]/kS$
	Capacity	$S^2/(S + k)$	KS/k	$kS/K(S + k)$

[a] Values for the apparent binding affinities and binding capacities that might be estimated from Scatchard plots similar to those in Figures 1–4 are summarized below. Site I corresponds to the neighborhood of $\beta = 0$; site II corresponds to the region where $b << f$. In addition, the ratio of the values for each parameter (i.e., affinity constant and binding capacity) for site I to the values for site II are presented. [b] In the case of site I, the derivations of the affinity constants and binding capacities were valid only for small values of i_0 (i.e., $i_0 << k$).

measured directly, the derivations in this paper are the ones with relevance to the commonly employed methodology of binding displacement studies. Furthermore, the upward concave Scatchard plot derived by Feldman (see Figure 2 of Feldman, 1972) and cited by Rodbard (1973) does not resemble those observed in actual studies with receptors; according to Feldman (1972), the hyperbolic Scatchard plot asymptotically approaches a horizontal line with a negative intercept on the ordinate. In contrast, Figure 2 of this paper more closely resembles those determined empirically (Hammond et al., 1972; Freychet et al., 1972; Kahn et al., 1974; Shlatz and Marinetti, 1972).

It is outside the scope of this paper to discuss fully the question of whether this model adequately explains the upward concavity of Scatchard plots of actual data reported from other laboratories. Nevertheless, I will mention briefly some of the results which support the applicability of the model to the interaction of at least one hormone, insulin, and its receptor. Cuatrecasas (1971a,b) and Cuatrecasas et al. (1971) obtained data which suggest that the insulin receptor binds iodoinsulin 10- to 100-fold more tightly than it binds insulin. They reported a dissociation constant of $k \simeq 10$ μU/ml for the binding of [^{125}I]iodoinsulin by the insulin receptor of liver and adipocytes. In contrast, concentrations of unlabeled insulin as large as 150–1000 μU/ml were required to displace 50% of the binding of [^{125}I]iodoinsulin when the total concentration of [^{125}I]iodoinsulin was 2.5–3.5 μU/ml. Lambert et al. (1972) have reported data with the insulin receptor which may be interpreted similarly. Of course, [^{125}I]iodoinsulin prepared in different laboratories may have different properties.

Those investigators who have studied the displacement of [^{125}I]iodoinsulin by unlabeled insulin have in general observed Scatchard plots which were concave upward (Freychet et al., 1972; Kahn et al., 1974). Some investigators have employed varying concentrations of labeled hormone of constant specific activity. For such studies, under the proper assumptions,[1] it can be shown that a linear Scatchard plot is predicted even if $k \ll K$. This difference in experimental methods may explain the apparent linearity of the Scatchard plots for [^{125}I]iodoinsulin binding reported by Cuatrecasas (1971a,b), Cuatrecasas et al. (1971), Kono and Barham (1971), and Gammeltoft and Gliemann (1973). Similarly, Crofford (1968) reported a linear Scatchard plot in his studies which employed methods not involving binding of labeled insulin to receptor.

I have considered briefly the binding of insulin to its receptor merely as one *possible* example of the applicability of the theoretical model developed in this paper. There are not yet sufficient data to establish with certainty whether insulin and iodoinsulin are bound by receptor with equal affinities. However, I do not intend the discussion of one particular example to divert attention from the possibility that the model may have wider applicability to systems involving other hormones and ligands. I hope that by exposing one potential artifactual cause of a nonlinear Scatchard plot, I may aid others in correct interpretation of binding data.

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[1] If $b = S/[1 + k(1 + F/K)/f]$, then $b = S'/[1 + k'/f]$ where $S' = S/\alpha$, $k' = k/\alpha$, and $\alpha = 1 + (k/K)(F/f)$. But for $k/K \ll f/F$, $\alpha \simeq 1$. Therefore, $b \simeq S/[1 + k/f]$. In the studies cited in the text, the condition $k/K \ll f/F$ was probably satisfied.