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COMMENTARY

THE CHOICE OF ERRONEOUS MODELS OF HORMONE–RECEPTOR INTERACTIONS: A CONSEQUENCE OF ILLEGITIMATE UTILIZATION OF SCATCHARD GRAPHS

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Scatchard graphs are widely used for analysing the binding of hormones to receptors. This type of analysis is valid for simple reversible equilibria between hormone–receptor complexes and free hormone and receptor. When all receptor sites have the same affinity for the hormone, the equation is that of a line, and from the slope and intercepts the total number of sites and the binding constant can be calculated. However, as recently discussed by Klotz [1], widely erroneous values can be deduced if a complete binding isotherm is not obtained. A survey of the recent literature by Nørby *et al.* [2] also reveals common misinterpretations of these plots when the data yield upward curvatures. In this paper, we wish to discuss other possible incorrect uses and faulty interpretations of linearized equations: the utilization of non-equilibrium experimental data. Indeed, our computer simulations show that the sole utilization of the Scatchard graphs yields no indication of the possible involvement of an irreversible step in the interaction mechanism, and can thus lead to the choice of an erroneous reversible model unless careful studies of the association and dissociation time courses are also performed.

The reaction between hormone (H) and receptor (R) can be a rather slow process. Moreover, the first reversible complex (HR) can irreversibly transform [3] into a second stable complex (Z): model 1.

$$H + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} HR \xrightarrow{k_{+2}} Z$$

Model 1.

If the concentrations of 'bound hormone' [B], i.e. [HR] + [Z], are determined before the reaction is completed, and the data are mistakenly used to build a Scatchard plot (i.e. [B]/[H] vs [B]), curves are obtained which, within the limits of experimental error, can be very easily confused with straight lines (Fig. 1a). Note that the absolute value of the slope (i.e. the 'association constant') increases with increasing values of k_{+2}, although the true association constant, i.e. k_{+1}/k_{-1}, is not modified. For any given value of k_{+2}, this absolute value also increases with increasing time (Fig. 1b). If the various initial hormone concentrations $[H_0]$ are all larger than the total receptor concentration $[R_0]$, the reaction is completed when $[Z] = R_0$ and, under these conditions, the line is vertical and the 'association constant' infinite.

This type of phenomenon can be easily detected by measuring the binding of H as a function of time. Upon completion of the reaction, either the free receptor or the free hormone has totally disappeared. A time course of the dissociation reaction supplies another significant indication: complete release of the bound hormone does not occur.

The situation can be much more complicated by possible spontaneous degradations of H and/or R during the incubation (model 2). This situation is often encountered when peptide hormones are incubated in the presence of total homogenates: hormones and receptors are non-specifically degraded by proteolytic enzymes [5, 6].

$$\begin{array}{ccccc} H & + & R & \rightleftharpoons & HR \rightarrow Z \\ {\scriptstyle k_{-3}}\downarrow & & \downarrow{\scriptstyle k_{+4}} & & \\ H' & & R' & & \end{array}$$

Model 2.

Contrary to what happens when the interaction obeys model 1, model 2 implies that for large values of time the reaction mixture contains H', R' and Z. This pseudo-equilibrium can be easily mistaken for a true equilibrium even if the experimenter carefully studies the time course of the forward reaction. Figure 2 displays computer-simulated time courses for reactions in which the free hormone (Fig. 2a) or the free receptor (Fig. 2b) is degraded during the incubation. Figure 3 shows the Scatchard graphs which are obtained with various hormone concentrations if the reaction is stopped after 1500 min. In all

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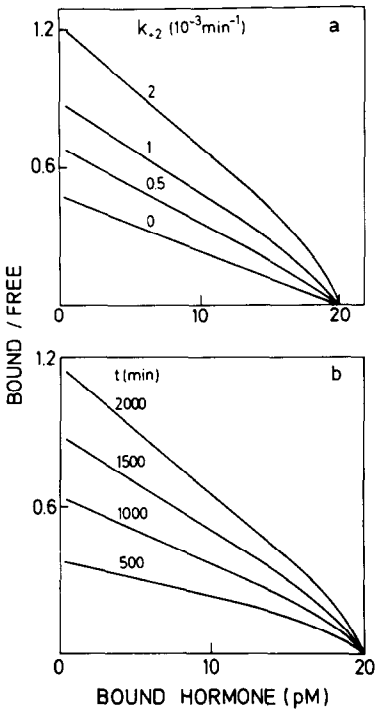

Fig. 1. Computer simulations of model 1 presented in the Scatchard coordinates. Model 1 is defined by the following set of differential equations $d(B)/dt = d(HR)/dt + d(Z)/dt$; $d(HR)/dt = k_{-1}(H)_t(R)_t - (k_{+2} + k_{-1})(HR)_t$; $d(Z)/dt = k_{+2}(HR)_t$. At time 0, B, HR and Z are equal to 0; B represents the bound hormone (B = [HR] + [Z]), which can be measured experimentally. $(H)_t$ and $(R)_t$ are defined as $(H_0 - (B)_t)$ and $(R_0 - (B)_t)$, respectively, where H_0 and R_0 represent total hormone and receptor concentrations. Saturation curves are simulated by numerical integration of the model, between time 0 and t, for increasing values of H_0. These curves are then represented in the Scatchard coordinates. Free hormone concentrations are calculated by the difference between total and bound hormone. Calculations and graphical representations are obtained by using the MLAB programme [4]. Conditions are as follows: H_0 is varied from 0 to 2000 pM; $R_0 = 20$ pM; $k_{+1} = 5 \times 10^{-5}$ pM^{-1} min^{-1}; $k_{-1} = 2 \times 10^{-3}$ min^{-1}. (a) Effect of increasing values of k_{+2}: $t = 1500$ min; $k_{+2} = 0$–2×10^{-3} min^{-1}. (b) Effect of increasing time: $k_{+2} = 1 \times 10^{-3}$ min^{-1}; $t = 500$–2000 min.

cases, graphs deviate from linearity only for low values of [B]/[H] (bound H/free H), i.e. for large hormone concentrations. It is very likely that in a real experimental situation these deviations will remain undetected. It is especially interesting to note that degradation of the free receptor apparently 'compensates' for the formation of the irreversible complex Z and that the curve obtained for the largest tested value of k_{+4} does not significantly deviate from a straight line.

Figure 4 displays a Scatchard graph obtained after a 1440 min incubation of ^{125}I-labelled human chorionic gonadotropin (hCG) with a homogenate of adult rat testis. The data show no significant deviation from linearity so that this hormone–receptor interaction might, at first sight, be considered as a simple reversible bimolecular reaction. The apparent association constant ($K_a = k_{+1}/k_{-1}$) calculated for a simple reversible equilibrium is 5.1×10^{10} M^{-1}. However, this model must be rejected on the basis of the dissociation time-course experiments (Fig. 5; [6]). Indeed, these experiments show that the interaction is partially irreversible. Further analysis demonstrates that both the association and dissociation kinetics are compatible with model 2 (Fig. 5). The values of the various kinetic constants derived from the experimental data by non-linear curve-fitting analysis are given in the legend of Fig. 5. These and slightly modified values have been used to draw Scatchard plots. The solid line in Fig. 4 best agrees with the experimental data. It is thus possible, in the present case, to obtain a good agreement between the two sets of independently obtained experimental data when model 2 is used.

It is interesting to note that the value of the apparent association constant computed from the data of

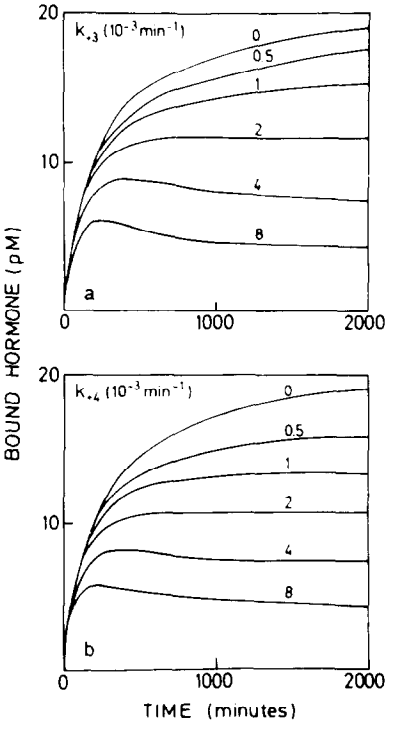

Fig. 2. Computer simulations of model 2. Association time courses. Model 2 is defined as model 1 (see legend of Fig. 1) with the exception that hormone and receptor degradation is assumed as previously described [6]. $d(H)/dt = -d(B)/dt - d(H')/dt$; $d(H')/dt = k_{+3}(H)_t$; $d(R)/dt = -d(B)/dt - d(R')/dt$; $d(R')/dt = k_{+4}(R)_t$. H′ and R′ are degraded hormone and receptor concentrations. At time 0, H′ and R′ = 0. These equations are integrated over increasing periods of time in order to simulate association curves. Conditions are as follows: $H_0 = 100$ pM; $R_0 = 20$ pM; $k_{+1} = 5 \times 10^{-5}$ pM^{-1} min^{-1}; $k_{-1} = 2 \times 10^{-3}$ min^{-1}; $k_{+2} = 2 \times 10^{-3}$ min^{-1}. (a) Degradation of the free hormone: $k_{+4} = 0$; k_{+3} varies from 0 to 8×10^{-3} min^{-1}. (b) Degradation of the free receptor: $k_{+3} = 0$; k_{+4} varies from 0 to 8×10^{-3} min^{-1}.

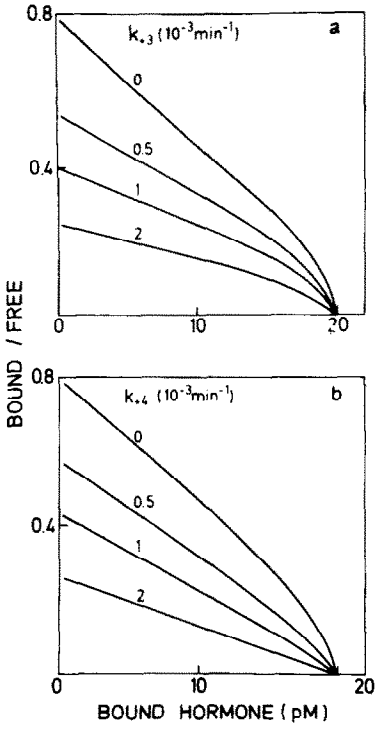

Fig. 3. Computer simulations of model 2 presented in the Scatchard coordinates. Scatchard plots are obtained by simulation as described in the legends of Figs. 1 and 2. H_0 varies from 0 to 2000 pM. Values of R_0, k_{+1}, k_{-1} are as in Fig. 2. $k_{+2} = 1 \times 10^{-3}$ min^{-1}. $t = 1500$ min. (a) Degradation of the free hormone: $k_{+4} = 0$; k_{+3} varies from 0 to 2×10^{-3} min^{-1}. (b) Degradation of the receptor: $k_{+3} = 0$, k_{+4} varies from 0 to 2×10^{-3} min^{-1}.

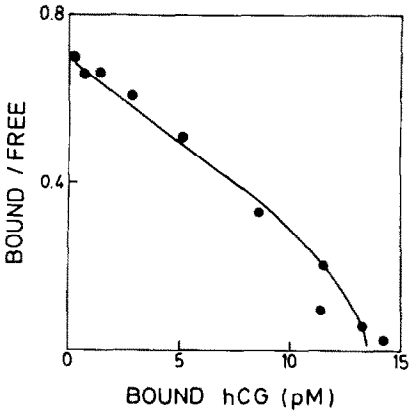

Fig. 4. Scatchard graphs obtained from actual experimental data for the binding of [^{125}I]hCG to a homogenate of rat testicular tissue. Increasing concentrations of ^{125}I-labelled human chorionic gonadotropin ([^{125}I]hCG) were incubated for 24 hr at 24° with rat testis homogenate (1500 $g \times 15$ min fraction), with slight modifications to the previously described method [6]. The incubation volume was 0.25 ml, containing 0.1 ml of tissue fraction. Bound and free hormones were separated by centrifugation. Hormone concentrations were calculated as described [6]. Each point is the mean of duplicate determinations. The solid line represents the Scatchard plot computed on the basis of model 2, in which the following values were used: $k_{+1} = 6.0 \times 10^7$ M^{-1} min^{-1}; $k_{-1} = 0.6 \times 10^{-3}$ min^{-1}; $k_{+2} = 3.5 \times 10^{-3}$ min^{-1}; $k_{+3} = 0.6 \times 10^{-3}$ min^{-1}; $k_{+4} = 0.25 \times 10^{-3}$ min^{-1}. $R_0 = 13.5$ pM. $t = 1440$ min.

Fig. 4 (5.1×10^{10} M^{-1}) is not very different from the k_{+1}/k_{-1} ratio derived from the kinetic experiments (6.8×10^{10} M^{-1}), or from the simulation (10×10^{10} M^{-1}). Thus, in the present case, the degradation of H and R roughly compensates for the accumulation of Z. As a consequence, an analysis of the data on the basis of a simple equilibrium does not necessarily yield an erroneous value for the association constant, but allows the choice of a wrong model for the interaction, which represents, in fact,

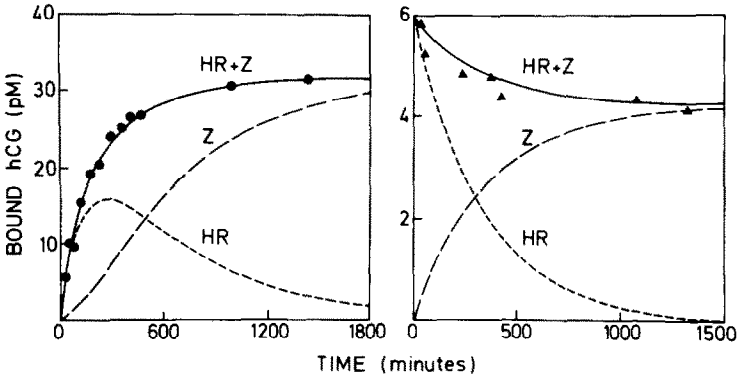

Fig. 5. Experimental data and non-linear curve-fitting analysis for the time course binding of [^{125}I]hCG to a rat testicular homogenate and of dissociation of the hormone–receptor complex. The association time course (left panel) was determined by incubating ^{125}I-labelled human chorionic gonadotropin ([^{125}I]hCG; $H_0 = 87$ pM) with rat testis homogenate ($R_0 = 42$ pM). Experimental conditions were as described in the legend of Fig. 4. The dissociation time course (right panel) was followed after addition of an excess of unlabelled hCG (Pregnyl®; 100 IU) to a mixture of [^{125}I]hCG and testis homogenate, previously incubated together for 30 min. Both sets of data were analysed simultaneously with the non-linear curve-fitting programme [4], using model 2. The lines represent the best fit of both sets of data (——, B; - - -, HR; — —, Z), obtained for the following values of the constants: $k_{+1} = 5.4 \pm 0.2 \times 10^{-5}$ pM^{-1} min^{-1}; $k_{-1} = 0.8 \pm 0.2 \times 10^{-3}$ min^{-1}; $k_{-2} = 2.1 \pm 0.7 \times 10^{-3}$ min^{-1}. The values of k_{+3} (0.8×10^{-3} min^{-1}) and k_{+4} (0.3×10^{-3} min^{-1}) had been determined previously [6] and were kept constant during the curve-fitting process.

a more distressing error. It is evident that data which fit a linear Scatchard plot also fit linear logit or Hill plots with a slope (Hill coefficient) of 1. Moreover, these double logarithmic plots are noteworthy for hiding deviations from linearity.

Similar results have been recently obtained in computer simulations of non-steady-state situations during enzyme-catalysed reactions [7]. When ternary mixtures of enzyme, substrate and irreversible inactivator are incubated, the progressive accumulation of inactivated enzyme induces a corresponding decrease of the reaction rate. This phenomenon can be easily detected by monitoring the appearance of product P as a function of time. But especially when the estimation of P is difficult and time-consuming, one is tempted to perform this estimation after a single incubation time when the amount of substrate transformed is low. If, under these conditions, Lineweaver–Burk ($1/v$ vs $1/[S]$), Hanes ($[S]/v$ vs $[S]$) or Eadie–Hofstee (v vs $v/[S]$) plots are illegitimately constructed using the wrong assumption that $[P]/t = v$, nearly linear graphs are obtained, although the various equations are those of curves [7, 8]. Computer simulations indicate that these curves can, within the limits of experimental errors, very easily be confused with lines [7]. The inactivator is then mistaken for a reversible inhibitor, and the value of the 'dissociation constant' of the enzyme–inhibitor complex which can be computed from these data understandably decreases with increasing incubation times at constant inactivator concentration. Again, the 'linearized' graphs by themselves yield no indication that a wrong model has been used to analyse the data. This mistake has been committed in the early studies of the inhibition of penicillin-sensitive enzymes by β-lactam antibiotics [9–12].

In conclusion, it is thus important to stress the fact that the obtention of linear Scatchard, Lineweaver–Burk, Hanes or Eadie–Hofstee graphs does not by itself demonstrate that the conditions necessary to allow the construction of these graphs have been fulfilled. A careful study of the time course of the reaction remains the best method to avoid choosing the wrong model.

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