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Irreversibility of the interaction of human growth hormone with its receptor and analysis of irreversible reactions in radioreceptor assays—Theoretical considerations

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Abstract. Kinetic studies of the binding and dissociation of [^{125}I]-human growth hormone to rabbit liver and mammary gland membrane receptors have showed that the binding of [^{125}I]-human growth hormone was largely irreversible to liver membrane receptors and completely to the solubilised mammary gland receptor. As Scatchard analysis assumes complete reversibility of the hormone-receptor interaction the validity of estimates of affinity and capacity of receptors derived by this analysis may be questionable.

Theoretical considerations show that in unimolecular irreversible interactions of hormone and receptor, a nonlinear (concave) or a linear Scatchard plot can be obtained. In linear Scatchard plots the capacity of the receptor obtained by extrapolation represents an overestimation of true capacity. This overestimation correlates with the value of the intercept in the Scatchard plot.

Keywords. Receptors; Scatchard plot.

Introduction

A widely accepted premise in endocrinology is that the interaction of hormone and receptor is completely reversible (Jacobs *et al.,* 1977; Roth, 1973). Based on this assumption Scatchard analysis of the binding data is widely used to determine the binding constant and capacity of the receptor preparation. However in several experiments the reaction in fact appeared to be at least partially irreversible (Van der Gugten, *et al.,* 1980; Powell and Hollander, 1978; Herington *et al.,* 1983; Moore *et al.,* 1983). In one such experiment excess human growth hormone (hGH), as well other hormones which have growth hormone like activity failed to displace all the bound [^{125}I]-hGH from the [^{125}I]-hGH-receptor complex. These observations prompted a systematic examination of the reversibility of interaction between hGH and its receptor as well as a detailed kinetic consideration of the validity of using Scatchard analysis to characterize the interaction.

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Abb reviations used: hGH, Human growth hormone; HR, hormone-receptor; PEG, polyethyleneglycol; IgG, immunoglobulin; H-R complex, hormone-receptor complex.

Materials and methods

Pregnant rabbit liver and rabbit mammary gland membrane receptors were prepared as described previously (Posner *et al.,* 1974). Rabbit mammary gland receptors were solubilized by extracting with 0·2% deoxycholate, the 100000 *g* pellet of a rabbit mammary gland homogenate. Iodination of hGH was carried out by established procedures (Thorell and Johansson, 1971). The specific activity of the [^{125}I]-hGH prepared was 80–120 μCi/μg of protein. Association and dissociation studies were carried out in Tris-HCl buffer, pH 7·6 containing 0·05M magnesium chloride and 0·1 % bovine serum albumin.

Dissociation of hormone-receptor complex

The hormone-receptor (H-R) complex was obtained by incubating the rabbit liver membrane receptor and [^{125}I]-hGH in the cold overnight (150 μg membrane protein/ml and 200000 cpm/ml in the incubation solution). After 20 h of incubation the suspension was centrifuged at 3000 *g* for 20 min, the supernatant discarded and the precipitate was quickly washed once with cold buffer. Under these conditions of incubation the nonspecific binding corresponded to less than 15 % of the total counts bound. This pellet was used as a source of hormone receptor complex for the study of dissociation.

To study the rate of dissociation, the H-R complex was uniformly dispersed in 3·5 ml of buffer containing 2 μg/ml hGH. At various time intervals 0·2 ml aliquots were removed, centrifuged and the supernatant counted for radioactivity. The radioactivity released into the supernatant represented the extent of dissociation.

Association of hormone and receptor

Rabbit liver membrane receptor (20 ml, 400 μg protein/ml) and [^{125}I] -hGH (5 ml, 2 $\times$ 10^6 cpm/ml) were mixed and the association was allowed to proceed at 4°C while gently stirring the mixture. Aliquots of 0·5 ml were withdrawn from this mixture at different time intervals, immediately centrifuged for 20 min and the supernatant discarded. To the precipitate 0·5 ml Tris HCl buffer pH 7·6 containing 2 μg of hGH was added and left at room temperature for 20 h. The suspension was centrifuged and the supernatant and the precipitate were counted separately. The radioactivity in the supernatant corresponds to the [^{125}I]-hGH that was released from the complex, and hence reversibly bound. The counts in the precipitate corresponded to the [^{125}I]-hGH irreversibly bound to the receptor. The data were then analysed for the binding characteristics by conventional kinetic analysis.

Kinetic analysis of association and dissociation data

In all the above experiments of association the conditions used are such that the concentration of the receptor was at least 10 fold the concentration of [^{125}I]-hGH used. Hence the reaction between hormone and receptor would be expected to follow pseudo first-order kinetics. All the data were analysed for first order reaction by Standard procedures.

Theoretical approach to analysis of non-reversible systems for Scatchard plot

Rationale: In our studies it was found that the Scatchard analysis of the binding of hGH to rabbit mammary gland receptor was linear despite the fact that the reaction was demonstrably irreversible (Van der Gugten *et al.,* 1980; Posner *et al.,* 1974; Shiu and Friesen, 1974). Thus it was deemed important to determine whether on theoretical grounds an irreversible reaction would lead to a linear Scatchard plot and if so how to interpret the data in such systems.

Theoretical considerations

If the reaction between hormone and receptor is assumed to be an irreversible one, the reaction would follow the kinetics of a second order for unimolecular reactions between hormone and receptor. Thus the extent of the conversion of the reactants to the product namely HR at any given time would be given by the following equation.

$$kt = \frac{1}{(a-b)} ln \frac{b(a-x)}{a(b-x)}.$$

a = Initial concentration of receptor; b = Initial concentration of hormone; k = Second order rate constant; t = time of the reaction; x = Concentration of HR complex at time t. The above equation can be transformed into the following form:

$$x = \frac{ab\, e^{kt(a-b)} - 1}{e^{kt(a-b)} a - b}. \tag{1}$$

The variables which determine the extent of conversion of the reactants, hormone and receptor to product HR would be *a, b, k* and *t.* Knowing the value for these variables, concentration of the complex formed can be obtained by substitution in the above equations.

Generating a theoretical Scatchard plot from radioreceptor assays

In a radioreceptor assay among the four variables (namely *a,b,k* and *t*) which determine the extent of binding, three variables that are always kept constant are *a, k* and *t.* The only variable that is altered in an assay is the concentration of the hormone itself, *b.* Thus if *a*, *k* and *t* are given arbitrary values (values comparable to those seen in receptor assays) concentration of the complex (x) formed at varying concentrations of the hormone can be obtained from eq. (1). This would correspond to the concentration of the hormone bound, *B.* Free hormone concentration (F) is then obtained by subtracting B from the initial concentration of the hormone, *b.* A theoretical Scatchard plot can then be drawn for binding data using the values of B and F obtained as outlined above.

Results

Figure 1 presents the data on the dissociation of the H-R complex. It is seen that at 25 h dissociation is complete. The data can be fitted into a first order reaction with the maximum counts dissociated being 90000 (the value obtained by extrapolating the

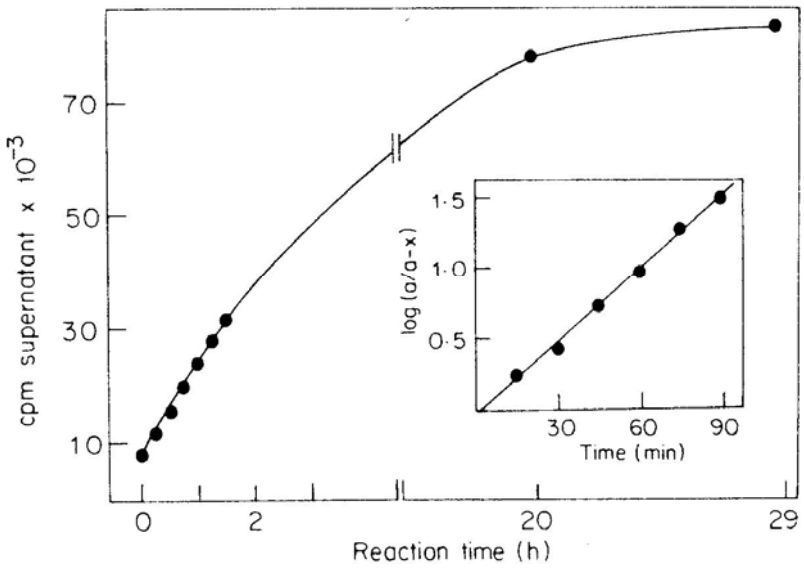

Figure 1. Time course dissociation of HR complex in the presence of excess cold hormone (2 μg/ml). The total counts in 200 μl of the HR complex were 200000 cpm. Inset shows the dissociation data fitted as a first order reaction, using a = 90000 cpm.

curve in the figure), indicating that the dissociation is occurring from a kinetically homogeneous H-R complex. The maximum dissociation corresponds only to 40 % of the total counts bound by receptor. The 60 % undissociated counts are too high to be accounted for by nonspecific binding.

Figure 2 represents the data on the association of [^{125}I]-hGH and its receptor. It is

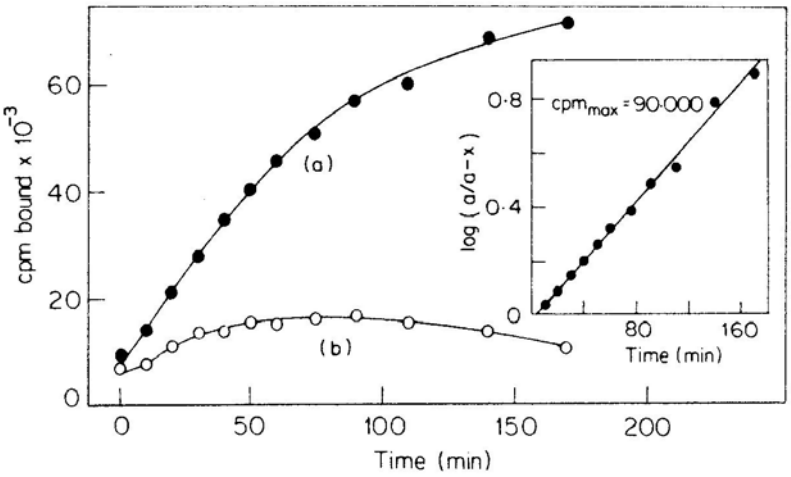

Figure 2. Association of hormone and receptor for reversible binding. The incubation mixture, (500 μl) contained 180000 cpm and the binding observed at 30 h was 80000 cpm. The inset shows the irreversible binding data fitted to a first order reaction with z = 80000 cpm. (o), Reversible binding. (●), Irreversible binding

seen that the irreversible binding (curve a) is far greater than the reversible binding (curve b). The concentration of the receptor used was 10 fold in excess of the concentration of the hormone. Total irreversible binding at 24 h reached a maximum of 80000 counts. With this as the active [^{125}I]-hGH capable of binding to the receptor, the irreversible binding could be fitted into a first order plot (given in the inset). It is apparent that the reversible reaction forms only 20 % of the total reaction of the hormone with the membrane receptor.

Figure 3 (upper panel) represents the total and irreversible binding of the hGH tracer with solubilised rabbit mammary gland receptor. It is apparent from the approxima tion of curve ‘a’ (total) to curve ‘b’ (irreversible) that the reaction is quantitatively irreversible. The lower panel in the figure shows the data fitted for a first order reaction for curve 'b'. Since in this particular case the concentration of the receptor is nearly 10 times the concentration of the hormone, the data conforms to a pseudo first order reaction.

Figure 4 presents typical plots of theoretically generated Scatchard plots. The value of the variables used in obtaining these plots are described in the legend. Two important

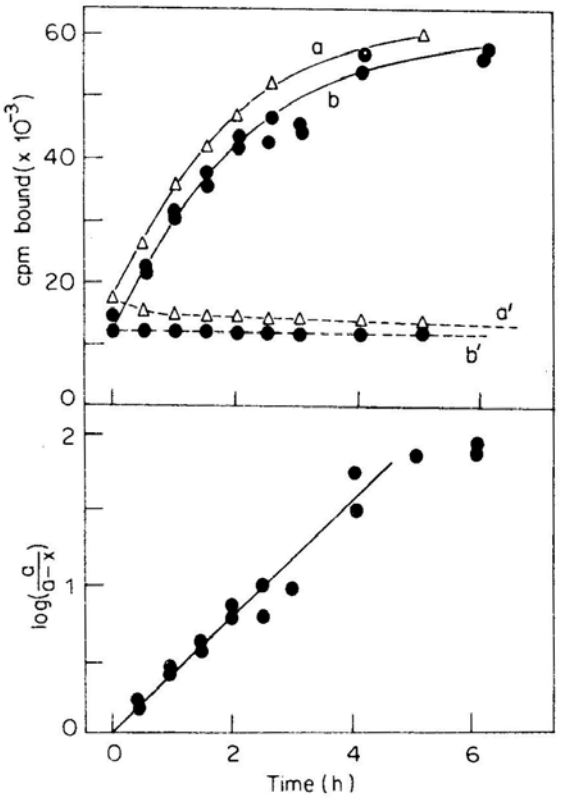

Figure 3. Kinetics of [^{125}I]-hGH binding to solubilised rabbit mammary gland receptor. Upper panel: Total (curve a) and irreversible (curve b) binding of [^{125}I]-hGH brought down by PEG and IgG-globulins. Lower panel shows the binding date fit to first order kinetics with a = 60000 cpm.

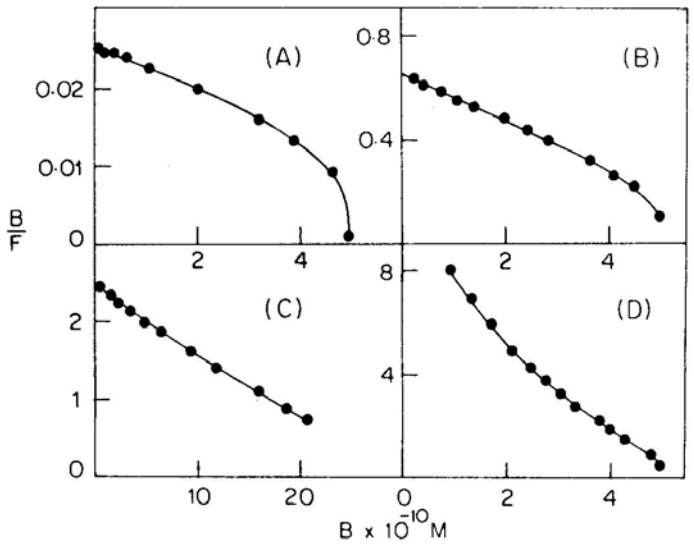

Figure 4. Theoretical Scatchard plots for irreversible systems. (A), $a = 5 \times^{-10}$ M, $k = 5 \times 10^6$/h, and $t = 10$h. (B), $a = 5 \times 10^{-10}$ M, $k = 5 \times 10^8$/h, and $t = 10$h. (C), $a = 25 \times 10^{-10}$ M, $k = 5 \times 10^7$/h, and $t = 10$h. (D), $a = 5 \times 10^{-10}$ M, $k = 5 \times 10^8$/h, and $t = 10$h.

general features observed in many of these plots were; (i) in majority of cases the initial component of the plot was linear (as in figure 4 A, B and C), and in such cases the capacity of the receptor obtained by extrapolation (as is normally done in Scatchard analysis) did not correspond to the true capacity of the receptor. In addition the extrapolated capacity did not appear to bear any simple relation to true capacity; (ii) in a few cases (figure 4D) the plot was curvi-linear.

From these data it is clear that an irreversible interaction between hormone and receptor can on theoretical grounds explain the experimentally observed linearity of Scatchard plots. In addition these results demonstrate that nonlinear Scatchard plots can be obtained under conditions of unimolecular reactions between hormone and receptor.

In order to understand the significance of Scatchard plots in irreversible systems more data was generated. The three variables which can be independently manipulated to obtain a Scatchard plot are *t, a* and *k.* Various Scatchard plots were generated by varying the value of one of the above variables at a time. Figures 5,6 and 7 present the data when *t*, *k* and *a* respectively are varied. The value of variables chosen were in the range normally seen in receptor assays for hGH and oPRL. ($k = 1 \times 10^8$/h; $a = 2{\cdot}5 \times 10^{-11}$ M and t = 10 h).

Figures 5, 6 and 7 demonstrate that most of the plots were linear in the low receptor saturation range, and became nonlinear (convex) as the receptor saturation increased. In most cases the linearity extended upto nearly 60–70 % saturation of the receptor. The only condition where the plot, under low receptor saturation, was non linear was when the *B*/*F* at low receptor saturation was higher than 3 (Figures 6C, D and 7A). In those specific cases the plot was concave. A second general feature of all these plots is that, the

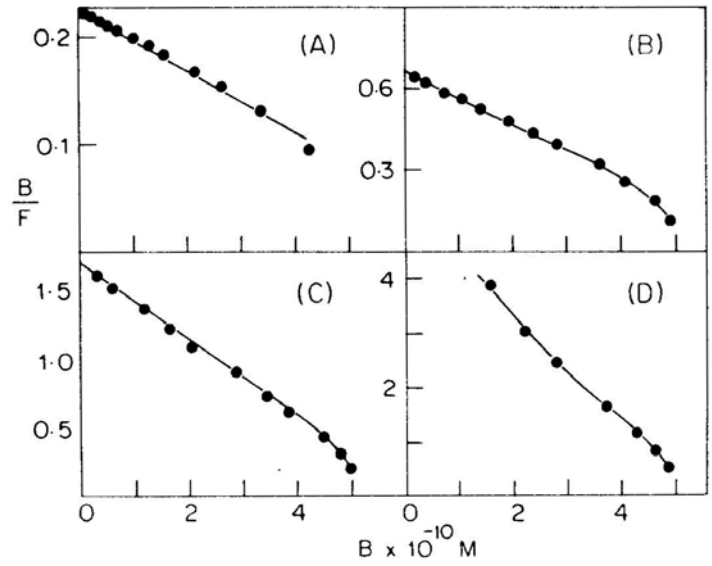

Figure 5. Theoretical Scatchard plots for an irreversible hormone and receptor reaction, obtained by changing the variable *t,* other constant variables were $k = 2 \times 10^8$/h and $a = 5 \times 10^{-10}$ M. (A), (B), (C) and (D) correspond to times of 2h, 5h, 10 h and 20 h respectively.

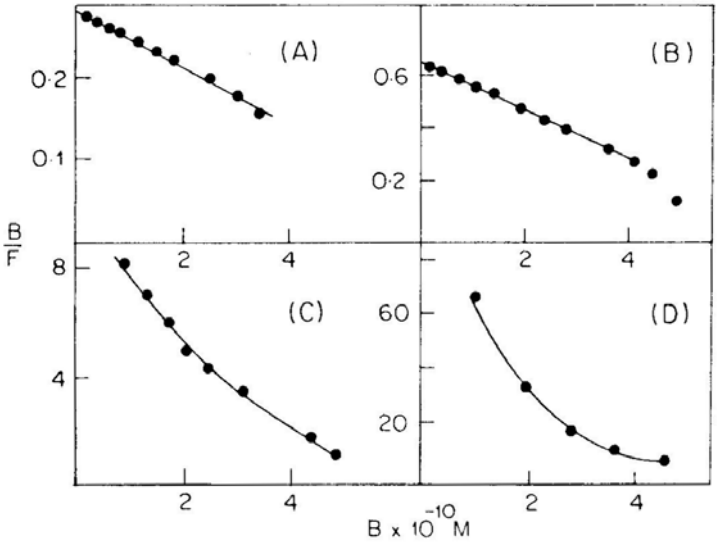

Figure 6. Plots are similar to those shown in figure 2, but in this case the rate constants have been varied. Constant variables were $t = 10$ h and $a = 5 \times 10^{-10}$ M. (A), (B), (C) and (D) correspond to k values of 5×10^7, 1×10^8 and 1×10^9/h respectively.

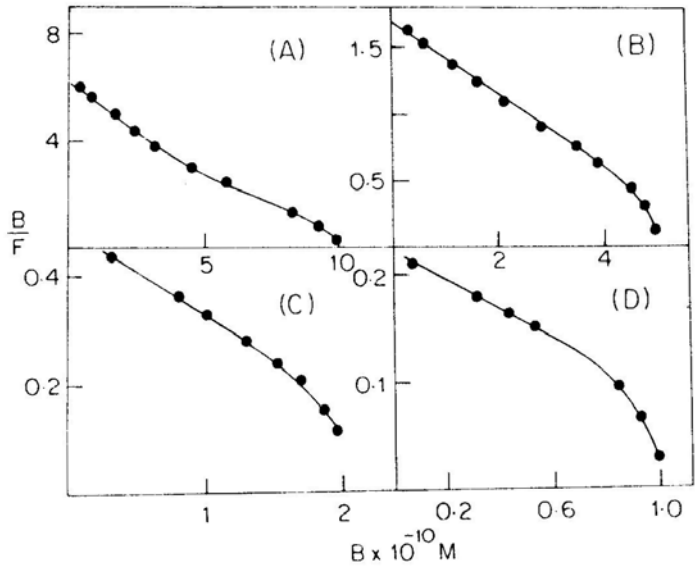

Figure 7. Plots are similar to those shown in figure 2 but in this case *a* is varied. Other variables were $k = 2 \times 10^8$/h and $t = 10$ h. (A), (B), (C) and (D) correspond to a value of (receptor concentration) 10×10^{-10} M, 5×10^{-10} M, 2×10^{-10} M and 1×10^{-10} M respectively.

apparent B_{max} obtained by extrapolation was always greater than the true receptor concentration (in plots which showed linearity under low receptor saturation). Overestimation was greater when $(B/F)^*_{max}$ was less. As $(B/F)_{max}$ increased the overestimation decreased somewhat, but the curve became nonlinear at low receptor saturation so that extrapolation was not possible (in figures 6C, D and 7A).

Overestimation in capacity (the latter obtained by extrapolation from the linear component of the plot) always decreased when the value of $(B/F)_{max}$ was high. Empirical attempts were made to find a relation between the overestimation of capacity and the $(B/F)_{max}$. It was found that a plot on log $(B/F)_{max}$ *vs* overestimation yielded an approximate linear relationship, no matter which values were assigned to the variables. Hence more theoretical Scatchard plots were generated by choosing the three variables (*k, t* and *a*) at random. Table 1 shows the variables selected and the values obtained for $(B/F)_{max}$ and overestimation of capacity. Figure 8 shows the plot of $ln(B/F)_{max}$ *vs* overestimation of capacity. It is obvious from the plot that log $(B/F)_{max}$ shows an inverse linear relationship to the overestimation. As $(B/F)_{max}$ decreased from 2 to 0·2 the overestimation increased from 18 to 60%.

* The B/F value obtained at 0 receptor saturation (intercept) by extrapolation of the linear part of the curve is defined as $(B/F)_{max}$ and is used subsequently.

Table 1. Relationship between estimates of receptor capacity and $(B/F)_{max}$.

Rate constant (K) $\times 10^8\,M^{-1}\,h^{-1}$	Time (h)	True receptor conc. (C) $\times 10^{-10}$ M	Estimated (C) $\times 10^{-10}$ M	Overestimation in (C) (%)	$(B/F)_{max}$
1·00	10·0	5·0	6·50	30	0·660
0·05	10·0	5·0	8·20	65	0·025
1·00	10·0	2·0	2·90	45	0·230
2·00	10·0	5·0	6·0	20	1·700
3·00	10·0	5·0	5·20	4	3·400
1·00	10·0	5·0	6·75	35	0·650
5·00	10·0	5·0	Nonlinear		
10·00	10·0	5·0	Nonlinear		
2·00	10·0	10·0	8·50	−15	6·400
2·00	10·0	5·0	6·00	20	1·700
2·00	10·0	2·0	2·80	40	0·500
2·00	2·0	5·0	8·00	60	0·220
2·00	10·0	5·0	6·00	20	1·70
2·00	3·5	5·0	7·70	54	.42
2·00	7·0	5·0	6·50	30	1·00
1·50	7·0	3·5	5·20	49	0·45
1·50	15·0	3·5	4·50	29	1·20
0·50	20·0	4·0	6·00	50	0·50
1·20	15·0	3·5	4·60	31	0·90
2·40	15·0	3·5	4·00	14	2·50

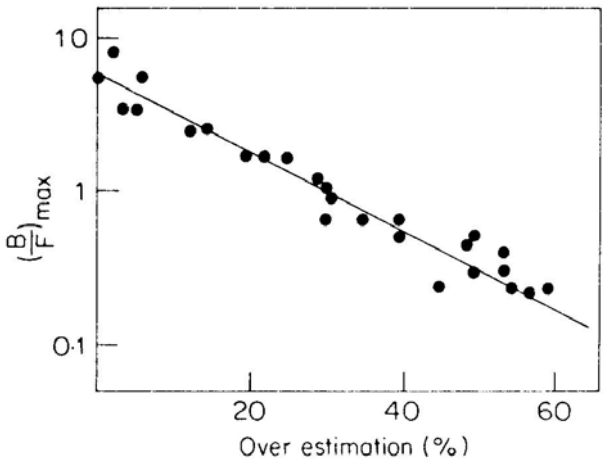

Figure 8. Plot of overestimation in receptor capacity (X-axis) against log $(B/F)_{max}$ (data taken from the table).

Discussion

The data presented clearly demonstrates the irreversibility of hormone and receptor intereaction, both with rabbit liver and rabbit mammary gland receptors. Similar

conclusions were derived in studies using the rat liver receptor system (Thorell and Johansson, 1971). The irreversibility of 60 % seen in the interaction of hormone with membrane bound receptor cannot be attributed to nonspecifically bound tracer because nonspecific binding does not exceed 15 %, under the conditions used to study the formation of H-R complex. The data on the association of hGH with rabbit liver receptor also shows some irreversibility. In fact, in this case the degree of irreversibility is 80% of the total hormone bound. Since the reversible and irreversible reaction with time do not follow a precursor-product relationship, it is concluded that the irreversible reaction is rapid if not instantaneous. This irreversibility is also clearly apparent in the case of solubilised mammary gland receptor and [^{125}I]-hGH (figure 3).

These results raise several questions, one of which concerns the validity of using Scatchard analysis for the determination of K_a and capacity measurements. In a reversible reaction the binding is fitted to a straight line to obtain estimates of K_a and capacity but if irreversibility is a feature of the H-R reaction the interpretation of Scatchard analysis may be questionable, and in some circumstances erroneous as reported elsewhere (Murthy and Friesen, 1985).

It is also clear (from figures 1–5) that in irreversible reactions of hormone and receptor, binding data can yield linear Scatchard plots. The data also demonstrate that if the assay is carried out such that the $(B/F)_{max}$ is very high (> 3) one can obtain a concave plot for a unimolecular irreversible reaction between hormone and receptor. Analysis of the Scatchard plot for irreversible reactions also indicates that the estimates of capacity may be inaccurate. However, an approximate estimation of the capacity can still be obtained because the average overestimation of the capacity obtained by extrapolation of the Scatchard plot can be computed from figure 8.

The significance of the slope in Scatchard analysis of binding data is different in reversible and irreversible systems as well. In the latter system the slope seems to be affected by all the three variables, *t, k* and *a,* whereas in reversible systems (Scatchard, 1949) it would be independent of all the three variables. The slope in an irreversible Scatchard plot would be a complicated function of *k, t* and *a* and does not represent any intrinsic characteristic like the binding constant obtained by Scatchard analysis of reversible reactions (Posner *et al.,* 1974).

A nonlinear Scatchard plot would indicate an interacting or heterogeneous system in reversible reactions (DeMeyts *et al.,* 1976; Kahn *et al.,* 1974; Deal and Guyda, 1983). However, as stated before in irreversible unimolecular systems under conditions of high $(B/F)_{max}$ nonlinear Scatchard plots also would be obtained. In the same system, a radioreceptor assay carried out under conditions of low $(B/F)_{max}$ would show a linear plot until 70% receptor saturation was reached. Thus if the reaction is irreversible, an interacting system can only be suggested when the Scatchard plot is nonlinear under both high and low $(B/F)_{max}$ condition of assay. Hence it is important to check the reversibility-irreversibility of the reaction before Scatchard analysis is attempted.

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References

Deal, C. L. and Guyda, H. J. (1983) *Endocrinology,* **133,** 1512.
DeMeyts, P., Bianco, A. R. and Roth, J. (1976) *J. Biol. Chem.,* **251,** 1877.
Gavin, G. R., Gordon, P., Roth, J., Archer, J. A. and Buell, D. N. (1973) *J. Biol. Chem.,* **248,** 2202.
Herington, A. C, Harrison, D. and Graystone, J. (1983) *Endocrinology,* **113,** 2032.
Jacobs, S., Shechter, Y., Bissel, K. and Cuatracasas, P. (1977) *Biochem. Biophys. Res. Commun.,* **77,** 980.
Kahn, C. R., Freychet, P., Roth, J. and Neville, D. M. Jr. (1974) *J. Biol. Chem.,* **249,** 2249.
Moore, M. V., Wohnlich, L. P. and Fix, J. A. (1983) *Endocrinology,* **113,** 2152.
Murthy, G. S. and Friesen, H. G. (1985) *J. Biosci.,* **7,** 135.
Posner, B. I., Kelly, P. A., Shiu, R. P. C. and Friesen, H. G. (1974) *Endocrinology,* **95,** 521.
Powell, B. L. and Hollander, V. P. (1978) *Endocrinology,* **103,** 710.
Roth, J. (1973) *Metabolism,* **22,** 1059.
Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.,* **51,** 660.
Shiu, R. P. C. and Friesen, H. G. (1974) *J. Biol. Chem.,* **249,** 7902.
Thorell, J. I. and Johansson, B. G. (1971) *Biochem. Biophys. Acta.,* **251,** 363.
Van der Gugten, A., Waters, M. J., Murthy, G. S. and Friesen, H. G. (1980) *Endocrinology,* **106,** 402.