Biochimica et Biophysica Acta, 406 (1975) 294–303

BBA 77078

QUANTITATIVE ASPECTS OF HORMONE-RECEPTOR INTERACTIONS OF HIGH AFFINITY.

EFFECT OF RECEPTOR CONCENTRATION AND MEASUREMENT OF DISSOCIATION CONSTANTS OF LABELED AND UNLABELED HORMONES

KWEN-JEN CHANG, STEVEN JACOBS and PEDRO CUATRECASAS

Department of Pharmacology and Experimental Therapeutics and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205 (U.S.A.)

(Received April 2nd, 1975)

SUMMARY

It is demonstrated that because of limitations in the magnitude of the specific activity of radiolabeled hormone derivatives, direct binding studies of hormone-receptor interactions of high affinity (10^{-9}–10^{-11} M, depending on whether ^{3}H- or ^{123}I-labeled hormones are used) will be subject to artifactual distortions due to the need to utilize high concentrations of the receptor. If the concentration of the receptor is not ten times lower than the true affinity constant, the apparent dissociation constant obtained from direct concentration binding curves will vary as a linear function of the receptor concentration. In addition, at high receptor concentrations saturability becomes difficult to demonstrate experimentally and the binding data yield apparently non-hyperbolic, sigmoidal curves which can be mistakenly interpreted to depict cooperative interactions. Similar artifacts related to receptor concentration are predicted for measurements of the hormone concentration dependence of biological processes (e.g. activation of adenylate cyclase, transport processes, etc.). Methods for detecting these effects, and correctly measuring affinities for labeled and unlabeled hormones under these conditions, are described. The implications for measuring the binding properties of hormone-receptor interactions are discussed, especially in reference to studies of the comparative analysis of receptor function in altered metabolic states and to studies relating the biological and binding properties of hormones.

INTRODUCTION

Direct binding isotherms, displacement curves and Scatchard analyses [1] are the graphical representations most commonly used to depict and analyze the parameters of hormone-receptor interactions. The concentration of unlabeled ligand which reduces the binding of the labeled ligand by 50 % is often used to approximate the dissociation constant, or to compare the relative affinities in different tissues or in

the same tissue from different metabolic states. This approach is correct only when the concentration of the receptor binding sites and of the labeled ligand are both very much lower than that of the dissociation constant of the system [2]. Thus, in very high affinity systems the use of the competitive displacement curve may be severely limited by the relatively low specific activity of the radioactively labeled ligands currently available [2].

The Scatchard plot [1] was originally described for low affinity systems. In high affinity systems Scatchard plots can easily be distorted by non-specific interactions [3, 4] or by even slight inaccuracies in the estimation of the free ligand concentration [2]. In these systems the best information is obtained from low saturation points which describe the high affinity process and which occur as far as possible from the low affinity or possible non-specific binding sites [4]. Scatchard plots are often obtained by conversion of competitive displacement curves on the assumption that the labeled and unlabeled ligands have the same affinity. It has recently been shown that a non-linear Scatchard plot will result if the affinity of the unlabeled ligands is different from that of labeled ligand [5]; in such cases the plots will be suggestive of multiple binding sites or cooperativity. Thus, in high affinity systems it is difficult to measure the dissociation constant of a ligand with an affinity different from that of the labeled ligand. In a binding saturation curve which utilizes only increasing concentrations of the labeled ligand, the concentration at which half-maximal binding is achieved usually approximates the dissociation constant. However, in high affinity systems this parameter is seriously affected by the concentration of receptor binding sites.

The present report presents the theoretical basis of the effect of receptor site concentration on binding saturation curves. Methods for detecting these anomalies, and for properly measuring affinity under these circumstances, are presented. In addition, a simple method for accurately measuring dissociation constants from competitive displacement curves with unlabeled ligands is described.

RESULTS

Effect of receptor binding site concentration on direct binding isotherms

Considering the reversible, bimolecular receptor-hormone interaction,

$$R + H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RH \tag{1}$$

where R represents the free receptor binding site*, H the free hormone or ligand and RH the complex, at equilibrium binding will follow the mass law expression,

$$\frac{[R_f][H_f]}{[RH]} = K_D = \frac{k_{-1}}{k_1} \tag{2}$$**

* R is defined as receptor binding site. Thus the following derivations equally apply to a receptor containing multiple, independent and equivalent, binding sites; and the concentration of receptor binding site will be equal to the value of the number of binding site per receptor molecular times the receptor concentration.

** The K_D and K_I are interchangeable with thermodynamic equilibrium constant and kinetic dissociation constant of hormone-receptor complex since the equilibrium constant equals the dissociation constant in the reactions described in the paper.

where K_D is the dissociation constant, $[R_f]$ and $[H_f]$ are the concentrations of free receptor binding sites and hormone, respectively. From Eqn 2 and since $[R_t] = [R_f]+[RH]$, the fractional occupancy (f) of receptor sites can be derived and will follow the Langmuir equation,

$$f = \frac{[RH]}{[R_t]} = \frac{[H_f]}{[H_f]+K_D} \text{ or } [H_f] = K_D\left(\frac{f}{1-f}\right) \tag{3}$$

Since $[H_t] = [H_f]+[RH]$ and $[RH] = f[R_t]$,

$$[H_t] = K_D\left(\frac{f}{1-f}\right) + f[R_t] \tag{4}$$

where $[R_t]$ is the total receptor binding site concentration. Dividing Eqn 4 by K_D yields the equation,

$$\frac{[H_t]}{K_D} = \frac{f}{1-f} + f\left(\frac{[R_t]}{K_D}\right) \tag{5}$$

Eqns 4 and 5 clearly indicate that the relationship between fractional occupancy, f, and $[H_t]$ is dependent upon the ratio of the total receptor binding site concentration to the dissociation constant ($[R_t]/K_D$). The results are computed and shown in different graphic forms in Figs 1–3. Fig. 1 shows receptor occupancy, f, as a theoretical function of labeled hormone or ligand concentration at various concentrations of receptor binding site. The linear plot of Eqn 5, as shown in Fig. 1, clearly shows that at the low saturation portion of the curve binding appears to be a linear function of the hormone concentration at the values of $[R_t]$ greater than K_D. The portion of linear dependence of binding is increased as the receptor binding site concentration increases. However, at values of $[R_t]/K_D < 0.1$, the last term of Eqn 5 is negligible, thus resulting in a typical Langmuir hyperbolic equation (Fig. 1),

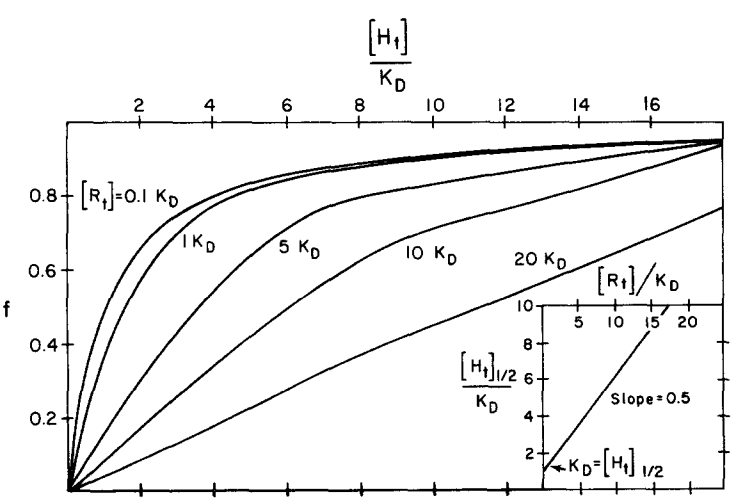

Fig. 1. Theoretical binding curves as a function of hormone concentration, computed from Eqn 5, $[H_t]/K_D = f/(1-f) + f([R_t]/K_D)$, at various values of $[R_t]/K_D$ showing effect of receptor concentration on binding. Insert is the graphical presentation of Eqn 8, $[H_t]_{1/2} = K_D = [R_t]/2$.

$$\frac{[H_t]}{K_D} = \frac{f}{1-f} \quad \text{or} \quad f = \frac{[H_t]}{[H_t]+K_D} \tag{6}$$

Under these conditions the hormone concentration at which half-maximal binding occurs closely approximates the dissociation constant, K_D.

It is clear that the concentration at which half-maximal binding is achieved (apparent dissociation constant) is equal to the true dissociation constant (K_D) only if the receptor binding site concentration is very much smaller than K_D. In very high affinity systems such conditions may be difficult to approach experimentally. For instance given a dissociation constant of 10^{-11} M, where the receptor binding site concentration would have to be less than 10^{-12} M, the amount of radioactivity bound to the receptor at half-maximal binding would be less than 1100 cpm (55 % counting efficiency) per ml if the hormone is labeled with carrier-free ^{125}I (specific activity, 2 Ci/μmol) at a molar ratio of one. Figs 1 and 2 clearly demonstrate that the apparent dissociation constant increases as the receptor binding site concentration is increased.

Since in high affinity systems it is difficult experimentally to achieve optimal conditions for studying binding by these methods, it is desirable to devise a means of correcting the contribution of receptor binding site concentration on the measurement of the dissociation constant. At $f = 0.5$, Eqn 5 becomes

$$\frac{[H_t]_{\frac{1}{2}}}{K_D} = 1 + \frac{[R_t]}{2K_D} \tag{7}$$

Where $[H_t]_{\frac{1}{2}}$ is the apparent dissociation constant. Rearranging Eqn 7 gives

$$[H_t]_{\frac{1}{2}} = K_D + \frac{[R_t]}{2} \tag{8}$$

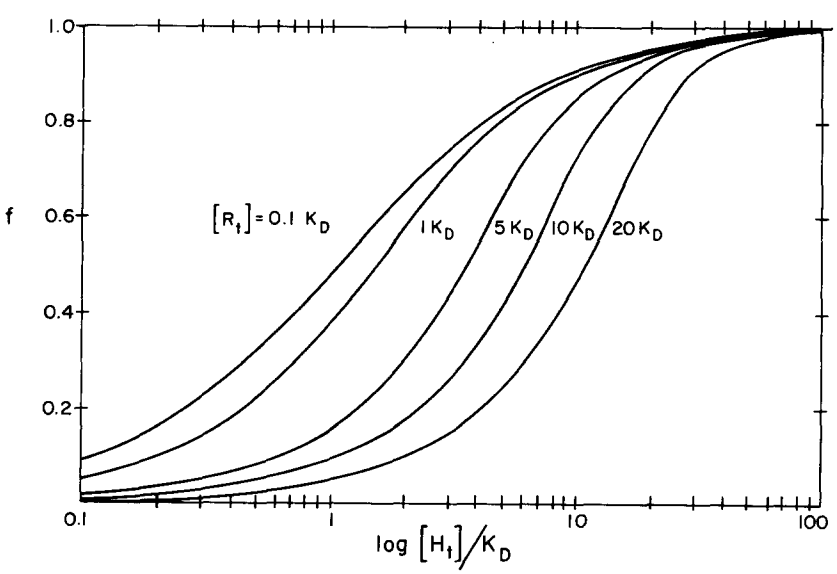

Fig. 2. Theoretical semilog plot of Eqn 5, $[H_t]/K_D = f/(1-f) + f([R_t]/K_D)$, showing artifactual apparent cooperativity and changes in the apparent dissociation constant with increasing concentrations of receptor.

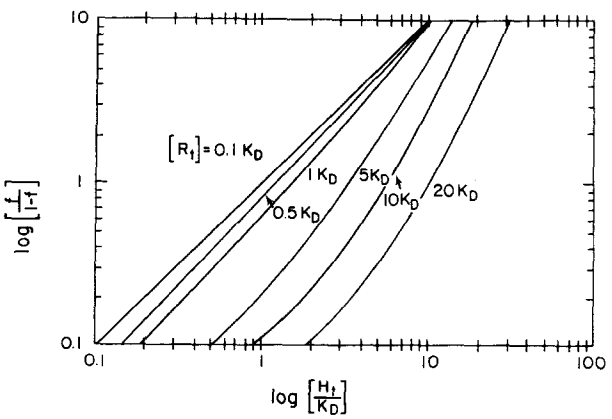

Fig. 3. Theoretical Hill plot of Eqn 5, $[H_t]/K_D = f/(1-f) + f([R_t]/K_D)$, showing apparent artifactual cooperativity and changes in the apparent dissociation constant with increasing concentrations of receptor.

Thus, the apparent dissociation constant is a linear function of the receptor binding site concentration with a slope of 0.5 and an ordinate intercept of K_D as shown in the insert of Fig. 1. Experimentally, the dissociation constant can be obtained by plotting the apparent dissociation constant vs the reciprocal of a series of dilution factors of receptor protein and extrapolating to the ordinate intercept*.

Another important effect of receptor concentration on binding isotherms is the appearance, at high receptor binding site concentrations, of artificial, apparently cooperative binding curves, as shown in semilog (Fig. 2) and Hill (Fig. 3) plots. The slope at half-maximal binding increases as the receptor binding site concentration is increased beyond the value of K_D.

Measurement of the dissociation constants of unlabeled hormones

A method is described here for measuring the dissociation constants of unlabeled competitive inhibitors (e.g. unlabeled hormones, analogs or antagonists) whose affinity differ from that of the labeled hormone. In a bimolecular reaction having independent (non-interacting) binding sites, the two ligands (labeled and unlabeled) compete for the same binding site as described by

$$R + H \rightleftharpoons RH \tag{9}$$

$$R + I \rightleftharpoons RI \tag{10}$$

where H and I are labeled hormone and inhibitor, respectively, and RH and RI are the labeled hormone-receptor complex and inhibitor-receptor complex, respectively. At equilibrium, the mass action expression in the absence of inhibitor is given by Eqn 11, and in the presence of inhibitor, at concentration $[I_t]$ by Eqns 12 and 13,

* If the concentration of free labeled undamaged hormone can be accurately measured, a plot of fractional occupancy vs the concentration of free hormone would yield a free hormone concentration at half-maximal binding equal to K_D.

$$K_D = \frac{[R_t - RH][H_f]}{[RH]} \tag{11}$$

$$K_D = \frac{[R_t - RH - RI][H'_f]}{[RH]} \tag{12}$$

and

$$K_I = \frac{[R_t - RH - RI][I_f]}{[RI]} \tag{13}$$

where $[H_f]$ and $[H'_f]$ are the concentrations of free, labeled hormone present when the same amount of labeled hormone is bound to the receptor in the absence and presence of inhibitor $[I_t]$, respectively. $[I_f]$ is the concentration of free inhibitor. Since the right terms of Eqns 11 and 12 are equal,

$$K_D = \frac{[R_t - RH][H_f]}{[RH]} = \frac{[R_t - RH - RI][H'_f]}{[RH]} \tag{14}$$

canceling [RH] and rearranging Eqn 14 yields equation

$$\frac{[R_t - RH]}{[R_t - RH - RI]} = \frac{[H'_f]}{[H_f]} \tag{15}$$

Defining $X = [H'_f]/[H_f]$ (dose ratio), substituting into Eqn 15 and rearranging it yields equation

$$[RI] = \frac{[R_t - RH][X-1]}{X} \tag{16}$$

Substitution of Eqn 16 for Eqn 13 yields equation

$$K_I = \frac{[I_f]}{X-1} \tag{17}$$

Since $[I_f] = [I_t] - [RI]$,

$$K_I = \frac{[I_t] - [RI]}{X-1} \tag{18}$$

Replacing [RI] of Eqn 18 by Eqn 16 yields equation

$$K_I = \frac{[I_t]}{X-1} - \frac{[R_t] - [RH]}{X} \tag{19}$$

which correlates $[I_t]$ as the function of X at a given value of $[R_t] - [RH]$. The dose ratio, X, can be estimated from the concentration vs binding curve at any given value of bound receptor by the ratio of $[H'_f]/[H_f]$ (Fig. 4). X can be approximately calculated from the ratio of $[H'_t]/[H_t]$ if the bound labeled hormone, [RH], is not more than 5 % of total labeled hormone.

The dissociation constant of the unlabeled hormone or its analogs can be estimated from Eqn 19 by knowing X, [RH], and $[I_t]$. Alternatively, a plot of $[I_t]/X-1$ vs $1/X$ can be constructed at several concentrations of $[I_t]$ (Fig. 5). A linear

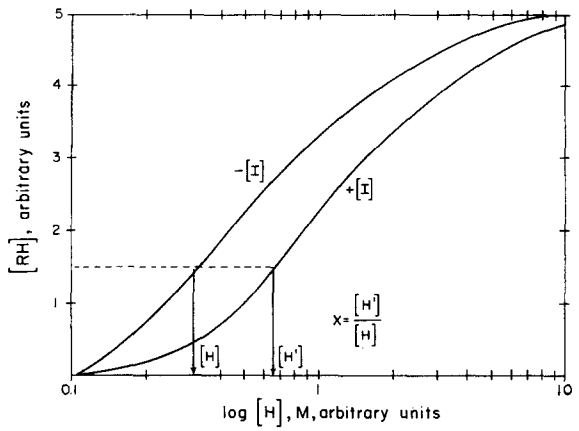

Fig. 4. Graphical presentation of the measurement of dose ratio, X, from concentration vs binding curves in the absence and presence of competitive inhibitor (i.e. unlabeled hormone). [H] can be either free labeled hormone concentration or total labeled hormone concentration if the bound labeled hormone is less than 5 % of total labeled hormone.

plot with a slope of $[R_t]-[RH]$ and an ordinate intercept equal to K_I is obtained. Thus, the dissociation constant of the unlabeled hormone and the total receptor concentration can be estimated easily without knowing the affinity of the labeled hormone.

Under condition where $[R_t]-[RH] \cong 0$, or when the slope of Fig. 5 is close to zero, Eqn 19 can be simplified to

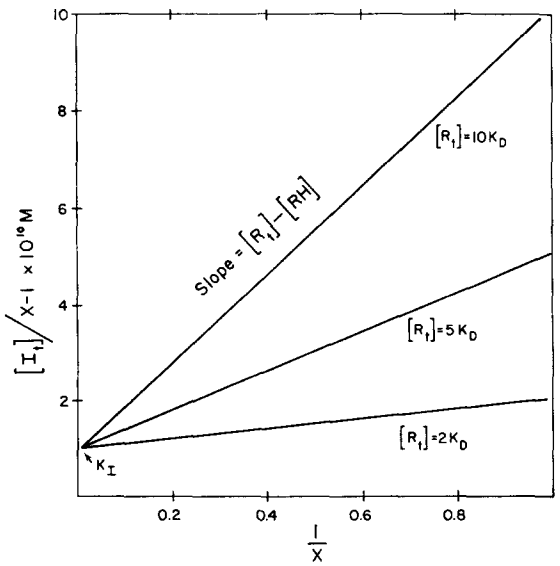

Fig. 5. Theoretical plot of $[I_t]/X-1$ vs $1/X$ computed from Eqn 19 $[I_t]/X-1 = K_I+([R_t]-[RH])/X$, when $[RH] = 10^{-10}$ M, $K_I = 10^{-10}$ M and $[R_t]$ at concentrations of $2 \cdot 10^{-10}$, $5 \cdot 10^{-10}$ and $10 \cdot 10^{-10}$ M.

$$K_I = \frac{[I_t]}{X-1} \tag{20}$$

which is the same as the Schild equation [6, 7]. In this case the plot of log $(X-1)$ vs log $[I_t]$ yields a linear function with a slope of 1 and an abscissa intercept of log K_I for the competitive inhibitor. If the unlabeled hormone or its analog behaves according to Eqn 19 or 20, it is safe to conclude that it competes for the same binding site as the labeled ligand.

DISCUSSION

In the last few years considerable progress has been made in the biochemical identification and characterization of hormone-receptor interactions, principally from binding studies which utilize radioactively labeled hormone derivatives [8]. Although not recognized heretofore in hormone binding studies, accurate determinations of the binding properties of such interactions depend to a large extent on the proper selection of the receptor concentration as well as on the magnitude of the specific radioactivity of the labeled hormone used. Since under ideal conditions the concentration of receptor must be at least 10-fold lower than the dissociation constant, very high affinity systems (e.g. dissociation constants of 10^{-10}–10^{-11} M) require the use of very low concentrations, and therefore quantities, of receptor. In such situations even the most highly radioactive hormones (e.g. those labeled with carrier-free ^{125}I at 2 Ci/μmol) will yield very low quantities of labeled hormone-receptor complexes unless inordinately large volumes are used for the assay [2]. When ^{3}H-labeled compounds with specific activities as high as 10 Ci/mmol are used, negligible quantities of radioactively labeled receptor complexes can be expected to form, and in such cases difficulties will occur even when the dissociation constant is as high as 10^{-9} M.

Because of the current unavailability of hormone derivatives of sufficiently high specific radioactivity, most hormone receptor studies must use relatively high concentrations of the receptor to permit experimental measurements. From the present studies it appears that such conditions may lead to erroneous thermodynamic estimates as well as to misleading mechanistic interpretations. The saturability of the binding curve, the apparent dissociation constant and the shape of the concentration vs binding curve can be affected markedly by the receptor concentration (Figs 1–3). The apparent dissociation constant is a linear function of the receptor binding site concentration with a positive slope of 0.5 (Fig. 1, insert, and Eqn 8). Furthermore, it is evident that at high receptor concentrations a "non-saturable" (experimentally) binding curve will be encountered (Fig.1). In this respect it is pertinent that saturation in hormone receptor binding studies is frequently difficult to demonstrate. In addition, at high receptor concentrations non-hyperbolic curves, probably sigmoid (Figs 1 and 2), occur which can be mistakenly interpreted as showing positive cooperativity because of apparent Hill coefficients of $n > 1$ at the point of half-maximal binding (Fig. 3). Although Scatchard plots [1] derived from such data are in principle not affected by receptor concentration and can be used to estimate affinity constants and saturation, very serious experimental problems arise with respect to the accuracy of the measurements of free hormone concentration when the receptor concentration is high [2]. Even minor degrees of radioligand heterogeneity, or of hormone degredation,

can introduce serious errors [3].

Analogous observations in the kinetic behavior of enzyme systems at high concentrations of enzyme have been described previously [9–11]. Other factors which can affect the concentration vs binding curve include ligand-ligand interactions (polymerization, self-aggregation, isomerization) [12, 13], receptor-receptor interactions [13–15], non-specific binding [12], and heterogeneity of receptor [16] or of labeled ligand. Non-hyperbolic binding curves or the lack of correlation between the hormone concentration dependence for binding and for a biological activation process (which are frequently done under different conditions of receptor concentration) could be misinterpreted as being indicative of cooperative receptor interactions or of the existence of "spare" receptors. The performance of such studies at two or more different receptor concentrations should permit easy detection of such artifacts.

The same reasoning developed in this report leads to the prediction that the same artifactual effects related to receptor concentration will be observed in studies of the hormone concentration dependence for a biological activation process. For example, underestimation of the affinity for hormonal activation of an in vitro enzymatic activity can occur if the enzyme (or receptor) concentration is high relative to the true affinity of the hormone tested. Specifically, in studies of the hormonal activation of adenylate cyclase high concentrations of membranes (i.e. enzyme and receptor) are frequently required to generate sufficiently high quantities of product for assay. This will be especially troublesome when short assay periods are used (to obtain zero-order conditions), or when the specific radioactivity of the substrate ATP is low, thus requiring an increase in the enzyme and receptor concentrations to generate sufficient product. Such conditions may easily lead to grossly discrepant correlations between the hormone concentration dependence for binding and for enzyme activation, a situation frequently encountered in the literature for hormone-adenylate cyclase systems. Analogous observations relating enzyme concentration with the apparent K_a for cyclic AMP-activated protein kinase and the affinity for cyclic AMP binding have been reported recently [17]. Misleading results may be observed in any situation where the insensitivity of the assay method, whether related to a binding, enzymatic or bioassay, compels the use of receptor concentrations which are high relative to the true affinity of the system.

The effects of receptor concentration are of particular significance when comparisons of the hormone receptor affinity or "saturation" (capacity) are to be made between metabolically distinct states (e.g. obesity, diabetes, etc.) or between tissues in the same or different species. Proper interpretations require that such binding studies be performed at precisely the same receptor (not "membrane") concentration, which is especially difficult with membrane preparations from tissues as complex as liver, or when heterogeneous (with respect to receptors) cell populations such as lymphocytes [3] are used. Correct interpretations in such studies will probably require the performance of binding studies at varying concentrations of receptor as well as hormone.

As described earlier [2], the use of native, unlabeled hormones in competitive displacement curves for estimating hormone receptor affinities is similarly influenced by the concentration of receptor and of the labeled hormone. In such approaches the same considerations of receptor concentration and of specific radioactivity of the hormone seriously limit the utility of the methods for quantitative analyses. For example, because the apparent K_D is increased at high receptor concentrations [2], the

sensitivity of competitive displacement methods will be poor and their practical application to "radioreceptor" assays for hormones will be severely limited. Furthermore, Scatchard analysis of such data will be affected by differences in the affinities of the native and labeled hormones [5].

If it is necessary to use a high concentration of receptor because of the limitations imposed by the low specific activity of the labeled hormone, there is no simple relationship between the binding of the labeled and unlabeled hormones if the affinities of these two species are different. Therefore, the use of Eqns 19 and 20 may be the most suitable method for the study of the behavior of the unlabeled hormone or its analogues. These equations consider and correct for the contribution of receptor binding site concentration, the function measured is independent of the affinity of the labeled hormone and experimentally complete saturation in the binding curves is not required. This method is recommended for comparative binding studies of tissues from metabolically distinct states or species. However, if the free, labeled hormone cannot be accurately estimated the limitation exists that the receptor-bound hormone must be less than 5 % of the total labeled hormone in order to approximate the dose ratio, X, by the value of $[H'_t]/[H_t]$. Furthermore, the equations derived in this paper are applicable only where binding equilibrium has been achieved.

ACKNOWLEDGEMENT

Supported by grants from National Institutes of Health (AM 14956), and The Kroc Foundation. K. C. is recipient of a postdoctoral fellowship from the Heart Association of Maryland, S. J. is a recipient of a U.S.P.H.S. postdoctoral fellowship. P. C. is recipient of a U.S.P.H.S. Research Career Development Award (AM 31464).

REFERENCES

1 Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
2 Jacobs, S., Chang, K.-J. and Cuatrecasas, P. (1975) submitted to Biochem. Biophys. Res. Commun.
3 Cuatrecasas, P., Hollenberg, M. D., Chang, K.-J. and Bennett, V. (1975) in Recent Progress in Hormone Research, Vol. 31, Academic Press, New York, in the press
4 Weber, H. G., Schildknecht, J., Lutz, R. A. and Kesselring, P. (1974) Eur. J. Biochem. 42, 475–481
5 Taylor, S. I. (1975) Biochemistry 14, 2357–2361
6 Schild, H. O. (1949) Br. J. Pharmacol. 4, 277–280
7 Grunlakshana, O. and Schild, H. O. (1939) Br. J. Pharmacol. 14, 48–58
8 Cuatrecasas, P. (1974) Annu. Rev. Biochem. 43, 169–214
9 Straus, O. H. and Goldstein, A. (1943) J. Gen. Physiol. 26, 559–585
10 Goldstein, A. (1944) J. Gen. Physiol. 27, 529–580
11 Cha, S. (1970) J. Biol. Chem. 245, 4814–4818
12 Cuatrecasas, P. and Hollenberg, M. D. (1975) Biochem. Biophys. Res. Commun. 62, 31–41
13 Nichol, L. W., Smith, G. D. and Ogston, A. G. (1969) Biochim. Biophys. Acta 184, 1–10
14 Monod, J., Wyman, J. and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88–118
15 Koshland, Jr, D. E., Nementhy, G. and Filmer, D. (1966) Biochemistry 5, 365–385
16 Klotz, I. M. and Munston, D. L. (1971) Biochemistry 10, 3065–3069
17 Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3580–3583