ESTIMATION OF HORMONE RECEPTOR AFFINITY BY COMPETITIVE DISPLACEMENT OF LABELED LIGAND: EFFECT OF CONCENTRATION OF RECEPTOR AND OF LABELED LIGAND

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SUMMARY. The concentration of ligand (e.g., hormone) at which a given fraction of bound labeled ligand is competitively displaced from its binding site (e.g., receptor) depends upon the concentration of labeled ligand and of binding sites as well as on the affinity of the ligand. The ligand concentration at which 50% of the bound, labeled ligand is displaced [K'_d(app)] is often taken (mistakenly) to be equal to the dissociation constant of the ligand. For an ideal bimolecular reaction, it is shown that K'_d(app) actually exceeds the dissociation constant by an amount equal to the labeled ligand concentration plus the binding site concentration minus three halves the concentration of labeled ligand that would be bound in the absence of unlabeled ligand. For both epidermal growth factor and insulin, the concentration of unlabeled hormone at which a given fraction of bound labeled hormone is displaced from placenta membranes is increased by increasing the labeled hormone concentration or the placenta membrane concentration. K'_d(app) will give spuriously high estimates of the dissociation constants for these hormones if measured at high labeled hormone or binding site concentrations. These considerations also have important implications for comparative studies of receptors in different species or metabolic states, and for the feasibility of "radioreceptor" assays.

The affinities of ligands (e.g., hormones) for their binding sites (e.g., receptors) are very frequently assessed by the competitive displacement of a labeled ligand which binds to the same site. The fraction of labeled ligand displaced by a given concentration of unlabeled ligand depends both on the binding site and the labeled ligand concentrations, as well as on the affinity of the ligand. Unless this is appreciated, the calculated ligand affinity can be seriously underestimated.

Under certain conditions, the concentration of ligand at which one half of the bound labeled ligand is displaced will equal the dissociation constant of the ligand. In this report a relationship is derived for an ideal bimolecular reaction, which relates this concentration to the dissociation constant, the binding site concentration and the labeled ligand concentration. The effect of varying the concentrations of binding sites and labeled ligand is illustrated with fractional competitive displacement curves for insulin and epidermal growth factor (EGF)[1] binding to placenta membranes.

MATERIALS AND METHODS. ^{125}I-Labeled insulin (220 MCi per mg), (1) and ^{125}I-labeled EGF (260 MCi per mg) (2) were prepared with chloramine T. Human placenta membrane was prepared, and insulin and EGF binding were assayed, as pre-

[1] EGF, epidermal growth factor.

viously described (1, 2) using Millipore filters.

RESULTS. In the simple case where both the labeled and unlabeled ligands bind reversibly to the same, single species of sites according to the reaction scheme $A + B \rightleftarrows C$, if there are no competing reactions, a relationship can be derived which describes the concentration of unlabeled ligand which displaces 50% of the bound, labeled ligand.

In the presence of only labeled ligand, at equilibrium

$$K_d = \frac{(R_o - RH)\ (H_o - RH)}{RH} \quad (1)$$

where K_d equals the dissociation constant for the labeled ligand, H_o the total concentration of all species of labeled ligand, R_o the total concentration of all species of binding sites, and RH the concentration of bound, labeled ligand in the absence of unlabeled ligand.

If enough unlabeled ligand is added to the system to displace 50% of the bound labeled ligand a new equilibrium is reached which is described by

$$K_d = \frac{(R_o - \frac{1}{2}RH - Rh)\ (H_o - \frac{1}{2}RH)}{RH/2} \quad (2)$$

where Rh is the concentration of bound, unlabeled ligand, and of course, $\frac{1}{2}RH$ is the concentration of bound labeled ligand under these conditions. Dividing equation 1 by equation 2 and solving for Rh,

$$Rh = (R_o - \tfrac{1}{2}RH) - \frac{(R_o - RH)\ (H_o - RH)}{2(H_o - \frac{1}{2}RH)} \quad (3)$$

The dissociation constant of the unlabeled ligand, K'_d, which may be different from that of the labeled ligand can be expressed as some fraction, n, of K_d. Therefore,

$$\frac{(R_o - \frac{1}{2}RH - Rh)\ (h_o - Rh)}{Rh} = K'_d = nK_d = \frac{n(R_o - \frac{1}{2}RH - Rh)\ (H_o - \frac{1}{2}RH)}{\frac{1}{2}RH} \quad (4)$$

where h_o equals the total concentration of all species of unlabeled ligand. Solving the first and last expressions of equation 4 for Rh,

$$Rh = \frac{(\frac{1}{2}RH)\ (h_o - Rh)}{n\ (H_o - \frac{1}{2}RH)} \quad (5)$$

The free, unlabeled ligand concentration at which 50% of the bound, labeled ligand will be displaced, $(h_o - Rh)$, will be defined by $K'_d(app)$. Substituting the value for Rh given by equation 5 into equation 3, and solving for $(h_o - RH)/n$,

$$K'_d(app)/n \overset{def}{=\!=} (h_o - Rh)/n = \frac{2(R_o - \frac{1}{2}RH)\ (H_o - \frac{1}{2}RH)}{RH} - \frac{(R_o - RH)\ (H_o - RH)}{RH} \quad (6)$$

Concentrating on the first term on the right side of equation 6

$$\frac{2(R_o-\frac{1}{2}RH)\ (H_o-\frac{1}{2}RH)}{RH} = \frac{2(R_o)(H_o) - (R_o)(RH)-(H_o)(RH) + (RH)^2/2}{RH} \quad (7)$$

Subtracting the quantity $(R_o + H_o - \frac{3}{2}RH)$ from the fraction of the right side of equation 7, and then adding it, gives

$$\frac{2(R_o-\frac{1}{2}RH)\ (H_o-\frac{1}{2}RH)}{RH} =$$

$$\frac{2(R_o)(H_o) -2(R_o)(RH) -2(H_o)(RH) + 2(RH)^2}{RH} + R_o + H_o-\frac{3}{2}RH \quad (8)$$

Factoring the first term on the right side of equation 8 and substituting into equation 6 results in

$$K'_d(app)/n = \frac{2(R_o-RH)\ (H_o-RH)}{RH} + R_o + H_o-\frac{3}{2}RH - \frac{(R_o-RH)\ (H_o-RH)}{RH} \quad (9)$$

Adding the first and last terms on the right side of equation 9, multiplying both sides of the equation by n, and substituting K_d for $(R_o-RH)\ (H_o-RH)/RH$ in accord with equation 1 gives

$$K'_d(app) = n(K_d+H_o+R_o-\frac{3}{2}RH) = K'_d + n(H_o+R_o - \frac{3}{2}RH) \quad (10)$$

It can be seen from equation 10 that when $K_d \gg R_o+H_o$, $K'_d(app) \cong K'_d$. If this condition is not met, $K'_d(app)$ will exceed K'_d. When $H_o \gg K_d+R_o$, $K'_d(app)$ will approximate nH_o. Similarly, when $R_o \gg K_d+H_o$, $K'_d(app)$ will approximate nR_o.

Curves of the fractional competitive displacement of ^{125}I-labeled EGF binding to placenta membranes are shifted to the right by increasing the membrane or the ^{125}I-labeled hormone concentration (Fig. 1). The unlabeled EGF concentration at which 50% of the bound, labeled EGF is displaced, $K'_d(app)$, is also increased substantially. Similar results are obtained for the displacement of ^{125}I-labeled insulin binding to membranes (Fig. 2).

DISCUSSION. Competitive displacement curves accurately reflect the affinity of a ligand only if the concentrations of labeled ligand and binding sites are substantially less than the dissociation constant for the labeled ligand. If these conditions are not met, the apparent affinity will be less than the true affinity.

Because of the limitations on the magnitude of the specific activity of labeled ligands (e.g., ^{3}H- or ^{125}I-labeled hormones), it may not be practical to assess the affinity of very high affinity systems (such as hormone-receptor complexes) by means of competitive displacement. For example, considering a system with a dissociation constant of 10^{-11} M, the ligand concentration required to displace 50% of the bound labeled ligand will reflect the dissociation constant with accuracy, only if both the binding site and labeled ligand concentrations are 10^{-12} or less. Under these conditions the concentration of bound, labeled ligand would be about 5×10^{-14} M. If the ligand were labeled

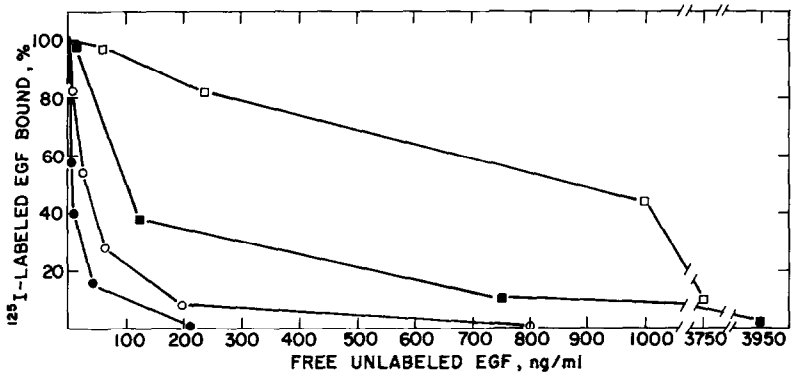

Fig. 1. Competitive displacement of ^{125}I-labeled EGF from placenta membranes by native EGF. Varying concentrations (23 μg per ml, ●; 70 μg per ml, 0; 338 μg per ml, ■; 2330 μg per ml, □) of placenta membranes were incubated in 0.2 ml of Krebs-Ringer-bicatbonate buffer, 0.1% albumin, pH 7.4, at 24° for 10 minutes with various concentrations of unlabeled EGF. ^{125}I-Labeled EGF (0.29 ng per ml, ●, or 1.8 ng per ml, 0, ■, □) was added and the incubation was continued for an additional 30 minutes at 24°. The suspensions were filtered through a Millipore filter to determine binding. Binding in the presence of 10^{-6} M EGF (nonspecific binding) has been subtracted. The concentration of free, unlabeled EGF was calculated as follows (cpm of ^{125}I-labeled EGF added minus total cpm bound) x (concentration of unlabeled EGF added)/(cpm of ^{125}I-labeled EGF added).

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with carrier free ^{125}I at a molar ratio of one, this would represent only about 250 dpm per ml.

Provided that the unlabeled and labeled ligands have the same affinity, the data used to plot competitive displacement curves can be evaluated by Scatchard plots (3). Estimations of affinity by Scatchard plot analyses are not invalidated by high concentrations of labeled ligand or binding sites. This method should be used in preference to competitive binding curves whenever it is suspected that either of these concentrations are not small compared to the dissociation constant. Under conditions of high ligand or binding site concentrations, however, other serious methodological problems may arise which can complicate proper analysis by Scatchard plots. For example, with very high concentrations of labeled ligand, where only a very small fraction (e.g., about 2 to 5%) of the total ligand is bound, the errors in estimating small changes in the concentration of the bound ligand may be large and magnified on Scatchard representations. On the other hand, if the binding site concentration is very high, such that a very large proportion of the labeled ligand is bound, accurate estimates of small changes in the concentration of free ligand may be prohibited by the presence of even small amounts of unbound, labeled

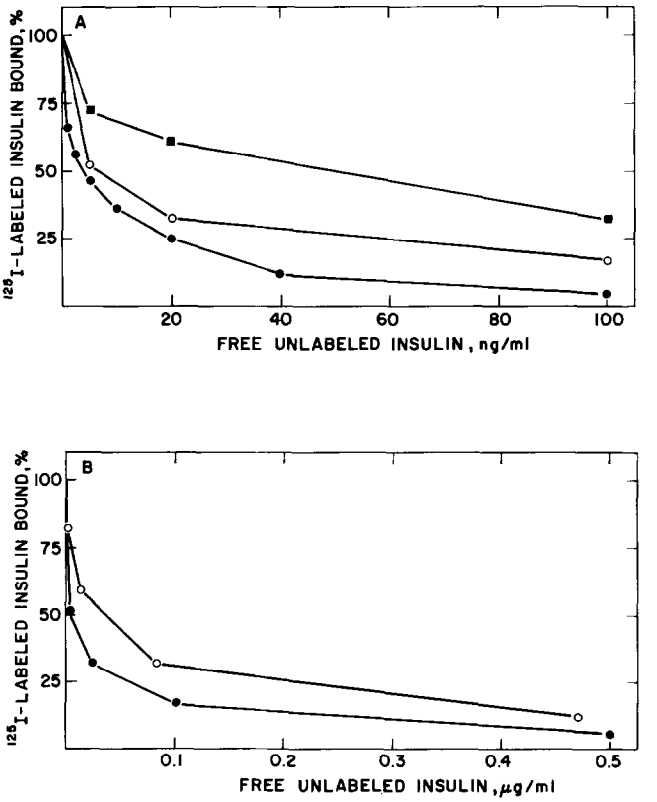

Fig. 2. Competitive displacement of ^{125}I-labeled insulin from placenta membranes by native insulin. A. Human placenta membranes, 46 micrograms per ml, were incubated (24°, 10 min) with varying concentrations of unlabeled insulin, and varying concentrations (0.35 ng per ml, ●; 1.1 ng per ml, 0; 9.4 ng per ml, ■) of ^{125}I-labeled insulin were added as described in Fig. 1. Calculations were performed as in Fig. 1. B. The indicated concentrations of unlabeled insulin, and ^{125}I-labeled insulin (1.09 ng per ml), were added to placenta membranes (46 μg per ml, ●; 1,170 μg per ml, 0) as described in Fig. 1. Calculations were performed as described in Fig. 1.

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ligand which may be chemically damaged or altered such that they do not bind properly. The latter situation is encountered commonly in studies of labeled peptide hormones.

When the unlabeled and labeled ligands do not have the same affinities, there is no simple way of determining the free, unlabeled ligand concentration (4). Under this condition Scatchard plots will not be linear (4, 5). Other conditions exist where Scatchard plots will not be linear. These include heterogeneity of binding sites (6), ligand-ligand interactions (7, 8), binding site-binding site interactions (7), heterogeneity of labeled ligand, non-

specific binding (8), inactivation or chemical transformation of the ligand or binding site during the binding reaction and reactions of the ligand with the binding site which are not of the form $A + B \rightleftharpoons AB$ (9). These conditions are contrary to the assumptions used to derive equation 10. When they exist equation 10 will not be valid.

Because differences in binding site concentration affect competition displacement curves, they should be used only cautiously when comparing the affinities of different tissues, different species or the same tissue in differing metabolic states (e.g., obesity, hyperinsulinemia). In such studies, what appears to be a difference in affinity, might result from a difference in binding site concentration. Similarly, the sensitivity of "radioreceptor" assays for measuring hormone concentrations in biological tissues will be limited by the concentrations of labeled ligand and membrane used in the assay. Such assays are based basically on competitive-displacement curves of the type illustrated in Fig. 1. To achieve experimental accuracy, it is necessary to work with a sufficiently high quantity of membrane-bound radioactivity. The relatively high membrane and radioligand concentrations which must be used will thus decrease the sensitivity of the assay.

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REFERENCES

1. Cuatrecasas, P. (1971) Proc. Nat. Acad. Sci. USA 68, 1264-1268.
2. O'Keefe, E., Hollenberg, M. D. and Cuatrecasas, P. (1974) Arch. Biochem. Biophys. 164, 518-526.
3. Scatchard, G. (1949) Anals N. Y. Acad. Sci. 51, 660-672.
4. Chang, K-J., Jacobs, S. and Cuatrecasas, P. (1975) Biochim. Biophys. Acta, in press.
5. Taylor, S. I. (1975) Biochemistry 14, 2357-2361.
6. Klotz, I. M. and Munston, D. L. (1971) Biochemistry 10, 3065-3069.
7. Nichol, L. W., Smith, G. D. and Ogston, A. G. (1969) Biochim. Biophys. Acta 184, 1-10.
8. Cuatrecasas, P. and Hollenberg, M. D. (1975) Biochem. Biophys. Res. Commun. 62, 31-41.
9. Swillens, S., Van Cauter, E., and Dumont, J. E. (1974) Biochim. Biophys. Acta 364, 250-259.