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The Bivalent Ligand Hypothesis

A Quantitative Model for Hormone Action

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SUMMARY

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A model is presented wherein a single hormone molecule is treated as bearing two distinct regions capable of interaction with the cell surface: a specificity determinant which binds with high affinity and specificity to one class of cell surface sites called receptor, and a response determinant which binds with much lower affinity and specificity to a second class of cell surface sites called effector. It is postulated that the steady-state response elicited by the hormone is proportional to the amount of hormone bound to effector and that, under ordinary conditions, the ratio of effector sites to receptor sites is substantially less than unity, perhaps on the order of 0.1. In this model, the formation of a hormone-receptor complex does not serve to initiate directly the process by which the response is elicited, but rather to increase selectively the apparent affinity of effector for a specific hormone by several orders of magnitude. It is shown that this model is capable of qualitatively and semiquantitatively rationalizing, in a unified manner, a broad variety of experimental data on the relationship between hormone binding and steady-state response elicitation, in both the absence and presence of cross-linking agents such as lectins and anti-hormone and anti-receptor antibodies.

1. INTRODUCTION

The elicitation of a biological response at the cellular level by hormone, and the dependence of the magnitude of the response upon the concentration of hormone, have been studied intensively for many years in a broad variety of hormone-cell systems. More recently, it has become generally recognized that the initial step in the process of response elicitation is the binding of hormone to sites on the cell surface, called receptors, which are specific for a particular hormone. Large numbers of experiments have been carried out to measure the dependence of specific hormone binding to intact cells, membranes, and solubilized receptors upon the concentration of hormone. These studies have revealed that the relationship between the concentration dependence of receptor binding and the concentration dependence of elicited response is highly variable, not only between different hormone-cell combinations, but also within a particular combination, depending upon experimental conditions (1). Numerous qualitative hypotheses and quantitative models have been put forward in attempts to correlate data on equilibrium binding and steady-state response elicitation (1, 2).

In this paper we present a new quantitative model, called the bivalent ligand model, for the mechanism of action of at least some classes of hormones. The bivalent ligand model is based upon the hypothesis that a hormone may simultaneously bind to, and thereby cross-link, two distinct and otherwise independent macromolecular species on the surface of the cell. By using this model we can rationalize and correlate a large body of experimental data, part of which is not amenable to interpretation within the context of previously proposed models.

In Section 2 we review basic principles governing the equilibrium binding of bivalent ligand to surfaces bearing one or more classes of binding sites. In Section 3 the basic bivalent ligand model of hormone action is introduced in quantitative terms, and general qualitative and quantitative features of binding and response isotherms calculated using this model are discussed. In Section 4 the model is extended to allow for the case in which hormone may be cross-linked by antibody, and general

This work is dedicated to the memory of Professor Willard F. Libby, teacher and friend.

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qualitative and quantitative features of binding and response isotherms calculated using the extended model are discussed. In Section 5 the bivalent ligand model is compared with previous quantitative models relating equilibrium binding to steady-state response elicitation, and the kinds of experimental observations which may be rationalized by both the previous models and the bivalent ligand model are reviewed. In Section 6 the extended model is used to interpret and correlate the results of recent experiments in which anti-hormone antibody is added to hormone-cell combinations. In Section 7 experimental data on the binding and response elicitation of "hormone-like" antibodies are interpreted within the context of the bivalent ligand model. In Section 8 the basic model is used to interpret in a unified manner the results of two otherwise unrelated studies of hormone binding to cell surfaces and solubilized membrane proteins. Section 9 concludes the paper with discussions of the role of cross-linking in hormone action, some kinetic consequences of the bivalent ligand model, and the distinction between agonists and antagonists.

2. EQUILIBRIUM RELATIONSHIPS GOVERNING BINDING OF BIVALENT LIGAND TO SURFACE SITES

We first summarize the primary features characterizing the symmetrically bivalent ligand, in which both valences bind to the same class of site on the cell surface, and then generalize to the case of the asymmetrically bivalent ligand, in which each valence is specific for a different class of surface site.

Symmetrically bivalent ligand. This case has been treated in the literature, particularly with reference to the binding of antibody to cell surface antigens (3–5). The results obtained below are equivalent to those obtained previously, but the derivation is presented to introduce notation and concepts which may be readily generalized to the case of the asymmetrically bivalent ligand. It is assumed, in accordance with a variety of experimental findings (6), that surface sites are free to diffuse in the plane of the surface.

For the simplest case of a single symmetrically bivalent ligand and a single class of surface sites, the following equilibria may be written:

$$\begin{aligned}[LS] &= K[L][S] \qquad \text{(step 1)} \\ [LS_2] &= \alpha K[LS][S] \qquad \text{(step 2)}\end{aligned} \tag{2.1}$$

The two reaction steps may be described in terms of their constituent standard enthalpy and entropy changes:

$$-RT \ln K = \Delta H_1^\circ - T\Delta S_1^\circ - RT \ln 2 \tag{2.2}$$

$$-RT \ln \alpha K = \Delta H_2^\circ - T\Delta S_2^\circ + RT \ln 2 \tag{2.3}$$

where R is the molar gas constant and T the absolute temperature. The factors $RT \ln 2$ result from the 2-fold degeneracy of the species LS, which is absent in the species L and LS_2. Since the two ligand valences and binding sites are identical by definition, we may set $\Delta H_1^\circ = \Delta H_2^\circ$. It then follows from Eq. 2.2 and Eq. 2.3 that

$$\ln \alpha = -(\Delta S_1^\circ - \Delta S_2^\circ)/R - 2 \ln 2 \tag{2.4}$$

To a first-order approximation, ΔS_1° corresponds to the difference between the translational and rotational entropy of a macromolecule moving in three dimensions and freely rotating about three mutually perpendicular axes, and the corresponding entropies of the ligand-site complex which is constrained to move in two dimensions and to rotate about a single axis. In the same approximation, ΔS_2° corresponds to the difference between the sum of the entropies of translation and rotation of a ligand-site complex and a free site, and the corresponding entropies of a site-ligand-site complex. It may be readily appreciated that while both S_1° and ΔS_2° are negative, ΔS_1° has a much larger absolute value, so that $\ln \alpha$ will in general be large and positive. We shall refer to α as the localization factor because it reflects the degree of localization of the second ligand valence in the vicinity of the surface due to the binding of the first valence to a surface site.

An alternate approach presented by Reynolds (5) is to consider the binding of ligand to the first site as a form of concentration of ligand in the vicinity of the surface, making the second valence much more accessible to free surface sites than was the first. Both this approach and the explicit consideration of entropy changes lead to the conclusion that, depending upon the detailed nature of the ligand-site interaction, the value of the localization factor may exceed 1 by many orders of magnitude.

It is emphasized that the concentrations of free and surface-bound species are not directly comparable, as the former are 3-dimensional volume concentrations whereas the latter are 2-dimensional surface concentrations or densities. This does not prevent us from writing dimensionally consistent and thermodynamically valid equilibrium relationships of the form of Eq. 2.1—as well as the more extensive sets of relationships to be encountered subsequently—so long as the appropriate dimensional units are assigned to association constants and localization factors.

The equilibrium average number of ligand molecules bound per site, $\bar{\nu}$, and the fractional site occupancy, FSO, may be written

$$\begin{aligned}\bar{\nu} &= ([LS] + [LS_2])/S_T \\ FSO &= (S_T - [S])/S_T\end{aligned} \tag{2.5}$$

where S_T, the total number of binding sites, is given by

$$S_T = [S] + [LS] + 2[LS_2] \tag{2.6}$$

In Fig. 1, $\bar{\nu}$ and FSO are plotted as functions of free ligand concentration for several values of α. Note that as α increases, two regions of binding affinity become resolved. The higher-affinity region corresponds to the formation of LS_2 and the lower-affinity region corresponds to the conversion of LS_2 to LS as additional ligand seeks to bind to the already fully occupied sites.

Asymmetrically bivalent ligand. For the simplest case of a single bivalent ligand and two classes of surface sites, the following equilibria may be written:

$$\begin{aligned}[LS^{(1)}] &= K_1[L][S^{(1)}] \\ [LS^{(2)}] &= K_2[L][S^{(2)}] \\ [LS^{(1)}S^{(2)}] &= \alpha K_1K_2[L][S^{(1)}][S^{(2)}]\end{aligned} \tag{2.7}$$

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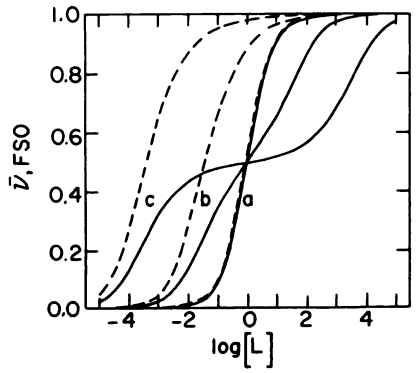

FIG. 1. *Binding of a symmetrically bivalent ligand and fractional site occupancy of a single class of surface sites as functions of free ligand concentration*

——, $\bar{\nu}$; - - -, *FSO*. Calculated using Eqs. 2.1 and 2.5 with $K = 1$. *a*, $\alpha = 0$; *b*, $\alpha = 100$; *c*, $\alpha = 10^4$.

The average number of ligand molecules bound per site is given by

$$\bar{\nu} = ([LS^{(1)}] + [LS^{(2)}] + [LS^{(1)}S^{(2)}])/(S_T^{(1)} + S_T^{(2)})$$

where

$$S_T^{(1)} = [S^{(1)}] + [LS^{(1)}] + [LS^{(1)}S^{(2)}] \qquad (2.8)$$

and

$$S_T^{(2)} = [S^{(2)}] + [LS^{(2)}] + [LS^{(1)}S^{(2)}]$$

If $K_1 = K_2$ and $S_T^{(1)} = S_T^{(2)} = 0.5$, the dependence of the binding isotherm upon the value of α will be identical with that shown in Fig. 1. In Fig. 2, binding isotherms are plotted for different ratios of $S_T^{(1)}$ to $S_T^{(2)}$. The inequality of $S_T^{(1)}$ and $S_T^{(2)}$ is associated with the appearance of a third region of intermediate binding affinity. The highest affinity region is due to the formation of $LS^{(1)}S^{(2)}$. The intermediate affinity region is due to the formation of $LS^{(1)}$ through combination of L with the $S^{(1)}$ which was in excess of $S^{(2)}$ and hence remained free after the formation of $LS^{(1)}S^{(2)}$. The low-affinity region is due to the conversion of $LS^{(1)}S^{(2)}$ to $LS^{(1)}$ and $LS^{(2)}$ as additional ligand binds to sites previously occupied by the bivalent complex.

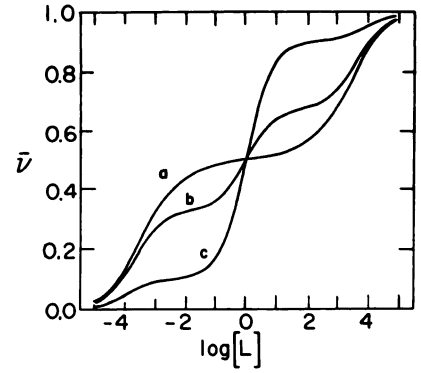

FIG. 2. *Binding of an asymmetrically bivalent ligand to two classes of surface sites as a function of free ligand concentration*

Calculated using Eqs. 2.6 and 2.7 with $K_1 = K_2 = 1$ and $\alpha = 10^4$. *a*, $S_T^{(1)} = S_T^{(2)} = 0.5$; *b*, $S_T^{(1)} = 0.67$, $S_T^{(2)} = 0.33$; *c*, $S_T^{(1)} = 0.9$, $S_T^{(2)} = 0.1$.

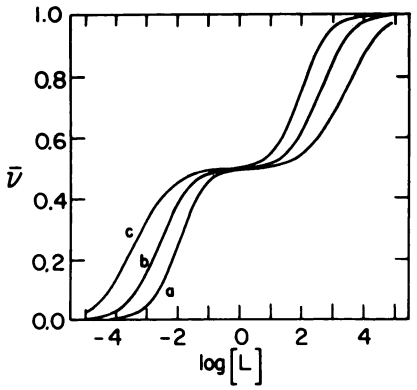

FIG. 3. *Binding of an asymmetrically bivalent ligand to two classes of surface sites as a function of free ligand concentration*

Calculated using Eqs. 2.6 and 2.7 with $K_1 = 100$, $K_2 = 0.01$, $S_T^{(1)} = S_T^{(2)} = 0.5$. *a*, $\alpha = 0$; *b*, $\alpha = 100$; *c*, $\alpha = 10^4$.

In Fig. 3 the dependence of the binding isotherm upon α for $S_T^{(1)} = S_T^{(2)} = 0.5$ and $K_1 = 10^4 K_2$ is shown. The effect of increasing α does not become apparent until the "splitting" of the isotherm into high and low affinity regions observed in Fig. 1 becomes comparable to or greater than that due to the intrinsic difference between K_1 and K_2.

In Fig. 4, the dependence of the binding isotherm upon α for $S_T^{(1)} = 0.9$, $S_T^{(2)} = 0.1$, and $K_1 = 10^4 K_2$ is shown. The major difference in the high-α and low-α isotherms occurs at low free ligand concentration, where $\bar{\nu}$ for the high-α isotherm is greater than that for the low-α isotherm at the same free ligand concentration. This difference represents the contribution of species $LS^{(1)}S^{(2)}$ to the high-α isotherm, which is absent from the low-α isotherm. Thus the high-α isotherm exhibits flattening at low saturations which might otherwise be attributed to binding site heterogeneity or negative cooperativity. Also plotted in Fig. 4 is the fractional occupancy of site class 2 as a function of free ligand concentration, defined as

$$FSO^{(2)} = (S_T^{(2)} - [S^{(2)}])/S_T^{(2)} \qquad (2.9)$$

It may be seen that a large localization factor has the effect of increasing the apparent affinity for ligand of an intrinsically low affinity class of binding sites by many orders of magnitude.

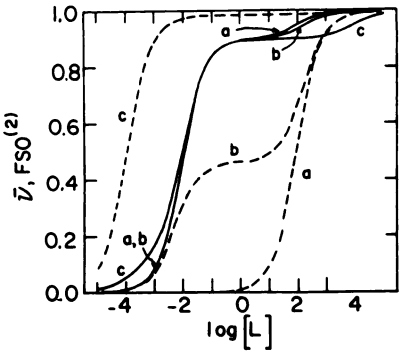

FIG. 4. *Binding of an asymmetrically bivalent ligand to two classes of surface sites and fractional occupancy of site class 2 as functions of free ligand concentration*

——, $\bar{\nu}$; - - -, $FSO^{(2)}$. Calculated using Eqs. 2.6 and 2.7 with $K_1 = 100$, $K_2 = 0.01$, $S_T^{(1)} = 0.9$, $S_T^{(2)} = 0.1$. *a*, $\alpha = 0$; *b*, $\alpha = 100$; *c*, $\alpha = 10^4$.

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This simple model of a single asymmetrically bivalent ligand and two species of surface sites may readily be extended and generalized in a variety of ways. One such extension, to allow for the possibility of cross-linking ligand, is presented in detail in Section 4, and other extensions are suggested in subsequent sections.

3. THE BIVALENT LIGAND HYPOTHESIS OF HORMONE ACTION: BASIC MODEL

It is suggested that some hormones, among them insulin and epidermal growth factor, might behave as asymmetrically bivalent ligands. One of the hormone valences, which we shall call the specificity determinant, is presumed to bind with high affinity and selectivity to a single homogeneous class of sites on the cell surface which we shall call *receptor*. The second hormone valence, which we shall call the response determinant, is presumed to bind with much lower affinity and specificity to one or more different classes of sites on the cell surface. It is proposed, as a first approximation, that the steady-state biological response associated with hormone binding is proportional to the extent of occupancy of one of these classes of low affinity sites, which we shall call *effector*. It is proposed that the ratio of receptor is less than 1 and may be quite small. The remaining classes of low-affinity sites, which may or may not be large in number relative to receptor, are assumed to be unrelated to biological response and are hence called non-effector. It may be shown[1] that the behavior exhibited by systems containing even large amounts of noneffector may be mimicked by an equivalent system containing receptor and effector only. The binding constants, localization factor, and ratio of effector and receptor characterizing the equivalent system are, in the most general case, functions of the ratio of noneffector to receptor in the real system. If it is assumed that this ratio is constant—as would be expected for a well characterized cell line—then the behavior of a system containing significant amounts of noneffector may be validly described in terms of the constant parameters of the equivalent system containing receptor and effector only.

We therefore postulate the following equilibria, denoting hormone by H, receptor by R, and effector by E.

$$\begin{aligned}[HR] &= K_R[H][R] \\ [HE] &= K_E[H][E] \\ [HRE] &= \alpha K_R K_E[H][R][E]\end{aligned} \tag{3.1}$$

It will be assumed that the value of K_E is so low that, at the highest concentrations of hormone encountered experimentally, $K_E[H] << 1$. The amount of HE formed at equilibrium will then be essentially proportional to the concentration of free hormone, and radiolabeled hormone bound as the non-receptor associated species will not be displaced by large excesses of unlabeled hormone. Nonspecific binding may thus be formally attributed to the formation of HE, and this species will not contribute to the calculation of specific binding isotherms.

[1] A. P. Minton, unpublished data. Calculations are available upon request.

The total amounts of receptor and effector are given by

$$\begin{aligned}R_T &= [R] + [HR] + [HRE] \\ E_T &= [E] + [HE] + [HRE]\end{aligned} \tag{3.2}$$

Given the values of K_R, K_E, R_T, E_T, and $[H]$, the concentrations of all species may be calculated using Eqs. 3.1 and 3.2. We define the average number of molecules of hormone bound per receptor to be

$$y_B = ([HR] + [HRE])/R_T \tag{3.3}$$

and the fractional elicited steady-state response to be

$$y_R = (E_T - [E])/E_T \tag{3.4}$$

In Fig. 5A binding and response isotherms are plotted for a constant $R_T{:}E_T$ ratio of 10:1 and a broad range of values of α. The plateau in the response isotherms observed at log $[H] = 0$ may be taken to represent the maximal level of fractional response observed experimentally, denoted by y_R^{max}. For smaller values of α, y_R^{max}, may be considerably less than the theoretical limiting value of 1.0.

We define the binding half-saturation concentration, denoted by $[H]_{50}^B$, to be equal to that concentration of free hormone at which $y_B = ½$. The response half-saturation concentration, denoted by $[H]_{50}^R$, is defined to be equal to that concentration of free hormone at which $y_R = y_R^{max}/2$. In Fig. 5B, log $[H]_{50}^B$, log $[H]_{50}^R$, and y_R^{max}, are plotted as functions of log α. An increase in α is associated with the following changes: (a) the appearance of enhanced binding at low receptor saturation (which might otherwise be attributed to site heterogeneity or negative cooperativity); (b) a decrease in the concentration of hormone required to elicit half-maximal response; and (c) an increase in the magnitude of the maximal response.

The basic BL model predicts that $[H]_{50}^B$ will always be equal to or greater than $[H]_{50}^R$, and that the response isotherm will be noncooperative. The model does not predict the appearance of positively cooperative binding isotherms for any combination or parameter values.

4. EXTENSION OF THE BIVALENT LIGAND MODEL TO ALLOW FOR CROSSLINKING OF LIGAND

In Section 6 we discuss the results of observations made on systems in which anti-hormone antibody has been added to hormone-cell combinations. In such a system one must allow for the possible existence of many species; these are defined and schematically depicted in Fig. 6.

The binding of antibody to hormone has two major effects upon the system.

1. The mass of the antibody-hormone complex is considerably larger than that of the free hormone, and the entropy decrease associated with initial binding of the complex to the cell surface, $\Delta S_1°$, will be larger than that associated with initial binding of antibody-free hormone. In addition, antibody may compete with receptor for binding to the specificity determinant of the hormone or may compete with effector for binding to the response determinant of hormone. Thus the association constant for initial binding of the antibody-hormone complex to

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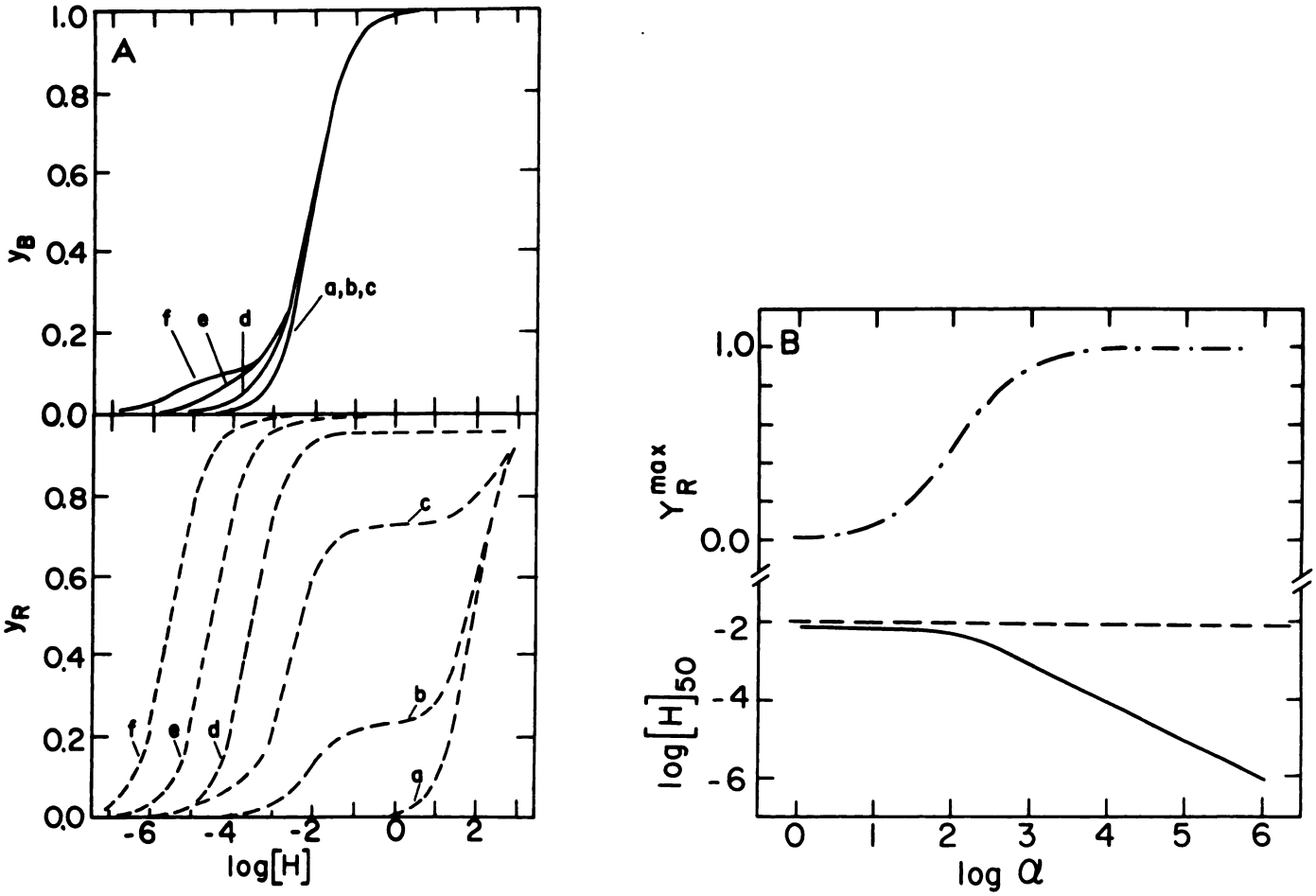

FIG. 5. *Hormone binding and elicited response as functions of free hormone concentration*

A. Calculated using Eqs. 3.1–3.4 with $K_R = 100$, $K_E = 0.01$, $R_T = 1.0$, and $E_T = 0.1$. ——, y_B; - - -, y_R. *a*, $\alpha = 0$; *b*, $\alpha = 30$; *c*, $\alpha = 300$; *d*, $\alpha = 3000$; *e*, $\alpha = 3 \times 10^4$; *f*, $\alpha = 3 \times 10^5$.

B. Log $[H]_{50}^R$ (——), log $[H]_{50}^B$ (- - -), and y_R^{max} (-·-·-) as functions of log α. Calculated using same equations and parameter values as in Fig. 5A.

ether receptor or effector will be reduced by a factor f for the complex AH and a factor f' for the complex AH_2, relative to the corresponding binding constant for antibody-free hormone. If the mass of the antibody is large relative to the mass of the hormone, then f' will be approximately equal to f, and both will be substantially less than 1.

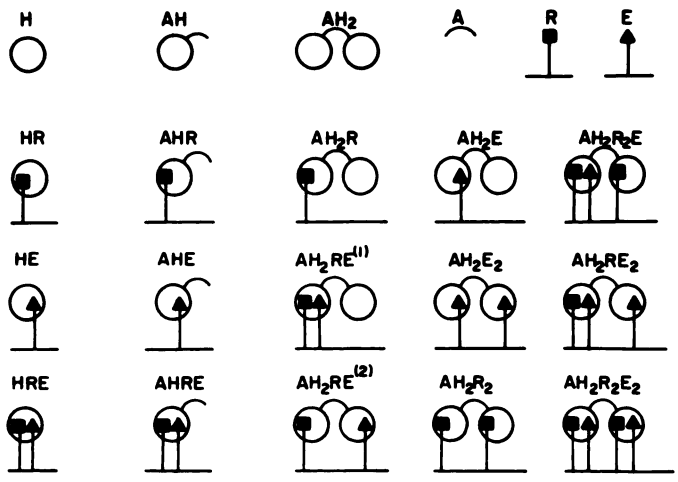

FIG. 6. *Possible species in a system containing symmetrically bivalent anti-hormone antibody (A), asymmetrically bivalent hormone (H), receptor (R), and effector (E)*

2. When the first hormone molecule of the complex AH_2 binds to the cell surface, the mobility of the second hormone molecule is diminished and the entropy decrease associated with binding of this molecule to cell surface is correspondingly diminished. Thus the association constant for initial binding of the second hormone molecule of the complex AH_2 to either R or E will be enhanced by a second localization factor α_A. It is presumed that the positions of the two hormone molecules in the complex AH_2 will not be as closely correlated as are those of the specificity and response determinants of a single native hormone molecule. Hence $\alpha > \alpha_A > 1$.

Experiments discussed in Section 6 suggest that under some conditions the binding of antibody to hormone may have an effect upon the value of α. Thus to increase the generality of the model we designate α_0 as the possibly distinct value of α associated with the formation of the antibody-free complex HRE.

Given the above assumptions, the following equilibrium expressions may be written:

$$[AH] = 2K_A\,[A]\,[H]$$
$$[AH_2] = K_A{}^2\,[A]\,[H]^2 \qquad (4.1)$$

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and

$$
\begin{aligned}
[HR] &= K_R\,[H]\,[R] \\
[HE] &= K_E\,[H]\,[E] \\
[HRE] &= \alpha_0\,K_R\,K_E\,[H]\,[R]\,[E] \\
[AHR] &= fK_R\,[AH]\,[R] \\
[AHE] &= fK_E\,[AH]\,[E] \\
[AHRE] &= f\alpha K_R K_E\,[AH]\,[R]\,[E] \\
[AH_2R] &= 2f'K_R\,[AH_2]\,[R] \\
[AH_2E] &= 2f'K_E\,[AH_2]\,[E] \\
[AH_2RE^{(1)}] &= 2f'\alpha K_R K_E\,[AH_2]\,[R]\,[E] \\
[AH_2RE^{(2)}] &= 2f'\alpha_A K_R K_E\,[AH_2]\,[R]\,[E] \\
[AH_2R_2] &= f'\alpha_A K_R^2\,[AH_2]\,[R]^2 \\
[AH_2R_2E] &= 2f'\alpha\alpha_A K_R^2 K_E\,[AH_2]\,[R]^2\,[E] \\
[AH_2E_2] &= f'\alpha_A K_E^2\,[AH_2]\,[E]^2 \\
[AH_2RE_2] &= 2f'\alpha\alpha_A K_R K_E^2\,[AH_2]\,[R]\,[E]^2 \\
[AH_2R_2E_2] &= f'\alpha^2\alpha_A K_R^2 K_E^2\,[AH_2]\,[R]^2\,[E]^2
\end{aligned}
\tag{4.2}
$$

where K_A is the equilibrium constant for association of hormone and antibody in solution, and the factor 2 is introduced to allow for degeneracy of asymmetrical species.

It is evident from the form of the above relations that the factors f' and α_A, being respectively less than and greater than 1, will tend to compensate each other to a greater or lesser extent. Since our incomplete knowledge of the structures of, and interactions between, antibody, hormone, and cell surface does not permit an *a priori* calculation of either of these factors, we can only note that it is possible, at least in principle, for the cross-linked hormone to bind to the cell surface with an affinity which is greater than, equal to, or less than that of the same hormone free of antibody. However, the model does predict that antibody-bound hormone which is not cross-linked (i.e., existing either as AH or Fab—H) will bind to the cell surface with an affinity which is less than that of cross-linked hormone, and ordinarily less than that of free hormone as well. An exception to this latter case may arise if $\alpha_0 < f\alpha$.

The total amounts of receptor and effector are given by

$$
\begin{aligned}
R_T = [R] &+ [HR] + [HRE] + [AHR] \\
&+ [AHRE] + [AH_2R] + [AH_2RE^{(1)}] \\
&+ [AH_2RE^{(2)}] + [AH_2RE_2] + 2([AH_2R_2] \\
&+ [AH_2R_2E] + [AH_2R_2E_2]) \\
E_T = [E] &+ [HE] + [HRE] + [AHRE] + [AH_2E] \\
&+ [AH_2RE^{(1)}] + [AH_2RE^{(2)}] \\
&+ [AH_2R_2E] + 2([AH_2E_2] + [AH_2RE_2] \\
&+ [AH_2RE_2] + [AH_2R_2E_2])
\end{aligned}
\tag{4.3}
$$

and the total amounts of hormone and antibody are given by

$$
\begin{aligned}
H_T &= [H] + [HA] + 2[AH_2] \\
&\quad + (\text{surface-bound } H) \\
A_T &= [A] + [HA] + [AH_2] + (\text{surface-bound } A)
\end{aligned}
\tag{4.4}
$$

In order to simplify solution of the equilibrium relationships 4.1–4.4, it will be assumed that the total amounts of hormone and antibody present are at least two orders of magnitude greater than the total binding capacity of the cell surface, and that as a result the concentration of free H, AH, and AH_2 will not be significantly depleted by the binding of (at most) a very small fraction of each species to the cell surface. In this case we may neglect the last term on the righthand sides of Eq. 4.4 relative to the sum of the first three terms in each equation, effectively decoupling Eqs. 4.1 and 4.4 from Eqs. 4.2 and 4.3. We may then solve the entire set of Eqs. 4.1–4.4 in the following manner. Given K_A, H_T, and A_T, we solve Eqs. 4.1 and 4.4 to obtain $[H]$, $[AH]$, and $[AH_2]$. Given the concentrations of these three species, K_R, K_E, α_0, α, α_A, f, f', R_T, and E_T, we then solve Eqs. 4.2 and 4.3 for the concentrations of all surface-bound species.

We may then calculate the binding and response isotherms using the following relations:

$$
\begin{aligned}
y_B = \{&[HR] + [HRE] + [AHR] + 2([AH_2R] \\
&+ [AH_2RE^{(1)}] + [AH_2RE^{(2)}] + [AH_2R_2] \\
&+ [AH_2R_2E] + [AH_2RE_2] + [AH_2R_2E_2])\}/R_T \\
y_R &= (E_T - [E])/E_T
\end{aligned}
\tag{4.5}
$$

In contrast to the basic model, the value of y_B in the extended model has a theoretical maximum of 2 rather than 1. However, results calculated using a wide variety of parameter values reveal the presence of a plateau in the binding isotherm at $y_B = 1.0$ which extends for one or more orders of magnitude in free hormone concentration. Hence we conclude that $y_B = 1.0$ corresponds to the experimentally accessible upper limit of specific hormone binding.

In Fig. 7, binding and response isotherms are plotted for three cases: *a*, the hormone is free of antibody; *b*, the hormone is entirely bound to antibody as the non-cross-

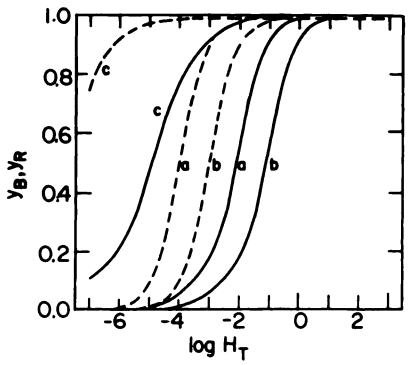

FIG. 7. *Binding and response as functions of total hormone concentration*

——, y_B; – – –, y_R. Calculated using Eqs. 4.1–4.4 with $R_T = 1$, $E_T = 0.1$, $K_R = 100$, $K_E = 0.01$, $\alpha_0 = \alpha = 10^4$, $\alpha_A = 10^2$, $f = f' = 0.1$. *a*, Antibody absent; *b*, all hormone present as AH; *c*, all hormone present as AH_2.

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linked complex AH; and c, the hormone is entirely bound to antibody as the cross-linked complex AH_2. We observe that the binding and response isotherms for cross-linked hormone are decidedly left-shifted relative to the corresponding isotherms for antibody-free hormone and that the binding isotherm for cross-linked hormone is somewhat less steep than the other two isotherms. This flattening effect was previously noted in the case of the symmetrically bivalent ligand reviewed in Section 2 (cf. high affinity region of Fig. 1). Also, the binding and response isotherms for the non-cross-linked antibody-bound hormone are right-shifted relative to the corresponding isotherms for antibody-free hormone, and the shape of the binding isotherm is not altered. Finally, the values of $[H]^B_{50}$ and $[H]^R_{50}$ for cross-linked hormone are several orders of magnitude smaller than the corresponding values for non-cross-linked antibody-bound hormone.

In a solution containing antibody and hormone, essentially all hormone will exist as H at limiting low concentrations of antibody ($A_T \rightarrow 0$), and essentially all hormone will exist as AH at limiting high concentrations of antibody ($A_T \rightarrow \infty$). The concentration of AH_2 will reach a maximum at intermediate ratios of antibody to hormone, but the mole fraction of hormone present as AH_2 cannot exceed 0.5 under equilibrium conditions. Thus, although the binding and response elicitation of hormone are greatly enhanced by cross-linking, the full effect of cross-linking is not observed in equilibrium mixtures of hormone and antibody. We subsequently discuss experiments involving antibody which has a hormone-like ability to elicit response. In contrast to experiments utilizing equilibrium mixtures of hormone and anti-hormone antibody, comparison of the hormone-like behavior of intact "anti-membrane" antibody and the Fab fragment derived therefrom should reveal the full effect of cross-linking.

In Fig. 8a–c, the dependence of binding and response upon antibody concentration has been plotted for two fixed total hormone concentrations and three values of the product $f\alpha_A$ (for $f' = f$). When $f\alpha_A > 1$ (Fig. 8a), one observes that the addition of antibody can elicit significant levels of response at hormone concentrations which are too low to elicit significant response in the absence of antibody. An increase in binding may or may not be observed, depending upon whether or not hormone at that particular total concentration binds significantly to the cell surface in the absence of antibody. When $f\alpha_A < 1$ (Fig. 8c), one observes no enhancement of either binding or response, but only diminishment corresponding to the conversion of hormone to the species AH, whose binding properties are impaired relative to those of H.

5. COMPARISON OF THE BIVALENT LIGAND MODEL AND PREVIOUS RELATED MODELS

Most previously proposed models which relate ligand binding (ligand = hormone or drug) to the steady-state magnitude of response elicited by ligand are based upon the hypothesis that response is proportional to the number of receptors to which ligand is bound (2). Such models cannot account for the variety of nonlinear relationships between binding and response which have been observed in various systems (1). Molecular hypotheses which quantitatively relate equilibrium binding of ligand to the steady-state magnitude of elicited response in a nonlinear fashion may be classified as follows:

Nonstoichiometric floating receptor hypotheses (7–9). The binding of ligand to receptor is associated with increased affinity of receptor for effector. The magnitude of elicited response is assumed to be proportional to the amount of receptor-effect complex.

Multiple activation hypotheses (10, 11). The binding of ligand to a single receptor molecule is associated with the potential activation of several molecules of effector. The magnitude of elicited response is assumed to be proportional to the amount of activated effector.

Multisubsite hypotheses (12). Ligand possesses two or more valences, each specific to a single subsite of the receptor. The magnitude of elicited response is assumed to be proportional to the number of sites in which all subsites are simultaneously occupied by the corresponding valences of a single ligand molecule.

The multisubsite model for a bivalent ligand differs from the bivalent ligand model presented here in two important respects: (a) subsites of different classes are by definition present in stoichiometrically equal quantities, and (b) response is presumed to be proportional to a particular occupancy state of all subsites taken together, rather than the occupancy state of one particular class of subsite.

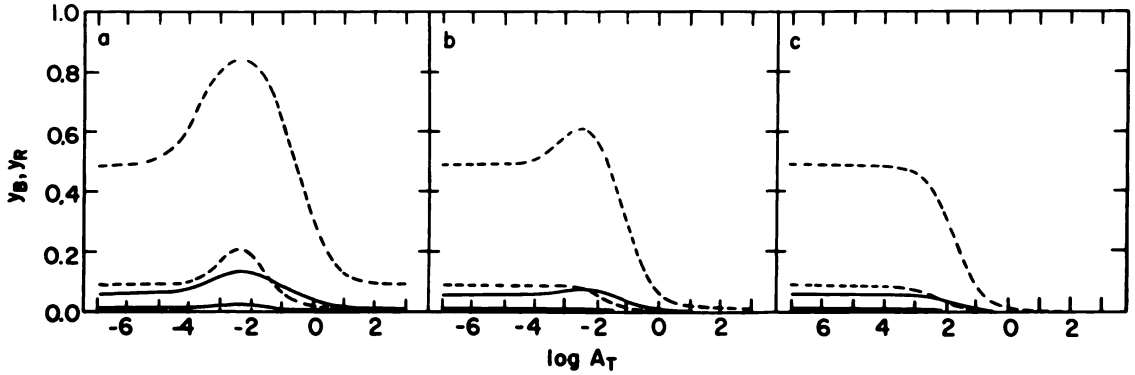

FIG. 8. *Binding and response as functions of antibody concentration for fixed total hormone concentration*
——, y_B; – – –, y_R. Calculated using Eqs. 4.1–4.4 with R_T, E_T, K_R, K_E, α_0, α, and α_A as in Fig. 7, and $K_A = 100$. The *upper* binding and response curves in each frame were calculated with $H_T = 10^{-4}$; the *lower* curves with $H_T = 10^{-5}$. a, $f = f' = 0.1$; b, $f = f' = 0.01$; c, $f = f' = 0.001$.

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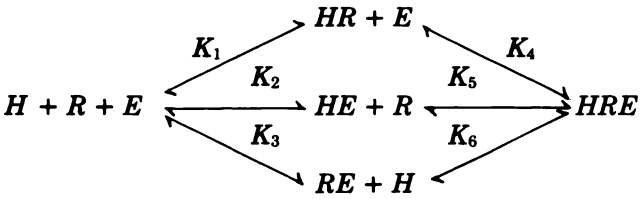
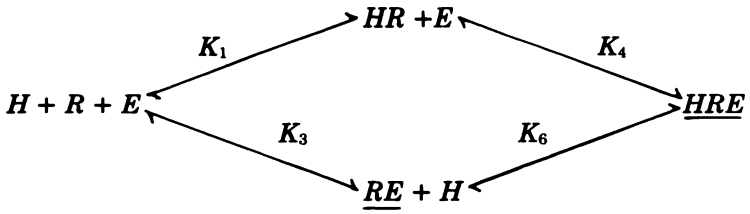
The multiple activation hypothesis represents a generalization of the floating receptor hypothesis in which the receptor molecule is permitted to interact with more than one molecule of effector. The bivalent ligand hypothesis most closely resembles, and may most easily be compared with, the simple floating receptor hypothesis in which receptor interacts with a single effector molecule. The relationship between models derived from these two hypotheses may be clearly shown by means of the three sets of equilibria depicted below.

Generalized association of three species:



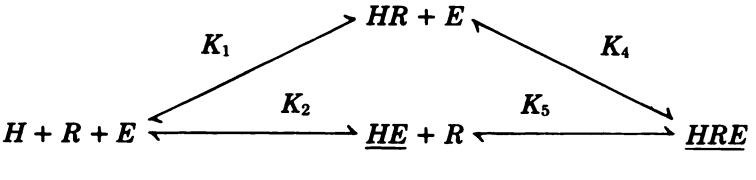
Floating receptor models:



$K_1 \gg K_3$

$(K_4 \ll K_6)$

Bivalent ligand model:



$K_1 \gg K_2$

$(K_4 \ll K_5)$

The steady-state biological response in each model is presumed to be proportional to the sum of the amounts of underlined species. In the floating receptor models, formation of the binary complex *HE* is excluded, whereas in the bivalent ligand model, formation of the binary complex *RE* is excluded. In both, floating receptor and bivalent ligand models, receptor, effector, and hormone may exist as two of three possible binary complexes and one ternary complex, and in both models the formation of the binary species *HR* is highly preferred over that of the second allowed binary species. Under conditions such that the concentration of the second allowed binary species is negligible relative to that of *HR* and *HRE* throughout the experimentally accessible range of free hormone concentration, the floating receptor and bivalent ligand models will be nearly identical from a functional standpoint, although obviously not from a structural standpoint. These are precisely the conditions under which these models are applied to the analysis of experimental data, so we may conclude that the floating receptor and bivalent ligand models will be capable for rationalizing many of the same experimental observations in nearly identical energetic and stoichiometric terms. Such observations include the following (7, 8):

1. Apparent site heterogeneity or negative cooperativity of hormone binding in some systems.
2. Lack of cooperativity with respect to response elicitation in the same systems.
3. Saturation of response at low levels of binding ("spare receptors") in the same systems.
4. Different effects of the same hormone in different target cells. This is attributed to a common receptor and differing effectors.
5. Similar response of one target cell to different hormones. This is attributed to the presence of more than one receptor and a common effector.

Even though there exist important similarities between the bivalent ligand and floating receptor models, fundamental differences remain. The most important of these is that in the bivalent ligand model, hormone is capable in principle of eliciting a response without binding to receptor, whereas in floating receptor (and multiple activation) models, hormone is not capable of doing so. In subsequent sections we see that this feature of the bivalent ligand model permits simple rationalization of several experimentally observed phenomena which would be difficult to explain using either the floating receptor or multiple activation models in their present forms.

6. THE EFFECT OF ANTIHORMONE ANTIBODIES ON HORMONE BINDING AND RESPONSE ELICITATION: BIVALENT LIGAND MODEL INTERPRETATION

In two recently reported studies, Schechter and coworkers (13, 14) have investigated the effect of the addition of antihormone antibodies to cell preparations containing concentrations of insulin or EGF[2] which were too low to elicit significant response in the absence of antibody. In both systems it was found that as the concentration of antibody increases, the elicited response increases substantially, reaches a maximal level, and then

[2] The abbreviations used are: EGF, epidermal growth factor; WGA, wheat germ agglutinin.

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declines with further addition of antibody. A parallel assay for hormone binding, carried out at much higher hormone concentrations, indicated that as the amount of anti-insulin antibody increases, the amount of insulin bound increases substantially, reaches a maximum, and declines in a manner which is comparable to the dependence of elicited response upon antibody concentration. In contrast, the binding of EGF to cells is either only modestly or not at all enhanced by the addition of anti-EGF antibody. When Fab fragments were added in place of the corresponding antibody, inhibition of binding and no response elicitation were observed.

These findings may be readily rationalized within the context of the bivalent ligand model if the following additional postulates are made.

1. In the case of EGF, the localization factor for antibody-bound hormone, α, is greater than the localization factor for antibody-free hormone, α_0. The high specificity of antibody for antigen suggests that the antigen-antibody complex contains a large number of discrete intermolecular interactions distributed about an extensive antibody-antigen interface. Just as short-range intermolecular interactions may confer additional structural rigidity upon molecules in a crystal lattice (15), we surmise that the hormone acquires additional structural rigidity upon complexation with antibody, which is reflected in a larger value of α. In the case of insulin, it is not necessary to postulate that $\alpha > \alpha_0$ in order to account for the observed results, but such an increase would not be inconsistent with these results.

2. The degree to which complexation with antibody interferes with the binding of hormone to the cell surface is greater in the case of EGF than in the case of insulin. Hence the values of f and f' are smaller for EGF than the corresponding values for insulin.

In accordance with these qualitative postulates, bivalent ligand model parameter values were chosen so as to mimic the qualitative features of the observations summarized above. In Fig. 9a, binding and response isotherms are plotted which simulate the behavior exhibited by the insulin/anti-insulin system. At a moderate hormone concentration corresponding to a fractional receptor saturation of 0.25 in the absence of antibody, the level of hormone binding first increases and then decreases as antibody is added. At a much lower hormone concentration, the level of response increases from near zero to a significant value and then decreases to near zero as antibody is added. In Fig. 9b, binding and response isotherms are plotted which simulate the behavior exhibited by the EGF/anti-EGF system. At a moderate hormone concentration corresponding to a fractional receptor saturation of approximately 0.55 in the absence of antibody, the level of hormone binding is initially unaffected and then decreases as antibody is added. At a much lower hormone concentration, the level of response increases significantly and then decreases to near zero as antibody is added.

According to the extended bivalent ligand model, the addition of Fab fragments to either hormone system in place of intact antibody would result in a monotonic change in the levels of binding and response from those characterizing the system in the absence of antibody to

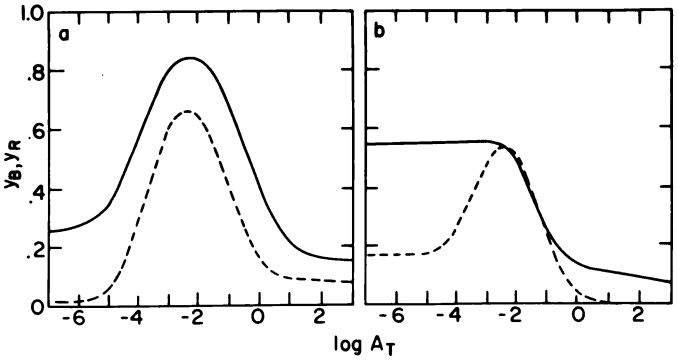

FIG. 9. *Binding and response as functions of antibody concentration*

——, y_B; – – –, y_R. Calculated using Eqs. 4.1–4.4 with R_T, E_T, K_1, K_2, K_A, α_0, and α_A as in Fig. 8, and $\alpha = 3 \times 10^5$.

a. Simulation of insulin/anti-insulin system: $f = f' = 0.3$. Binding curve calculated for $H_T = 2 \times 10^{-3}$, response curve calculated for $H_T = 1 \times 10^{-6}$.

b. Simulation of EGF/anti-EGF system: $f = f' = 0.0003$. Binding curve calculated for $H_T = 1 \times 10^{-2}$, response curve calculated for $H_T = 2 \times 10^{-5}$.

levels similar to those characterizing the system in the presence of a large excess of intact antibody. Thus neither binding nor response would be significantly increased at any added concentration of Fab, in agreement with experiments reported in refs. 13 and 14.

Schechter and co-workers (13) reported the preparation of an EGF derivative by cleavage of the single-polypeptide chain with CNBr. The resulting derivative, called CNBr-EGF, contains two shorter polypeptide chains linked by disulfide bonds. This derivative binds to cell surfaces with an affinity which is roughly one order of magnitude lower than that of native hormone, and does not elicit a significant response even when bound. The addition of anti-(native) EGF antibody to cell preparations containing the derivative results in essentially full restoration of native activity over a narrow range of antibody concentrations.

We may rationalize these findings within the context of the bivalent ligand model by adopting the following additional postulates.

1. The cleavage of the polypeptide chain of EGF results in a lowering of the affinity of the specificity and response determinants of the hormone for receptor and effector, respectively, by roughly a factor of 10. Ten-fold reductions in K_R and K_E could easily result from mild structural perturbations of the determinants.

2. The cleavage of the polypeptide chain of EGF results in a considerable lowering of the localization factor for antibody-free hormone, α_0. An effect of this sort would be expected if the specificity and response determinants resided on the two different segments of polypeptide chain created by the CNBr cleavage.

In Fig. 10 are plotted binding and response isotherms for simulated "native" and " modified" hormone, calculated in accordance with the postulates listed above.

We additionally postulate that the ability of antibody-bound CNBr-EGF to elicit a normal response may be attributed to the fact that the antibody is complementary to native, rather than modified, hormone. It is likely that

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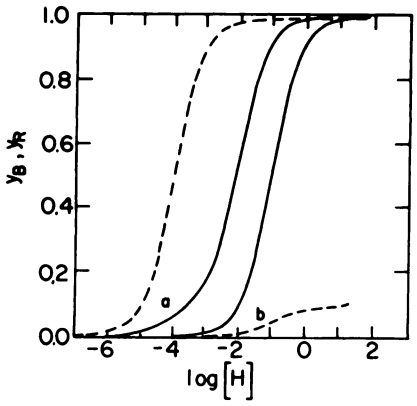

FIG. 10. *Simulated binding and response isotherms*
——, y_B; - - -, y_R. Calculated using Eqs. 3.1–3.4 with $R_T = 1$, $E_T = 0.1$. *a*, "Native hormone": $K_R = 100$, $K_E = 0.01$, $\alpha = 10^4$; *b*, "modified hormone": $K_R = 10$, $K_E = 0.001$, $\alpha = 100$.

this antibody will tend to bind the derivative in such a manner that the tertiary structure of the complexed derivative will be similar to if not identical with that of the native EGF molecule, and that the structural rigidity of the antibody-bound derivative will be comparable to that of the antibody-bound native hormone. Therefore, the value of α characterizing CNBr-EGF which is bound to antibody is assumed to be comparable to the value of α characterizing native EGF which is bound to the same antibody.

In Fig. 11 is plotted the simulated dependence of response upon antibody concentration for a fixed concentration of CNBr-EGF, calculated in accordance with the above postulates. At intermediate antibody concentrations, a large measure of elicited response is indeed restored, in qualitative agreement with the experimental observation. However, the width of the simulated response peak is much broader than that observed experimentally, indicating that not all factors contributing to the shape of this peak have been adequately taken into account in the present formulation.

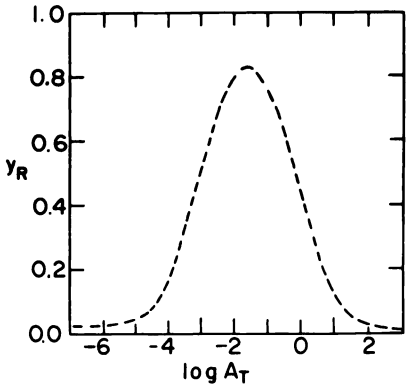

FIG. 11. *Response as a function of antibody concentration at fixed total hormone concentration*
Calculated using Eqs. 4.1–4.4 with $R_T = 1$, $E_T = 0.1$, $K_R = 10$, $K_E = 0.001$, $K_A = 100$, $\alpha_0 = 100$, $\alpha = 1 \times 10^5$, $\alpha_A = 100$, $f = f' = 0.0003$, and $H_T = 3 \times 10^{-2}$.

7. THE HORMONE-LIKE PROPERTIES OF ANTIMEMBRANE ANTIBODIES: BIVALENT LIGAND MODEL INTERPRETATION

Antibodies isolated from sera of some unusual patients exhibiting insulin-resistance syndrome were shown to compete with insulin for binding to cell surfaces and to elicit an insulin-like response when added to cells in the absence of hormone (16). More recently, antibodies have been obtained from the sera of rabbits which have been immunized by injection with plasma membranes, solubilized intrinsic membrane proteins, and partially purified receptor (17, 18). These antibodies also elicit an insulin-like response when added to liver or fat cells in the absence of insulin. The degree to which these response-eliciting antibodies compete with insulin for binding to the cell surface is highly variable; some compete poorly or not at all. The noncompeting antibodies retain the ability to bind to and elicit an insulin-like response from trypsinized cells which lack the ability to bind or respond to insulin. The Fab fragments prepared from these antibodies lack the ability to elicit an insulin-like response, but when anti-Fab antibody is added to cell preparations containing Fab, the ability to elicit response is partially restored.

Let us postulate that the preparation used to immunize the test animal (or the intact cells responsible for the pathological autoimmune response) contains a variety of antigenic determinants, among them the hormone-binding site of effector. The serum of the immunized animal or patient will then contain a heterogeneous population of antibodies. Each antibody species should behave as a classical, symmetrically bivalent ligand which can bind two identical antigenic groups with high affinity. One fraction of the antibody population (which may comprise a very small fraction of total antibody) is presumed to be specific for the hormone-binding site of effector, and it is moreover assumed that, when this anti-effector antibody binds to effector, a response will be elicited which is similar to that elicited by hormone. According to this picture, the competition of antibody with hormone for the receptor binding site on one hand, and the elicitation of hormone-like response by antibody on the other hand, are essentially independent phenomena, related only by the relative sizes of the receptor-specific and effector-specific fractions of total antibody.

It is not difficult to perceive how some fraction of antibody which has arisen through immune response to complex antigens containing many protein components (including effector) might be specific for effector. Even the "highly purified" preparation of solubilized receptor reported by Jacobs and co-workers (17) may contain small amounts of effector. In Section 8, experimental evidence for the formation of receptor-effector complexes in solubilizing buffer containing Triton X-100 are reviewed. In any event, Jacobs and co-workers (17) reported that polyacrylamide gel electrophoresis of the purified receptor preparation revealed the presence of "... a major ... band with an apparent molecular weight of 135,000 and minor bands of lower molecular weight." One of these "minor" bands could well have been effector, and hence it is not unreasonable to suggest that immu-

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nization with this preparation could yield effector-specific, as well as receptor-specific, antibodies.

The effect of replacing anti-effector antibody by anti-effector Fab fragments may be simulated by setting α in Eq. 2.2 equal to zero, thus reducing antibody to a monovalent ligand. In Fig. 1, one can see how a decrease in α results in a decrease in site occupancy at a fixed concentration of ligand. Since anti-effector antibody by definition binds only to effector, the elicited response is in this instance proportional to site occupancy. Hence we see that the replacement of anti-effector antibody by anti-effector Fab will result in a substantial reduction or elimination of the elicited response observed at low antibody concentrations.

The addition of anti-Fab antibody to a mixture of cells and Fab fragments may in principle permit the formation of hybrid species in which the antibody binds one anti-effector Fab fragment and one Fab fragment specific for another antigen. Since both Fab fragments are presumed to bind with comparable affinities to their respective antigenic determinants, the asymmetrically bivalent hybrid species will bind to effector (and the second determinant) with greater apparent affinity than the Fab fragments alone (cf. Fig. 2). In this manner the addition of anti-Fab antibody may elicit response in a system containing Fab at a concentration which is too low to elicit response in the absence of antibody (18, 19).

8. ADDITIONAL EXPERIMENTAL OBSERVATIONS: BIVALENT LIGAND MODEL INTERPRETATION

Very recently, Maturo and Hollenberg (20) reported that, when a crude preparation of rat liver membrane proteins solubilized with Triton X-100 is chromatographed on Sepharose, two peaks of specific insulin-binding activity are eluted. Material eluted in one of these peaks exhibits an insulin-binding isotherm and apparent Stokes' radius identical with those of highly purified insulin receptor. Material eluted in the second peak exhibits a larger apparent Stokes' radius and an insulin-binding isotherm characterized by two apparent classes of sites. One of the site classes has an insulin binding constant equal to that of purified receptor ($[H]^B_{50} = 7 \times 10^{-10}$ M]; the second class of sites has a much higher affinity for insulin ($[H]^B_{50} \ll 6 \times 10^{-11}$ M) and a binding capacity of approximately 5–10% that of the first class.

Maturo and Hollenberg (20) attributed the second peak to a complex of insulin receptor and a second macromolecular species, tentatively identified as a glycoprotein on the strength of its ability to bind concanavalin A. The glycoprotein is presumed to be incapable of specifically binding insulin when isolated from receptor.

When material from the second peak is rechromatographed, the first peak reappears together with the second. We interpret this finding as indicating that the receptor-glycoprotein complex is labile and at equilibrium only in the presence of isolated receptor and glycoprotein. It is presumed that the complex is observable as a discrete chromatographic species because the half-time for complex dissociation is comparable to or greater than chromatographic elution times.

According to this interpretation, material eluted in the peak ascribed to complex will, at equilibrium, presumably contain not only complex, but also isolated receptor and glycoprotein as well. The binding isotherm associated with this peak is then readily interpreted as the superposition of two simple binding isotherms, corresponding respectively to isolated receptor and receptor-glycoprotein complex.

If the glycoprotein component is identified as effector, then all of the preceding observations are consistent with either floating receptor or bivalent ligand models. However, Maturo and Hollenberg (20) also reported that glycoprotein isolated from receptor possessed insulin-binding activity which was characterized as nonspecific, since high concentrations of unlabeled insulin did not compete with labeled insulin for binding to glycoprotein. Whereas such behavior is not accounted for by the floating receptor model, it is characteristic of the formation of the complex *HE* in the bivalent ligand model, as described under Section 3.

In the simplest expression of the bivalent ligand model (shown diagramatically under Section 5) the complex *RE* is not presumed to comprise a significant fraction of total receptor or effector in the absence of hormone. However, the model may be slightly modified to accommodate the formation of receptor-effector complexes in the absence of hormone, providing that effector which is complexed to receptor does not contribute to response elicitation in the absence of hormone binding. The presence of preformed complexes of receptor and effector will result in the enhancement of the value of the parameter α, as described below.

In Section 2 it was pointed out that the localization factor α reflects a difference between two stepwise entropy changes (Eq. 2.5). The second of these entropy changes, ΔS_2^0, corresponds to the reduction in the entropy of surface translation and rotation due to cross-linking by ligand. When receptor and effector have been cross-linked or otherwise complexed prior to hormone binding, the entropy of surface translation and rotation of receptor and effector will already have been reduced below the corresponding entropies of the non-cross-linked or uncomplexed species. Therefore, additional cross-linking by means of hormone will result in an additional entropy decrease which is smaller in magnitude than if the receptor and effector have not been cross-linked prior to hormone binding. Thus ΔS_1^0-ΔS_2^0 will be larger in magnitude (i.e., more negative), and α will be larger for hormone binding to preformed complexes of *R* and *E* than for hormone binding to *R* and *E* which are not complexed prior to binding of hormone.

Several years ago it was reported that low concentrations of WGA, a bivalent lectin, enhance the binding of insulin to fat cells, membranes, and solubilized membrane preparations (21). We may interpret these observations as follows. Since both receptor and effector are presumed to be glycoproteins (20, 21), the addition of WGA may yield three types of cross-linked species, *R-W-R*, *R-W-E*, and *E-W-E*. To a first approximation the relative abundances of these three species will be in accordance with a binomial distribution, and since the ratio of *E* to *R* is estimated to be 1:10 or smaller, we may

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for all practical purposes ignore *E-W-E* relative to the other two cross-linked species. We shall presume that at low concentrations WGA does not complete with insulin for the insulin-binding sites on either *R* or *E*.[3] This would be expected if WGA bound to the glycoproteins with highest affinity to sites containing glycosidic chains, whereas insulin bound to the proteins at sites free of such chains.

The complex *R-W-R* is not of direct interest to us because it is expected to behave as normal *R* with respect to hormone binding. It was argued above that the value of α is greater for binding of hormone to preformed complexes of *R* and *E* than for binding of hormone to *R* and *E*, which is not complexed prior to binding of hormone. If this is true, then the complex *R-W-E* should exhibit a higher affinity toward insulin at low insulin concentrations than *R* and *E* which are not cross-linked by WGA. The addition of WGA would thus enhance insulin binding at low insulin concentrations, as reported (21).

Essentially the same argument can be used to explain the similar effect observed in preparations of solubilized membrane proteins. According to the results of Maturo and Hollenberg (20), some but not all of the receptor and effector present in these preparations will already be complexed. We would expect the addition of WGA to increase (through cross-linking) the proportion of receptor and effector which is complexed, creating additional high-affinity insulin-binding species and thus enhancing hormone binding at low hormone and WGA concentrations.

We note that the accessibility of effector to the external cross-linking agent WGA is an intrinsic feature of the bivalent ligand model. By contrast, the floating receptor and multiple activation models do not picture effector as being exposed to the exterior surface of the cell membrane, but rather as a protein which interacts with receptor either within the membrane or on its interior surface. Such models cannot readily rationalize the effect of WGA upon hormone binding to intact cells.

9. DISCUSSION

In the preceding sections we have shown how a variety of experimental observations may be rationalized and correlated in a straightforward manner through the use of the bivalent ligand model together with reasonable additional postulates. In the present section we discuss additional implications of this model.

Justification of model assumptions: an illustrative example. It was assumed in Section 3 that the magnitude of the steady-state biological response associated with hormone binding is proportional to the occupancy of effector sites, and that this occupancy may be calculated using an equilibrium formalism.

The first of these assumptions is made in the interest of simplicity and neglects the possibility that the mechanism by which binding of hormone to effector leads to the ultimate measured response introduces additional nonlinear relations. Molecular mechanisms such as enzyme cascades, which yield nonlinear relationships between binding and response, have been explored (22), and in the absence of experimental data such mechanisms cannot be excluded. However, one of the major results of the present work is the demonstration that nonlinear relationships between binding and response elicitation, of the type observed in a variety of experimental systems, may be accounted for by the present model without having to postulate additional nonlinear mechanisms subsequent to cell surface site occupancy.

The assumption that the steady-state occupancy of effector sites may be calculated using an equilibrium formalism implies that the first irreversible reaction step in the nonequilibrium response mechanism (unspecified here) must be subsequent to hormone-surface site association and dissociation reactions.

The following example of a "hormone"-modulated rate process is presented as an example of a simple mechanism which satisfies the above two assumptions.

We postulate the existence of a membrane enzyme *E* which converts substrate *S* to product *P* only when "hormone" *H* is bound.

$$H + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HE$$

$$HE + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HES \qquad (9.1)$$

$$HES \xrightarrow{k_3} HE + P$$

The fractional occupancy of enzyme by hormone is given by

$$y_E \equiv ([HE] + [HES])/E_T \qquad (9.2)$$

where E_T, the total concentration of enzyme, is $[E] + [HE] + [HES]$. The rate of the enzyme-catalyzed reaction is given by

$$\text{Rate} = k_3[HES] \qquad (9.3)$$

By applying the steady-state condition to the rate equations governing Eq. 9.1, the steady-state values of $[HE]$ and $[HES]$ are obtained, which, when substituted into Eqs. 9.2 and 9.3, yield

$$y_E = \frac{(1 + K_2[S])K_1[H]}{1 + (1 + K_2[S])K_1[H]} \qquad (9.4)$$

and

$$\text{Rate} = k_3E_T\frac{K_2[S]}{(1 + K_2[S])}y_E \qquad (9.5)$$

where $K_1 \equiv k_1/k_{-1}$ and $K_2 \equiv k_2/(k_{-2} + k_3)$.

While Eq. 9.4 was calculated using steady-state rather than equilibrium assumptions, the dependence of y_E upon $[H]$ is identical with that which would be calculated if it were assumed that *H* and *E* are in equilibrium with *HE*, with an equilibrium association constant $K_E = K_1(1 + K_2[S])$. The dependence of site occupancy upon hormone concentration may thus be calculated via an equilibrium formalism, recognizing that the association constant employed is an apparent rather than true equilibrium con-

[3] At higher concentrations WGA does compete for the insulin-binding site on *R* and presumably *E* as well, since it can elicit an insulin-like response in the absence of insulin (21).

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stant. Equation 9.5 shows that, for fixed $[S]$, the reaction rate (or biological response) is proportional to the site occupancy so calculated.

The above example establishes the existence of at least one molecular mechanism satisfying the two assumptions set down at the outset of this subsection, thereby verifying that these assumptions are physically reasonable.

Dissociation kinetics of asymmetrically bivalent hormones. DeMeyts and co-workers (23) have developed an assay for a property which they regard to be negative cooperativity. In this assay a labeled hormone (insulin) is equilibrated with cells at a low level of equilibrium receptor saturation. The cells are then rapidly transferred to large quantities of buffer, and the dissociation of labeled hormone from the cells is monitored as a function of time. It is found that, if the buffer contains a saturating concentration of unlabeled hormone, the rate of dissociation of labeled hormone from the cells is significantly greater than if the buffer contains no unlabeled hormone. In addition, DeMeyts and co-workers (24) have obtained insulin derivatives which bind more weakly to cells than native insulin. The binding isotherms of these derivatives, unlike those of native insulin or most other insulin derivatives, are characteristic of a single homogeneous class of binding sites (24). When cells binding labeled insulin are diluted into buffer containing saturating concentrations of these insulin derivatives, little or no acceleration of the rate of dissociation of labeled hormone as compared with control (buffer alone) is observed (23, 24). According to DeMeyts and co-workers, these derivatives lack a "cooperativity-inducing" region.

These observations of DeMeyts and co-workers (23) may be qualitatively accounted for if it is assumed that the "cooperativity-inducing" region is equivalent to the response determinant proposed in the bivalent ligand model. Hormone lacking this determinant, or possessing one with substantially reduced affinity for effector, will bind to cells more weakly than native hormone at low concentrations and will exhibit a simple single-site binding isotherm. A quantitative analysis of dissociation kinetics[4] indicates that the ability of unlabeled hormone to accelerate the rate of dissociation of labeled native hormone is significantly impaired or abolished if the unlabeled hormone lacks a response determinant or if the affinity of response determinant for effector is substantially reduced.

The role of cross-linking in hormone action. In Sections 6 and 7 we discussed two experimentally observed phenomena: (a) The addition of anti-hormone antibody to systems containing low concentrations of hormone can elicit a substantial response, whereas anti-hormone Fab fragments at the same concentrations do not. (b) Anti-membrane antibody can elicit a hormone-like response, whereas anti-membrane Fab fragments at the same concentrations do not. These observations have led to proposals (13, 14, 18, 19) that the cross-linking of aggregation of receptor plays an essential role in the elicitation of response by hormone. Our interpretation of the significance of cross-linking is rather different. Instead of invoking hormone-mediated cross-linking of receptors, we postulate that hormone itself cross-links receptor and effector. Moreover, the activation is not contingent upon the establishment of this crosslink per se, but rather upon the interaction between the response determinant of the hormone and the complementary binding site on effector. According to the bivalent ligand model, the cross-linking of hormone by anti-hormone antibody substantially increases the apparent affinity of hormone for the cell surface by a process of localization which is entirely analogous to the manner in which the binding of specificity determinant to receptor increases the apparent affinity of response for effector.

Full agonists, partial agonists, and antagonists. The following definitions are taken from Mackay (2). A *full agonist* is defined to be a ligand (hormone or drug) which elicits the maximal level of steady-state response obtainable from a given target cell or tissue under a given set of experimental conditions. Different full agonists may be characterized by different affinities for the target, so that different concentrations of different full agonists may be required to elicit a fixed fraction of maximal response. However, a sufficiently great concentration of any full agonist will by definition elicit the identical maximal response. A *partial agonist* is defined to be a ligand which elicits a maximal response which is less than that elicited by a full agonist. An *antagonist* is defined to be a ligand which elicits no response from the target, but which reduces the ability of agonists to elicit response, presumably by competing with agonist for the site(s) of ligand binding.

It has been proposed (see, for example, ref. 1) that full agonists, partial agonists, and antagonists are distinguished by the degree to which binding of ligand to receptor is associated with an alteration of the structure of receptor and a concomitant alteration of the reactivity of receptor toward other cellular components (such as effector in the floating receptor model).

The bivalent ligand hypothesis provides an appealingly simple alternative to the above approach. In Section 3 it was shown that, as the value of the localization factor α characterizing a particular bivalent ligand increases, the magnitude of the maximal response elicited by that ligand increases from a limiting low value of zero to a limiting high value of one (relative units). In Fig. 5B (*upper curve*) one may distinguish three regions. For $\log \alpha < 1$, $y_R^{\max} \simeq 0$, and the ligand will behave as an antagonist. For $1 < \log \alpha < 3$, $0 < y_R^{\max} < 1$, and the ligand will behave as a partial agonist. For $\log \alpha > 3$, $y_R^{\max} \simeq 1$, and the ligand will behave as a full agonist.

Different members of a series of structurally related ligands may be distinguished by different values of K_R and K_E as well as different values of α, and variation in these parameters will influence the observed relationships between binding and elicited response as well. However, the observation that variation in a single parameter, α, may by itself suffice to distinguish between these three classes of ligands remains an intriguing feature of the bivalent ligand model. Moreover, it suggests that chemical modifications of a full agonist which result in a "loosening" or derigidification of its structure might convert the ligand to either a partial agonist or antagonist depending upon the severity of the modification.

[4] A. P. Minton, manuscript in preparation.

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Conclusion. The notion that a hormone molecule may possess discrete regions for binding and response elicitation is not new: such proposals have been made with respect to the polypeptide hormones adrenocorticotropin (25) and glucagon (26). The conceptual novelty of the present model lies in the notion that receptor and effector comprise distinct and independent classes of surface sites, and in the respective roles assigned to these two site classes. Receptor does not itself mediate postbinding events associated with response elicitation, as commonly assumed. This function is assigned to effector, a species which in the absence of receptor would exhibit low affinity and specificity for binding of hormone. In the present model, receptor is perceived to function solely as a highly efficient and selective "concentrator" of the desired ligand in the immediate vicinity of the ligand-binding site of effector.

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