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Multi-subsite Receptors for Multivalent Ligands

Application to Drugs, Hormones, and Neurotransmitters

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SUMMARY

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Detailed structure-activity relationships of many drugs and hormones indicate that ligand-receptor binding involves the interaction of several functional regions on the ligand with complementary receptor "subsites." However most hormone receptor binding models have been based on a simple bimolecular reaction obeying the mass action law. We present a quantitative reinterpretation in pharmacological terms of the theory of flexible polyvalent ligand binding. The model explains: 1) the occurrence of complex binding isotherms showing both apparent heterogeneous and cooperative binding sites; 2) bell-shaped dose response curves; 3) the properties of full and partial agonists; 4) how a given antagonist can be either "competitive" or "noncompetitive" depending on concentration used. The classical simple bimolecular interaction between drug and receptor is a limiting case of the model, when steric hindrance completely prevents multiple receptor occupancy, or when the ligand and the receptor interact in an all-or-none mode.

INTRODUCTION

Pharmacological studies of drugs, neurotransmitters, and hormones have long suggested that their receptors could contain several binding regions or "subsites," each interacting with specific functional regions on the ligand molecule. Recent studies have provided direct evidence that ligand-receptor interactions are not simple bimolecular reactions obeying the mass action law, but instead are complex multistep processes (1-6). In well studied drug-receptor systems, for which many analogues are available, structure-activity relationships indicate that agonists and antagonists share some common functional groups, while other functional groups are unique to agonists or to antagonists (7-9). This suggests that multiple specific contacts are required to elicit the agonist response, and further, that antagonists fail to bind simultaneously to all the same subsites as the agonist and thus inactively occupy the receptor area.

As shown below, a ligand-receptor system with multiple contacts of hormone and receptor may easily be mistaken for true heterogeneity of binding sites, because both models share several experimental properties. The existence of specific states or classes of receptors with preferential affinity for either agonists or antagonists has been considered for the cholinergic receptor (9), the opiate receptor (10) and the *alpha*-adrenergic receptor (11). For physiological systems, separate receptors mediating op-

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posite agonistic and antagonistic effects would allow for various kinds of dose-response curves, including biphasic patterns with high-dose inhibition (12), although other mechanisms could explain bell-shaped (biphasic) dose response curves, e.g., specific desensitization (13) or receptor cross-linking (14).

The mathematical theory of the binding of polyvalent ligands, i.e., nucleic acids (15–18), polymers (19, 20), polypeptides (21), and antigens (22, 23), has also been described. However, the appraisal and interpretation of these theories in pharmacological terms has not been substantially undertaken. Most theoretical models for hormone-receptor binding and activation still ignore the possibility of multiple interactions.

We have examined the equilibrium binding properties of small flexible ligands interacting with multi-subsite receptors. Binding curves may reveal apparent heterogeneity and positive or negative cooperativity, even for a single class of homogeneous receptor. The flexibility of the ligand and the steric hindrance at the receptor area constitute the major factors which determine the properties of the model. Agonists may produce full or partial activation of the target system, depending on the stability of the fully interacting conformation, thus providing a mechanism which determines the intrinsic drug activity. Reversibly binding antagonists can decrease the apparent affinity of a competing agonist (competitive antagonism), but can also decrease its maximal response (noncompetitive antagonism).

Models for flexible ligand binding can explain many pharmacological properties of drug and hormone receptors which are often attributed to more complex mechanisms involving steps subsequent to binding. Multi-subsite receptor models thus constitute an important general approach to drug receptor interactions.

THE MODEL

The model for multiple subsite receptor (multi-subsites) may be defined as follows:

1) The term "subsite" refers to functional parts of the receptor able to interact with the ligand. The "receptor area" is composed of several of these subsites. The ligand contains specific functional regions or groups which can be "recognized" by the corresponding specific subsites on the cell surface receptor area. In general, these functional regions may differ from each other, unlike the case for immunoglobulins and polymers, where all binding sites are identical.

2) The interaction of the functional segments of the ligand with their corresponding subsites need not be simultaneous, as in the case of rigid molecules interacting in a "lock and key" mode (24). In most cases, the ligand would be flexible and could exist in several conformations both in the free and the bound states. Alternatively, the receptor area might be flexible, with conformational changes upon binding of a rigid ligand ("induced fit").

3) Of the various bound conformations or states, we assume that the fully interacting state, with all functional regions bound to their corresponding subsites, would activate the effector component. The other partially bound states might also partially activate the effector. Thus, the hormonal response would not be a function of total receptor occupancy but rather of the concentration of the active pool(s).

4) Due to the flexibility of the ligand, two or more ligands may be partially bound to the same receptor area when the ligand is at high concentration, each preventing the other from full activation of the system. Multiple occupancy of the receptor has been described for the case of *alpha*-bungarotoxin binding to the purified cholinergic receptor (2).

5) While the actual number of physical interactions of a ligand with its receptor may be quite large, even for small molecules, only a small number of functional regions are required for modeling purposes. The binding of each functional region of the ligand to its subsite may require several simultaneous physical interactions. The functional regions of the ligand are thus distinguished by their ability to bind separately to their corresponding subsites, owing to the flexibility of the molecule.

As shown in Figure 1, each reaction step between the binding pools is characterized by a microscopic equilibrium constant L_i.

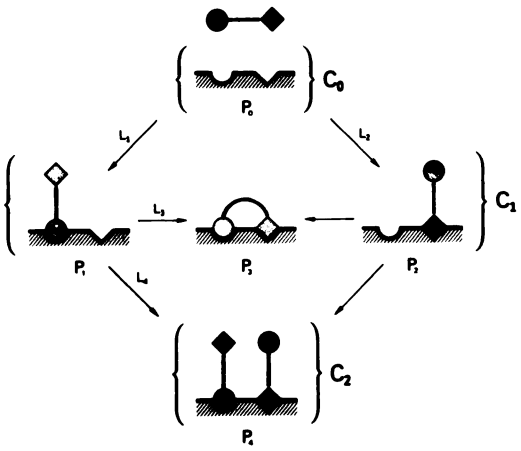

FIG. 1. *Binding reaction scheme for a divalent ligand interacting with two subsites*

The microscopic equilibrium constants L_i refer to each individual reaction. Unlabeled reaction arrows correspond to microscopic constants which can be calculated from the specified L_i. The binding pools in each horizontal row have the same number of ligands bound and are grouped into pool classes C_i.

The binding pools P_j could be grouped into classes C_i with i ligand molecules bound per receptor area. In Figure 1, the binding pools are grouped by rows into classes. In this example, C_1 contains three pools, including the fully interacting "active" pool, P_3. The equilibrium concentration of the pool classes C_i are determined by macroscopic stoichiometric constants K_i, which in turn are defined by the reaction scheme and the microscopic reaction constants, L_i.[2] Experimentally, only total ligand binding, B, and the active pool(s) can be measured. This restriction implies that the microscopic equilibrium constants, L_i, are experimentally indeterminate. However, the values of the macroscopic constants, K_i, may be inferred from experimental data. The mathematical statement of the model is provided in the Appendix.

[2] One must distinguish between the microscopic constants (e.g., $L_1 = P_1/E \cdot F$), the macroscopic constants for pool classes (K as used throughout the present manuscript), e.g., $K_2 = C_2/(E \cdot F^2)$, and the "stepwise" constants as used by Fletcher and others for the Adair model, e.g., $K_2 = C_2/(C_1 \cdot F)$. The use of K as defined here relates any pool to the empty receptor and free ligand, and simplifies the equations especially in more complex cases. Our K_j corresponds to B_j in Eq. 11 of (40).

EXAMPLES

A study of the simpler cases of multisubsite receptors indicates the general properties which can be expected from more complex cases. When n = 1, there is only one functional subsite and the ligand-receptor system behaves as a homogeneous system obeying the mass action law. There is only one binding pool, the active one. Any ligand able to bind to the receptor area should be an agonist. This simple model is unable to accommodate competitive antagonists or partial agonists without additional *ad hoc* assumptions.

When there are two subsites (n = 2) as in Fig. 1, the expressions for each binding pool P_j concentrations as a function of the empty receptor concentration E and the free ligand concentration F are as follows:

$$P_1 = L_1 E \cdot F \tag{1}$$

$$P_2 = L_2 E \cdot F \tag{2}$$

$$P_3 = L_1 L_3 E \cdot F \tag{3}$$

$$P_4 = L_1 L_4 E \cdot F^2 \tag{4}$$

The binding pools are grouped into three classes, containing 0, 1 or 2 bound ligands:

Typical examples of binding isotherms are shown in Figure 2A, with corresponding

$$C_0 = P_0 \tag{5}$$

$$C_1 = P_1 + P_2 + P_3 = K_1 E \cdot F \tag{6}$$

$$C_2 = P_4 = K_2 E \cdot F^2 \tag{7}$$

where the macroscopic stoichiometric constants K_1, K_2 are combinations of the reaction constants:

$$K_1 = L_1 + L_2 + L_1 L_3 \tag{8}$$

$$K_2 = L_1 L_4 \tag{9}$$

When only pool P_3 is active, the active pool concentration, A, and its stoichiometric constant are:

$$A = P_3 = K_A E \cdot F \tag{10}$$

$$K_A = L_1 L_3 \tag{11}$$

while the total concentration of ligand bound, B, is:

$$B = C_1 + 2 \cdot C_2 \tag{12}$$

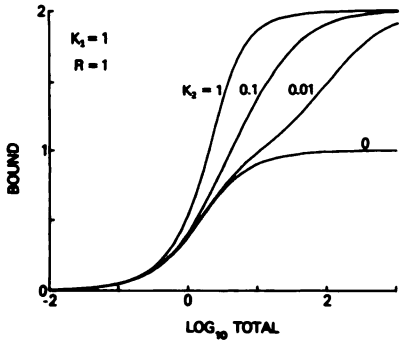

FIG. 2A. *Binding isotherms for varying degrees of steric hindrance in the case of two subsites (Fig. 1)*

A. Total receptor concentration R is 1. The macroscopic stoichiometric constants K_i are defined by equations 8, 9. When $K_2 = 0$, pool class C_2 is empty and steric hindrance totally prevents multiple receptor occupancy. As K_2 increases, the "mean" affinity increases, the curve shifts to the left, and the steepness increases.

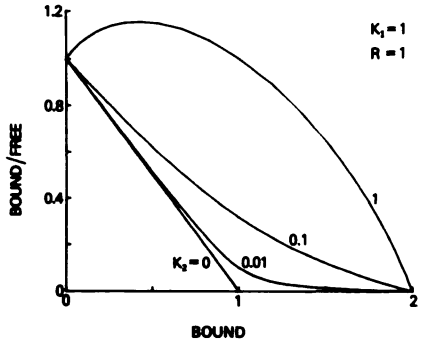

FIG. 2B. *Scatchard plot of the binding data shown in Fig. 2A*

When steric hindrance incompletely prevents multiple receptor occupancy ($K_2 = 0.01, 0.1$), the plot is concave up, suggesting apparent heterogeneity or negative cooperativity of binding sites. When $K_2 = 1$, multiple occupancy is favored instead of being prevented and the plot shows a downward concavity. For $K_2 = 0.25\ K_1^2$ one would obtain a linear Scatchard plot.

Scatchard plots (25) in Figure 2B. If only one ligand molecule could bind to a receptor area, then C_2 would be empty and $K_2 = 0$. In that limiting case, only pool class C_1 would contribute to the observable binding and maximum binding $B_{max} = R$, the total receptor concentration. If steric hindrance between two ligand molecules approaching the receptor area does not totally exclude simultaneous binding in pool class C_2 (i.e., $K_2 > 0$), the binding isotherm will show two apparent components (i.e. apparent binding

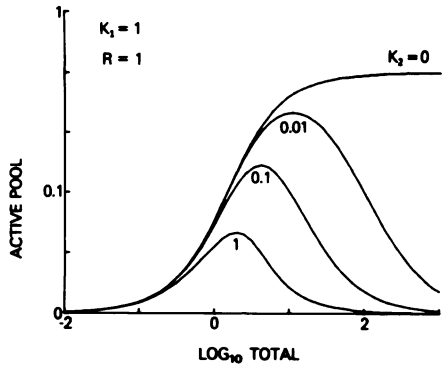

FIG. 2C. *Active pool concentration (P_3) as a function of total agonist concentration in the example of Fig. 2*

When steric hindrance does not totally prevent multiple occupancy, the activity profile is biphasic. The peak of the bell-shaped activity curve coincides with the point where the binding isotherm reaches 50% of maximal binding capacity in Fig. 2A.

classes), with a total binding capacity equal to $2 \cdot R$. A staggered binding isotherm (Fig. 2A) or a "concave-up" Scatchard plot (Fig. 2B) would ordinarily indicate heterogeneity, but in the case of multisubsites, may arise from steric hindrance ($K_1 = 1$, $K_2 = 0.01$), as predicted (19, 20).

If pool class C_2 (multiple receptor occupancy) were favored over pool class C_1 (single receptor occupancy) by a "cooperative" mechanism, one would expect to observe a steeper binding isotherm (Fig. 2A, $K_2 = 1$) or a "concave-down" Scatchard plot (Fig. 2B, $K_2 = 1$). The term "cooperativity" refers to any mechanism by which the affinity of a ligand for some of the subsites is changed by occupancy of other subsites. Lack of cooperativity is the same as lack of steric hindrance (i.e. $L_2 = L_4$) in the present model.[3]

[3] To obtain a linear Scatchard plot when $K_1 = 1$, K_2 would be 0.25. In general, $K_2 = \frac{1}{4} K_1^2$. If $L_3 = 0$ and $L_1 = L_2$, then a linear Scatchard plot is obtained when $L_2 = L_4$, that is, when binding to one subsite is independent of occupancy of the other. However, when $L_3 > 0$, i.e., when pool P_3 can form, then the condition $L_2 = L_4$ will not lead to a linear Scatchard plot. Thus, if one takes $L_2 = L_4$ as a criterion for non-cooperativity on a microscopic level, then we have the paradoxical result that a non-cooperative system with homogeneous subsites may have a non-linear Scatchard. Thus, use of the term "cooperativity" without reference to a particular model, e.g., stepwise, subsites, etc., may lead to confusion.

In the example of Fig. 2, the shape of the binding isotherm is completely determined by the relative values of the macroscopic stoichiometric constants K_1 and K_2. Pool classes C_1 and C_2 increase according to the first and the second power of free hormone concentration, respectively. At low ligand concentration, pool class C_2 may be small compared to C_1, due to steric hindrance. However, C_2 will predominate at higher ligand concentration, because of the higher order of the binding reaction (cf. Appendix, Eq. A2). Thus, the two apparent binding components (or stages) in Fig. 2A do not correspond to the separate filling of the two subsites (i.e., P_1, P_3), but rather to the separate filling of pool classes C_1 and C_2. In general, if there were n subsites and if multiple occupancy of the receptor were feasible, then one might find up to n apparent binding components in the binding isotherm.

At high agonist concentrations, the maximally active binding state. P_3 (contained in C_1) will eventually be replaced by binding pools with multiple receptor occupancy (C_i, for $i > 1$). Figure 2C shows that the expected active pool concentration is a biphasic function of the total ligand concentration whenever C_2 can form, i.e., $K_2 > 0$. The peak of the bell-shaped curve coincides with the point of 50% maximum ligand binding, when n = 2 (Eq. A9). The maximum size of the active pool decreases as K_2 increases, i.e., as the probability of multiple ligand binding is increased. Although the bell-shaped curves appear nearly symmetrical in Figure 2C, they may also be asymmetrical with a flat dome when multiple receptor occupancy is restrained by a low K_2 (due to steric hindrance or negative cooperativity) (Fig. 3). A non-linear coupling function relating active pool concentration and the observed response will not alter the dose which gives the peak response, as long as the function is monotonic. However, such a non-linear function may change the shape of the dose-response curve considerably.

The size of the active pool relative to the other pools in the pool class C_1, is determined by the ratio K_A/K_1 (Fig. 3). A high ratio (e.g., 90/100) favors a large maximum for the active pool while a low ratio (e.g., 10/100) yields a relatively small active pool size. The ratio K_A/K_1 (between 0 and 1) can be regarded as the "intrinsic activity coefficient" for the agonist. When $K_A/K_1 = 1$, the ligand is a full agonist. When $K_A/K_1 = 0$, the ligand still binds to the receptor area, but is inactive (antagonist).

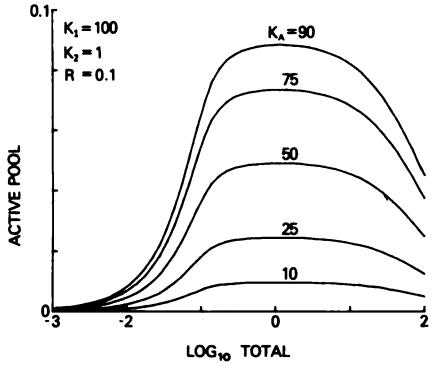

FIG. 3. *Dose-activity profile for various degrees of stability of the active pool in the case of a divalent agonist binding to two subsites (Fig. 1) in the presence of steric hindrance ($K_2 = 1$, $K_1 = 100$)*

Total receptor concentration $R = 0.1$. The equilibrium constant K_A (or L_1L_3) varies from 10 to 90, so that the active pool contributes from 10% to 90% of the pool class with single occupancy of the receptor (C_1, second row of Fig. 1).

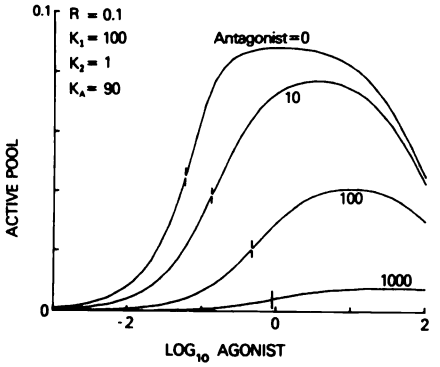

FIG. 4. *Dose-activity profiles for a divalent agonist in the presence of increasing concentrations of a monovalent antagonist (only one functional region)*

Both ligands bind with the same affinity ($L_1 = 1$ for both) to the common subsite. The binding properties of the agonist alone are the same as the most potent agonist in Fig. 3 ($K_A = 90$). The vertical bars indicate the ED_{50} of the rising limb of the biphasic activity curves with increasing amounts of agonist. The monovalent ligand behaves as a mixed competitive-noncompetitive antagonist.

Figure 4 indicates the effect on the dose response curve of the presence of an inac-

tive ligand (antagonist), which can bind to only one subsite ($L_2 = L_3 = 0$, for the antagonist). The presence of the antagonist both increases the apparent ED_{50} of the agonist and decreases the maximum concentration of the active pool. Thus, a univalent inactive ligand can behave as both a competitive and a noncompetitive antagonist. This dual effect of antagonists has been described in several pharmacological systems and has been attributed to the presence of spare or excess receptors (26).

More complex cases of agonist-antagonist interactions could be considered. For example, the antagonist could also bind to a third, accessory subsite (9), in addition to a receptor subsite shared with the agonist (Fig. 5). The accessory site could account for a higher affinity for antagonists than for agonists. However, the antagonistic properties of such a ligand would be qualitatively similar to those illustrated in Figure 4.

In cases where the functional region of the ligand can be identified and cleaved without altering their geometry, the properties of the functional fragments could be compared with those of the intact molecule. Figure 6 shows the competitive binding of intact ligand and its fragments. In this example, cleavage of the ligand into a mixture

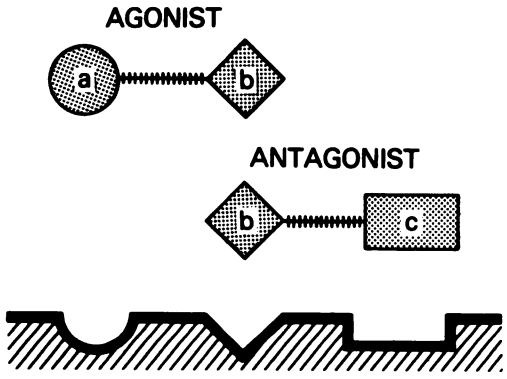

FIG. 5. *Schematic diagram for a divalent agonist competing with a divalent antagonist in binding to a receptor consisting of two subsites for the active region of the receptor and a third accessory subsite specific to the antagonist*

The binding curves are similar to those shown in Figure 4, except that the antagonist may have a much higher overall affinity for the receptor area, due to its interaction with the accessory subsite.

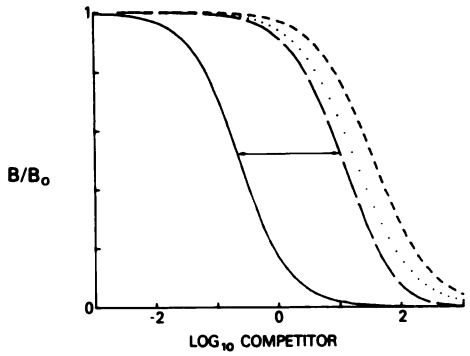

FIG. 6. *Competitive binding of a divalent ligand with mixtures of its fragments, each containing only a single binding moiety (a or b)*

The total receptor concentration $R = 0.1$. The displaced ("labeled") divalent ligand ($K_1 = 20.3$, $K_2 = 0.002$, $K_A = 20$) has concentration $H^* = 0.1$. The competitors used are the intact ligand (solid line), fragment a (dotted line), fragment b (short dashed line), or a mixture of equal amounts of the fragments a and b (long dashed line).

of its functional fragments results in a 50-fold increase in the 50% maximal inhibitory concentration (ID_{50}). The increase in ID_{50} following cleavage of a bifunctional ligand could be even larger and is generally equal to $1/(1 - K_A/K_1)$ in the presence of a very high degree of steric hindrance. Thus for a very highly efficacious agonist (K_A/K_1 nearly 1), the increase in the ID_{50} following cleavage of the ligand into fragments could be extremely large.

Figure 7 shows the equilibrium "competition" curves for a labeled full agonist, which competes for receptor with increasing doses of unlabeled full agonist, or partial agonists having a decreased affinity for one subsite. Thus, as one weakens the affinity for one subsite, the displacement curves have a progressively higher ED_{50}, and are progressively flatter.

DISCUSSION

The occurrence of bell-shaped dose-response curves is one of the salient properties of the present model. Such high dose inhibition of activity dose-response curves has been described in several receptor systems (27–32, 14). Besides the mechanism described herein, such bell-shaped curves could be due to receptor desensitization or nonspecific exhaustion of the response sys-

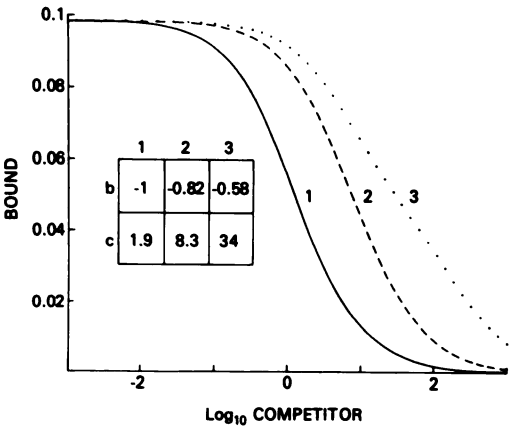

FIG. 7. *Competitive binding of a labeled divalent ligand (K_1 = 12, K_2 = 1, K_A = 10), and an unlabeled species with the same K_i or with ligands which have the binding affinity (L_2, L_3, L_4) for one subsite reduced by a factor of 10 or 100, resulting in values of K_1, K_2, and K_A of 2.1, 0.1, 1 and 1.11, 0.01, 0.1, respectively*

The weaker agonists have curves which are shifted to the right ($c = ED_{50}$) and have a reduced slope factor (b). The curves are adequately fit by the four parameter logistic equation (41).

tem (12, 13) or to receptor cross-linking (14). In the case of desensitization, one would expect that the agonist concentration at the peak response would vary systematically with incubation time (28), while it should be independent of incubation time for the present mechanism. For the adrenal angiotensin receptor, certain other agonists, e.g., ACTH, can elicit a response larger than the peak response observed for angiotensin (33), indicating that mechanisms other than desensitization or exhaustion of biosynthetic precursors are probably involved.

For *in vivo* systems, independent pharmacological receptors mediate opposite effects, e.g., the *alpha* and *beta* adrenergic receptors. In such systems, complex dose-response curves would be expected (12). Similarly, complex interactions of intracellular mediators of hormone action may also lead to biphasic dose-response curves (34).

The present model predicts that the peak response is obtained when an integral fraction of the true maximal binding capacity has been filled (B_{max}/n) (Eq. A9). In the case of two subsites, the maximum response would be observed when 50% of the "sites" would be occupied, if both hormone binding and action were measured under strictly identical conditions. Such a relationship could be tested experimentally.

More general examples could be considered by allowing binding pools other than the fully interacting pool (P_3) to be partly active. In general, activity might be given by

$$\text{Activity} = \sum_j \alpha_j P_j \quad (0 \leqq \alpha_j \leqq 1) \qquad (13)$$

where α_j is the intrinsic pool activity. This would allow for basal activity (due to P_0) and also an asymmetry of the high-dose inhibition, which may then plateau at an intermediate value determined by the activity coefficient for P_4 (Fig. 1), and not decrease to zero. If the coupling between the fraction of "active" receptors and the observable response were not proportional, then the shape of the response curve would differ from those shown in Figs. 2C and 3. Non-proportional coupling could flatten the peak into a broad plateau with a delayed downward segment.

Equation A4 of the model is mathematically equivalent to the stepwise model for multiple classes of binding sites (35). In fact, many mathematical models for ligand-protein interaction have been proven to be mathematically interchangeable with the stepwise model (36) first proposed by Adair for oxygen binding to hemoglobin.

Fisher *et al.* (37, 38) have postulated a ligand exclusion (or steric) mechanisms as an alternate explanation for apparently allosteric effects of regulatory enzymes. Their experimental data (38) are compatible with simultaneous occupancy of the catalytic area by the substrate and the inhibitor binding to distinct but interwoven subsites. In contrast to the predictions of the present model (Fig. 4), they implied that only non-competitive inhibition would be observed. The discrepancy may be due to the implicit exclusion of pool P_4 (class C_2) in most of their analyses, or due to use of a less comprehensive quantitative analysis of the model than is provided here (cf. APPENDIX).

Distinction between heterogeneous, cooperative receptor sites and a homogeneous multisubsite receptor on the basis of equilibrium binding and activity curves is experimentally difficult because both models

share most of their observable properties. The existence of a biphasic dose-response curve would favor the present model, although additional experiments would be required to exclude desensitization models. Many studies which have been interpreted as indicating apparent heterogeneity of receptor sites for agonists and antagonists, would also be compatible with multiple subsites constituting a single "receptor area." Ultimately, the use of purified receptors should provide a definitive approach for discriminating between macroscopic (independent receptors) and microscopic (multisubsites) heterogeneity. Masking of subsites using covalent reagents should provide additional information.

The presence of multiple interactions with a multisubsite receptor does not preclude other complex mechanisms after the binding step. However, the interpretation of complex hormone-receptor interactions could benefit from consideration of detailed binding mechanisms. Although structure-activity studies provide detailed descriptions of functional regions for drugs and hormones, essentially all theoretical models developed to date have been primarily concerned with a simple binding reaction. Bimolecular reactions obeying the mass action law could be considered as a limiting case of the present model, when the active fully interacting state (P_3) is infinitely more stable than the partially interacting states and when multiple occupancy of the receptor area is impossible.

In conclusion, the present model provides a simple, plausible molecular mechanism which may explain many properties of drug, hormone, and neurotransmitter receptor systems. Consideration of multiple interactions with subsites on a receptor area provides a much more versatile scheme than simple bimolecular interactions. Quantitative testing of the applicability of the present model could be obtained by least-squares curve fitting, using well defined methods developed for the stepwise model at equilibrium (35), and employing several of the methods developed for the analysis of n-ligand, m-binding site systems (39). Further, studies of the time course of binding and activation may be predicted quantitatively by a "kinetic" extension of the present model.

ACKNOWLEDGMENTS

Charles De Lisi kindly reviewed the manuscript and provided many helpful suggestions. The numerical analysis was based in part on the methods of Feldman (39).

APPENDIX

Mathematical Formulation of the Multi-Subsite Model

A macroscopic stoichiometric constant may be defined for each individual pool as the product of the equilibrium constants L_i for the sequence of reactions leading from the free receptor pool to that binding pool. Pools can be grouped into classes, C_i according to the number of ligand molecules bound per receptor. The collective stoichiometric constant K_i for the class C_i is the sum of the individual microscopic constants of the pools in that class. The total concentration of ligand bound is

$$B = \sum_{i=0}^{n} i \cdot C_i \tag{A1}$$

where n is the number of subsites and C_0 is the empty receptor pool. The pool class C_i and the macroscopic stoichiometric constant K_i are related by:

$$K_i = \frac{C_i}{(E \cdot F^i)} \tag{A2}$$

where F is the concentration of free hormone and E (or C_0) is the concentration of empty receptors:

$$E = \frac{R}{\left(\sum_{i=0}^{n} K_i F^i\right)} \tag{A3}$$

with $K_0 = 1$, and R being the total concentration of receptors. The concentration of hormone bound B as a function of free hormone F is given by:

$$B = \frac{R \cdot \left(\sum_{i=0}^{n} i \cdot K_i F^i\right)}{\left(\sum_{i=0}^{n} K_i F^i\right)} \tag{A4}$$

When $n > 1$, the apparent binding capacity of the receptor for the ligand differs from the receptor concentration:

$$B_{max} = \lim_{F \to \infty} B = n \cdot R \tag{A5}$$

For curve fitting purposes, the total hormone con-

centration H should usually be considered as the independent variable, instead of free hormone concentration F. Substituting $(H - B)$ for F in Eq. A4 and re-arranging gives:

$$S_0 + \sum_{i=1}^{n} (-1)^i S_i B^i + S_{n+1} = 0$$

where

$$\begin{aligned} S_0 &= \sum_{i=1}^{n} i \cdot K_i H^i R \\ S_i &= \binom{n}{n+1-i} K_n H^{n+1-i} \\ &\quad + \sum_{j=0}^{n-i} \left[\binom{i+j-1}{j} K_{i+j-1} \right. \\ &\quad \left. + (i+j) \cdot \binom{i+j}{j} K_{i+j} H^j R \right] \\ S_{n+1} &= (-1)^{n+1} K_n B^{n+1} \end{aligned} \tag{A6}$$

The active pool belongs to the pool class C_1 containing $(2^n\text{-}1)$ binding pools with one ligand molecule per receptor. An additional macroscopic stoichiometric constant K_A is defined for the active pool A:

$$A = \frac{R \cdot K_A F}{\left(\sum_{i=0}^{n} K_i F^i \right)} \tag{A7}$$

If $n > 1$ and $K_i \neq 0$ for all $i > 0$, the active pool concentration is a unimodal function of the free hormone concentration. Pool A reaches a maximum for a free hormone concentration $\hat{F}$ satisfying:

$$\sum_{i=0}^{n} (i - 1) \cdot K_i \hat{F}^i = 0 \tag{A8}$$

When $F = \hat{F}$, the concentration of bound ligand is given by:

$$B = R = \frac{B_{max}}{n} \tag{A9}$$

The model can be easily extended to the case of two ligands competing for the same receptor, but with possibly differing affinities for the receptor subsites. The binding pools are grouped into pool classes C_{ij} of the same binding stoichiometry, with i molecules of the first ligand and j molecules of the second ligand bound per receptor area. The corresponding macroscopic stoichiometric constants K_{ij} are accordingly defined as:

$$K_{ij} = \frac{C_{ij}}{(E \cdot F_1^i F_2^j)} \tag{A10}$$

where F_1 and F_2 are the free concentration of each ligand and E is the concentration of empty receptors:

$$E = \frac{R}{\left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} K_{ij} F_1^i F_2^j \right)} \tag{A11}$$

with $K_{00} = 1$, and $K_{ij} = 0$ for $i + j > n$.

The total concentration of each ligand bound B_1 and B_2 is

$$\begin{aligned} B_1 &= \frac{R \cdot \left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} i \cdot K_{ij} F_1^i F_2^j \right)}{\left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} K_{ij} F_1^i F_2^j \right)} \\ B_2 &= \frac{R \cdot \left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} j \cdot K_{ij} F_1^i F_2^j \right)}{\left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} K_{ij} F_1^i F_2^j \right)} \end{aligned} \tag{A12}$$

while the active pool concentration is defined as:

$$A = \frac{R \cdot (K_{A_1} F_1 + K_{A_2} F_2)}{\left(\sum_{i=0}^{n} \sum_{j=0}^{n-i} K_{ij} F_1^i F_2^j \right)} \tag{A13}$$

where K_{A_1} and K_{A_2} are the macroscopic stoichiometric constants for each ligand.

REFERENCES

1. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. & Gilman, G. (1977) Relationship between the beta-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* **252**, 5761–5775.
2. Maelicke, A., Fulpius, B. W., Klett, R. P. & Reich, E. (1977) Acetylcholine receptor; responses to drug binding. *J. Biol. Chem.* **252**, 4811–4830.
3. Yoda, A. (1973) Structure-activity relationships of cardiotonic potassium-dependent adenosine triphosphate; I. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of magnesium and phosphate. *Molec. Pharmacol.* **9**, 51–60.
4. Yoda, A., Yoda, S. & Sarrif, A. M. (1973) Structure-activity relationships of cardiotonic steroids for the inhibition of sodium-and potassium-dependent adenosine triphosphatase. *Molec. Pharmacol.* **9**, 766–773.
5. Taylor, P. W., King, R. W. & Burgen, A. S. V. (1970) Kinetics of complex formation between human carbonic anhydrases and aromatic sulfonamides. *Biochemistry* **9**, 2638–2645.
6. Neumann, E. & Chang, H. W. (1976) Dynamic properties of isolated acetylcholine receptor protein, kinetics of the binding of acetylcholine and Ca ions. *Proc. Natl. Acad. Sci. USA* **73**, 3994–3998.
7. Mukherjee, C., Caron, M. G., Mullikin, D. & Lefkowitz, R. J. (1976) Structure-activity relation-

ships of adenylate cyclase-coupled beta-adrenergic receptors; determination by direct binding studies. *Molec. Pharmacol.* **12**, 16–31.

8. Feinberg, A. P., Creese, I. & Snyder, S. H. (1976) The opiate receptor; a model explaining structure-activity relationships of opiate agonists and antagonists. *Proc. Natl. Acad. Sci. USA* **73**, 4215–4219.
9. Ariens, E. J. & Simonis, A. M. (1967) Cholinergic and anticholinergic drugs, do they act on common receptors? *Ann. N.Y. Acad. Sci.* **144**, 842–868.
10. Pert, C. B. & Snyder, S. H. (1974) Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Molec. Pharmacol.* **10**, 868–879.
11. Greenberg, D. A. & Snyder, S. H. (1978) Pharmacological properties of [^{3}H]dihydroergocryptine binding sites associated with alpha-noradrenergic receptors in rat brain membranes. *Molec. Pharmacol.* **14**, 38–49.
12. Szabadi, E. (1977) A model of two functionally antagonistic receptor population activated by the same agonist. *J. Theor. Biol.* **69**, 101–112.
13. Waud, D. R. (1968) Pharmacological receptors. *Pharmacol. Rev.* **20**, 49–88.
14. Colquhoun, D. (1968) The rate of equilibration in a competitive n drug system and the auto-inhibitory equations of enzyme kinetics: some properties of simple models for passive sensitization. *Proc. Roy. Soc. B.* **170**, 135–154.
15. Eigen, M. & Porschke, D. (1970) Co-operative non-enzymatic base recognition; I. Thermodynamics of the helix-coil transition of oligoriboadenylic acids at acidic pH. *J. Mol. Biol.* **53**, 123–141.
16. Schwarz, G. & Engel, J. (1972) Kinetics of cooperative conformational transitions of linear biopolymers. *Angew. Chem. Internat. Edit.* **11**, 568–675.
17. Delisi, C. & Crothers, D. M. (1971) Theory of the influence of oligonucleotide chain conformation on double helix stability. *Biopolymers* **10**, 1809–1827.
18. Britten, R. J. & Davidson, E. H. (1976) Studies on nucleic acid reassociation kinetics; empirical equations describing DNA reassociation. *Proc. Natl. Acad. Sci. USA* **73**, 415–419.
19. Laiken, N. & Nemethy, G. (1970) A model for the binding of flexible ligands to the surfaces of proteins and other macromolecules; I. Statistical-mechanical treatment. *J. Phys. Chem.* **74**, 4421–4441.
20. Laiken, N. & Nemethy, G. (1971) A new model for the binding of flexible ligands to proteins. *Biochemistry* **10**, 2101–2106.
21. Engel, J. & Winklmair, D. Equilibrium and kinetics of cooperative binding and cooperative association. in *Enzymes: Structure and Function VIII FEBS Meeting*, (J. Drenth, R. A. Oosterbaan, and C. Veeger, Eds.) American Elsevier 1972, 29–44.
22. Crothers, D. M. & Metzger, H. (1972) The influence of polyvalency on the binding properties of antibodies. *Immunochemistry* **9**, 341–357.
23. Delisi, C. & Metzger, H. (1976) Some physical chemical aspects of receptor-ligand interactions. *Immunol. Commun.* **5**, 417–436.
24. Burgen, A. S. V., Roberts, G. C. K. & Feeney, J. (1975) Binding of flexible ligands to macromolecules. *Nature* **253**, 753–755.
25. Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660–672.
26. van Rossum, J. M. & Ariens, E. J. (1962) Receptor-reserve and threshold-phenomena; II. Theories on drug action and a quantitative approach to spare receptors and threshold values. *Arch. Intern. Pharmacodyn.* **136**, 385–413.
27. Brown, E., Graham, J. D. & Taha, S. A. (1973) Fade and desensitization in guinea-pig ileum and vas deferens. *Eur. J. Pharmacol.* **22**, 64–74.
28. Frankhuijzen, A. L. (1975) Analysis of ergotamine-5H-T interaction on the isolated rat stomach preparation. *Eur. J. Pharmacol.* **30**, 205–212.
29. Mukherjee, C., Caron, M. G., Coverstone, M. & Lefkowitz, R. J. (1975) Identification of adenylate cyclase-coupled beta-adrenergic receptors in frog erythrocytes with (-)[^{3}H]alprenolol. *J. Biol. Chem.* **250**, 4869–4876.
30. Kaumann, A. J. & Birnbaumer, L. (1974) Studies on receptor-mediated activities of adenylate cyclases; IV. Characteristics of the adrenergic receptor coupled to myocardial adenylyl cyclase: stereospecificity for ligands and determination of apparent affinity constants for beta-blockers. *J. Biol. Chem.* **249**, 7874–7885.
31. Catt, K. J. & Dufau, M. L. (1973) Interactions of LH and LCG with testicular gonadotropin receptors. *Adv. Exp. Biol. Med.* **36**, 379–418.
32. Douglas, J., Saltman, S., Fredlund, P., Kondo, T. & Catt, K. J. (1976) Receptor binding of angiotensin II and antagonists correlation with aldosterone production by isolated canine adrenal glomerulosa cells. *Circ. Res.* **38**, Suppl. II: 108–112.
33. Fredlund, P., Saltman, S. & Catt, K. J. (1975) Aldosterone production by isolated adrenal glomerulosa cells; stimulation by physiological concentrations of angiotensin II. *Endocrinology* **97**, 1577–1586.
34. Van Cauter, E., Hardman, J. G. & Dumont, J. E. (1976) Implications of cross inhibitory interactions of potential mediators of hormone and neurotransmitter action. *Proc. Natl. Acad. Sci. USA* **73**, 2982–2986.

35. Fletcher, J. E., Spector, A. A. & Ashbrook, J. D. (1970) Analysis of macromolecule-ligand binding by determination of stepwise equilibrium constants. *Biochemistry* **9,** 4580–4587.
36. Magar, M. E. & Steiner, R. F. (1971) Equivalence of certain models in protein ligand equilibria and the possibility of distinguishing between them. *J. Theor. Biol.* **32,** 495–506.
37. Fisher, H. F., Gates, R. E. & Cross, D. G. (1970) A ligand exclusion theory of allosteric effects. *Nature* **288,** 247–249.
38. Collen, A. H., Cross, D. G. & Fisher, H. F. (1974) Two-step binding of adenosine diphosphate to L-glutamate dehydrogenase; effect on the binding of reduced nicotinamide adenine diphosphate and on enzymatic catalysis. *Biochemistry* **13,** 2341–2347.
39. Feldman, H. A. (1972) Mathematical theory of complex ligand-binding systems at equilibrium, some methods for parameter fitting. *Analyt. Biochem.* **48,** 317–338.
40. Fletcher, J. E. & Spector, A. A. (1977) Alternative models for the analysis of drug-protein binding. *Mol. Pharmacol.* **13,** 387–399.
41. DeLean, A., Munson, P. J. & Rodbard, D. (1978) Simultaneous analysis of families of sigmoidal curves; application to bioassay, radioligand assay, and physiological dose-response curves. *Amer. J. of Phy. Endocrin., Met. and Gastrointestinal Phy.* Vol. 4, E97-E102.