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# COOPERATIVE PROPERTIES OF HORMONE RECEPTORS IN CELL MEMBRANES

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The binding of many polypeptide hormones to cell surface receptors does not appear to follow the law of mass action. While steady–state binding data are consistent in many cases with either heterogeneous populations of binding sites or interactions of the type known as negative cooperativity, study of the kinetics of dissociation of the hormone receptor complex allows an unambiguous demonstration of cooperative interactions. Negative cooperativity, which seems to be widespread among hormone receptors, provides exquisite sensitivity of the cell at low hormone concentrations while buffering against acutely elevated hormone levels. The molecular mechanisms underlying the cooperativity are still largely unknown. Cooperativity may stem from a conformational transition in individual receptors or involve receptor aggregation in the fluid membrane (clustering) or more extensive membrane phenomena. Thus, new models of hormone action must be considered which integrate the progress in our knowledge of both the complex mechanisms regulating hormone binding to their surface receptors, and the dynamic properties of the cell membrane.

## INTRODUCTION

Numerous ligands, in their binding to macromolecules, disobey the law of mass action (1) and exhibit cooperative properties, i.e. the binding of one ligand molecule affects the binding affinity of other ligand molecules, subsequent binding being either facilitated (positive cooperativity) (2, 3) or impaired (negative cooperativity) (4, 5).

Such aberrant binding isotherms may occasionally be found in the inorganic world: Langmuir, in his classical study of the binding of gases to platinum surfaces (6), noted that the binding of some gas molecules may accelerate the dissociation of other gas molecules from the platinum surface; he derived an equation to describe the resulting isotherm which is formally identical to the equation derived by Adair for binding of oxygen to hemoglobin (7).

As far as biologically important macromolecules (hemoglobin, enzymes) are concerned, cooperative binding was often found to be associated with remarkable properties of these molecules, involving structural transitions in complex multisubunit structures; the set of properties defining "allosteric" molecules was first clearly depicted in the celebrated article describing the "Monod-Wyman-Changeux" model (2). A variety of models have since been proposed to describe the behavior of biological regulatory

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proteins (3–5, 8–12). Such models have very early been applied to the structure of cell membranes considered as "lattices" (or subunit structures with an "infinite" number of sites) to explain the apparent amplifying properties of biological membranes in response to ligand binding, including "graded" or "all or none" responses (13–16). These models emphasize the highly ordered aspect of cell membranes, in contrast with more recent models which place accent on membrane fluidity and movements of surface components (17, 18). In the recent past, the importance of these concepts in the regulation of hormone binding and action has started to be recognized.

## QUANTITATIVE ASPECTS OF HORMONE BINDING TO RECEPTORS

The first step in the action of polypeptide hormones is binding to specific receptors at the surface of their target cells (19, 20). This property is shared by catecholamines and a number of polypeptide growth factors, whereas steroid and thyroid hormones form complexes with cytoplasmic and nuclear binding structures. We will consider in this article only the surface receptors of polypeptide hormones.

With the introduction of labeling methods for hormones that preserve their biological properties and chemical integrity, it has become possible to study and characterize directly the binding of polypeptide hormones to their receptors in the cell membrane (19, 20). In general, the reaction of a hormone with its receptor is rapid, saturable, and reversible. A steady state is achieved in a reasonable time, and quantitative analysis can be performed according to methods established for protein-ligand reactions at equilibrium (21, 22). In some cases, the concomitant occurrence of degradation of the free hormone and of the receptors needs to be carefully controlled and the data corrected accordingly (23). Another correction factor comes from the binding of the hormone, besides the specific receptor sites, to an unsaturable compartment defined as "nonspecific" binding (24). After these corrections, the most common method of analyzing the data is to plot the bound/free ratio of the labeled hormone (B/F) as a function of the concentration of hormone that is bound to the receptors (B). This plot is usually refered to as the "Scatchard plot" (25, 26). The numerous assumptions to be fulfilled for the validity of this analysis have been stressed (20, 22, 27).

In some studies, this plot was linear, for example with growth hormone (28), gonadotropins (29) and calcitonin (30), suggesting the presence of a single homogeneous class of binding sites; in that case, the affinity constant, $K_a$, can be derived from the slope of the line and the binding capacity (or total receptor sites concentration) from its abcissa intercept.

For most hormones, however, curvilinear plots, concave upwards, were obtained. This can be due to a variety of causes, the two best known being either the presence of multiple classes of binding sites with differing affinities or the existence of site-site interactions of the type qualified as "negative cooperativity." In the latter case, the receptor sites do not have a fixed affinity, rather, the affinity of the receptors decreases as a function of the occupancy of the receptors population. Steady-state data alone can be fitted by both models (31). Most authors studying hormone receptors assumed that site-site interactions were absent and used exclusively the "multiple classes of sites" model. The Scatchard plot was then submitted to multicompartmental analysis and values of $K_a$ and binding capacities were derived for the subclasses of sites (21). This approach

has proved useful in providing an easy method for comparison of data obtained in various systems, with different hormones in different laboratories (20).

The interaction of insulin with its receptors is a typical example of curvilinear Scatchard plot (Fig. 1). The quantitative analysis of this system using a multiple classes of sites model has been extensively discussed and the results critically reviewed by Kahn et al. (21). Discrepancies were found between the results yielded by applying the above methods to steady-state data and the results obtained from kinetic data (28). A cooperative model, however, was not considered, and we decided to test it experimentally. Using an original method based on the kinetics of dissociation of insulin from its receptors in human cultured lymphocytes, we demonstrated site-site interactions of a type consistent with negative cooperativity (32). While using the same method, we were unable to find evidence of cooperativity in the binding of growth hormone to the cultured lymphocytes, consistent with the linear Scatchard plot (32).

In the present study, we demonstrate that negative cooperativity is widespread among hormone receptors, discuss its physiological significance for hormone action, and consider various models which may explain the observed phenomena.

## KINETIC DEMONSTRATION OF NEGATIVE COOPERATIVITY

The basic experimental design assumes that in the case of negative cooperativity, the decreased affinity will result at least partially from an increased dissociation rate. When this is the case, it is possible to detect the cooperative interactions by studying the

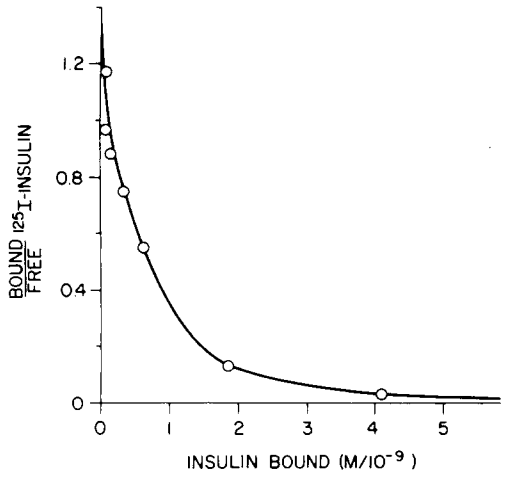


Fig. 1. Scatchard plot of insulin binding to receptors in human cultured lymphocytes $^{125}$I-Porcine insulin ($7 \times 10^{-12}$ M) was incubated for 90 min at 15°C with $1.7 \times 10^6$ cultured lymphocytes/ml (line IM-9) in the absence and presence of various concentrations of unlabeled insulin. At steady state, the cells were sedimented and radioactivity in cell pellet was counted. The bound/free ratio of labeled hormone is plotted as a function of hormone bound to the cells; "nonspecific binding," which constituted less than 5% of the total binding, has been subtracted.

dissociation of a tracer size of labeled hormone from the receptors under two conditions: at an "infinite" dilution of the hormone-receptor complex, and at an "infinite" dilution of the complex in the same medium containing an excess of unlabeled insulin. If site-site interactions of a destabilizing type are induced by hormone binding (negative cooperativity), unlabeled hormone filling empty receptor sites will speed up the dissociation of the labeled hormone bound to other receptor sites.

Slight differences in methodology are required for whole cells and membrane preparations. Cells at high concentration in a single batch are reacted with the labeled hormone at low concentration so that only a minority of the receptor sites are occupied by tracer. Association is monitored by centrifugation of aliquots of the incubation mixture in a Beckman microfuge. When a steady state of occupancy is achieved, the cells are centrifuged at 4°C, the supernatant which contains the unbound $^{125}$I-hormone is discarded, and the pellet is resuspended immediately up to the initial volume with ice-cold buffer. At this point, only a small minority of receptors are filled with labeled hormone while most receptors are unoccupied; the free hormone concentration at this time is effectively 0. Aliquots (100 $\mu$l) are immediately distributed in two sets of tubes; half contain 10 ml of hormone-free buffer (= "dilution only"), half contain 10 ml of buffer to which a saturating concentration of unlabeled hormone has been added (= "dilution + cold hormone"). To monitor the dissociation, duplicate tubes from each set are centrifuged at 4°C, 700 × g for 2 min at regular intervals; the supernatants are discarded and the pellets counted.

When membranes instead of whole cells are used, the method is identical, except that for studying dissociation the content of the dilution tubes is filtered through Millipore filters (0.45 $\mu$l), and the membranes which are retained on the filter are washed once with ice-cold buffer and counted.

The principles on which this experiment is based are explained in detail elsewhere (33). Briefly, in the absence of site-site interactions, the dissociation rate of the labeled hormone should be identical under the two conditions studied as long as the dilution factor is sufficient to prevent rebinding of the labeled species to the empty receptor sites.

The dissociation rate of growth hormone from its receptors in human cultured lymphocytes is indeed identical in both conditions. In sharp contrast, unlabeled insulin, when filling empty receptor sites in the same cells, markedly speeds up dissociation of the already bound $^{125}$I-insulin, demonstrating site-site interactions among insulin receptors consistent with negative cooperativity (32) (Fig. 2).

It is important to verify that the dilution factor in such experiments is effectively "infinite," i.e. sufficient to prevent significant reassociation of the labeled hormone; otherwise, the presence of the unlabeled insulin would decrease this reassociation by (a) filling the empty sites, and (b) isotopic dilution of the label, thus giving the false impression of an accelerated dissociation rate. In this case, however, the initial rate of dissociation should be identical, which is not the case for insulin; further, experimental controls for the absence of rebinding have been described elsewhere (32, 33).

## ALTERNATIVE MODELS

In the interpretation of the results in any new system, the following alternative models should be considered besides site-site interactions.

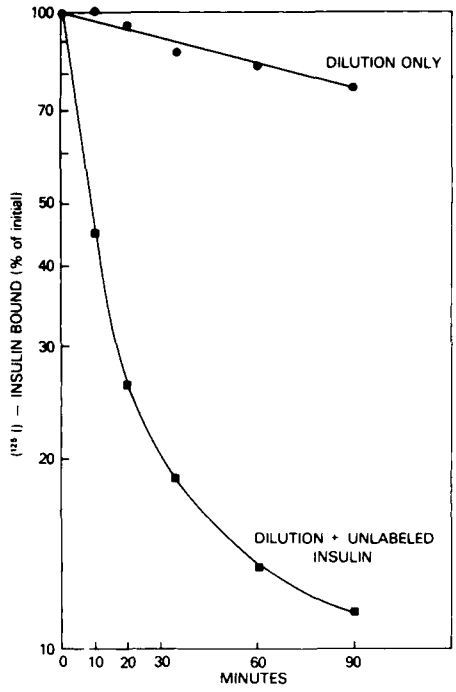


Fig. 2. Negative cooperativity in the dissociation of $^{125}$I-insulin from human cultured lymphocytes. [$^{125}$I] Insulin ($1.7 \times 10^{-11}$ M) was incubated for 30 min at 15°C with $5 \times 10^7$ cells/ml, after which the cells were sedimented at 4°C, the supernatant was replaced by an equal aliquot of chilled fresh medium, the cells were resuspended, and aliquots (0.1 ml) were transferred to a series of tubes that contained 10 ml of medium in the presence and absence of unlabeled hormone ($1.7 \times 10^{-7}$ M, 15°C). At intervals, two tubes of each set were centrifuged, and the radioactivity in the cell pellet was counted. The radioactivity on the cells, expressed as a percentage of the radioactivity present at t = 0, is plotted as a function of the time elapsed after the dilution of the system. Each point is a mean of duplicates; duplicates differed by less than 5%. The radioactivity at t = 0 was measured at the completion of the incubation, both before and after the first sedimentation step; both results were the same, indicating that trapped radioactivity and dissociation during the analyses were insignificant.

## Ligand-Ligand Interactions

**Polymerization of the hormone.** If the unlabeled hormone can form dimers or polymers of a higher order with the labeled hormone bound to the receptor sites, and if the polymer has a lower affinity for the receptor, accelerated dissociation of the label will be observed in the presence of concentrations of unlabeled hormone at which polymerization becomes significant. In the case of insulin and its receptors, we considered this hypothesis unlikely on the basis of the following facts.

(a) We observe accelerated dissociation of $^{125}$I-insulin in the presence of concentrations of insulin as low as $10^{-10}$ M (32); 20% of the maximal increase is obtained with $5 \times 10^{-10}$ M, concentration at which there is 1 dimer for 26,000 monomers (34). Blundell and co-workers (35) have calculated that at $10^{-8}$ M, pH 8.0, conditions at which our observed cooperative effect is quasimaximal, the final mole fraction of dimers would only be 0.00214.

(b) The "cooperative" effect in fact decreases at insulin concentrations $>10^{-7}$ M, at which dimers become a significant proportion of the molecular species (32, 36): the fall in effect superimposes with the theoretical proportion of dimers, suggesting that dimers can bind to the receptor, but that they do not induce the site-site interactions.
(c) Nondimerizing insulin species, tetra (nitrotyrosine)-insulin (37) and guinea pig insulin (38–41) induce the accelerated dissociation in strict correlation with their relative ability to bind to the insulin receptor sites (32, 36). Since they do not dimerize, no fall in effect is observed at high concentrations. This is consistent with the fact that the area of the insulin monomer which seems responsible for the cooperativity is covered in the dimer (see below).

In a recent article (42), Cuatrecasas and Hollenberg confirmed our initial report of insulin-induced acceleration of dissociation, but challenged our interpretation by attributing this effect to insulin dimerization. They not only overlooked the above controls that we had reported (32, 36), but further supported their view with erroneous quotations of the literature. We did not use insulin concentrations "which are all supersaturating with respect to receptor binding;" in fact, saturation of a small percentage of the sites induces significant cooperativity (32). Zimmerman et al. have not "failed to demonstrate dimerization" of guinea pig insulin due to the low insulin concentration used in sedimentation equilibrium studies (39); in a more recent study they have indeed clearly demonstrated that guinea pig insulin remains monomeric even at concentrations of 3 mg/ml (40); Cuatrecasas and Hollenberg are apparently unaware of the publication of this more complete study. Finally, Pekar and Frank (34) did not report a dissociation constant for dimerization of $7 \times 10^{-7}$ M; they reported an equilibrium constant of $1.4 \times 10^5$ 1/mole, which yields a dissociation constant of $\sim 7 \times 10^{-6}$ M. This agrees well with the value reported by Goldman and Carpenter (43) (not quoted by Cuatrecasas and Hollenberg) and is consistent with the fall in cooperative effect observed in our studies. In summary, the accelerated dissociation is due to the insulin monomer and, if anything, is lost with the dimer.

Less specific types of ligand-ligand interactions, such as steric hindrance of the receptor site or electrostatic repulsion, would explain curvilinear Scatchard plots, through a progressively decreasing association rate, but not an accelerated dissociation rate of already bound labeled molecules when the complex is diluted in the presence of an excess unlabeled molecule.

The binding of flexible ligands can also yield curvilinear Scatchard plots (44); this model does not fit the highly structured tertiary and quaternary conformation of insulin and does not explain the accelerated dissociation of one ligand molecule by others.

### Unstirred Layers ("Noyes-Whitney" Layers, Diffusion Boundary Layers)

The role of liquid stationary films as diffusion barriers has been considered in various areas of research, from batch adsorption of gases (45, 46) to membrane transport kinetics (47, 48). Conceivably, rebinding of the labeled hormone inside an unstirred layer surrounding the cell could occur even in an infinite dilution; isotopic dilution of the labeled hormone by unlabeled hormone in the unstirred layer could induce an increase in the apparent dissociation rate.

This phenomenon can be ruled out in the case of insulin receptors since dissociation of $^{125}$I-labeled growth hormone from receptors on the same cells is not accelerated by

unlabeled growth hormone (32). Furthermore, the high degree of structural specificity of the "cooperative" effect (see below) cannot be explained by the existence of an unstirred layer.

## NEGATIVE COOPERATIVITY IN OTHER HORMONE RECEPTORS

Following our initial report, a number of investigators, using this kinetic method, have confirmed the existence of negatively cooperative interactions, not only among insulin receptors in a variety of tissues, but also among receptors for thyrotropic hormone, nerve growth factor, and catecholamines (Table I and Figs. 3–5). This phenomenon is

**Table I. Kinetic Demonstration of Negative Cooperativity in Receptors for Hormones, Growth Factors and Neurotransmitters**

| Receptor for | Tissue Preparation | Reference |
|---|---|---|
| Insulin | Human cultured lymphocytes | De Meyts, Roth, Neville, Gavin and Lesniak, 1973 (32) |
| | Human blood monocytes | Bianco, Schwartz and Handwerger, 1974 (62) |
| | Human blood granulocytes | Fussgänger, Kahn, Roth and De Meyts, 1975 (63) |
| | Turkey erythrocytes | Ginsberg, Kahn and Roth, 1975 (submitted for publication) |
| | Turkey erythrocyte membranes | Ginsberg, Kahn and Roth, 1975 (submitted for publication) |
| | Rat liver membranes | De Meyts, Bianco and Roth, 1976 (33) |
| | Mouse liver membranes | Soll, Kahn and Neville, 1975 (64) |
| | Chicken liver membranes | De Meyts, Kahn, Ginsberg and Roth, 1975 (55) |
| | Guinea-pig liver membranes | De Meyts, Kahn, Ginsberg and Roth, 1975 (55) |
| | Human cultured placental cells | Podskalny, Chou and Rechler, 1975 (65) |
| | Human placenta membranes | Cuatrecasas and Hollenberg, 1975 (42) |
| | Cultured human fibroblasts | Rechler and Podskalny, (submitted for publication) |
| Nerve growth factor | Sympathetic and dorsal root ganglia | Frazier, Boyd and Bradshaw, 1974 (61) |
| | Embryonic heart and brain | Frazier, Boyd, Pulliam, Szutowicz and Bradshaw, 1974 (66) |
| Epidermal growth factor | Human placenta membranes | Cuatrecasas and Hollenberg, 1975 (42) |
| Thyroid-stimulating hormone | Thyroid membranes | Lee, Winand and Kohn, 1975 (67) |
| | Retro-orbital membranes | Lee, Winand and Kohn, 1975(67) |
| β-Adrenergic | Frog erythrocyte membranes | Limbird, De Meyts and Lefkowitz, 1975 (68) |

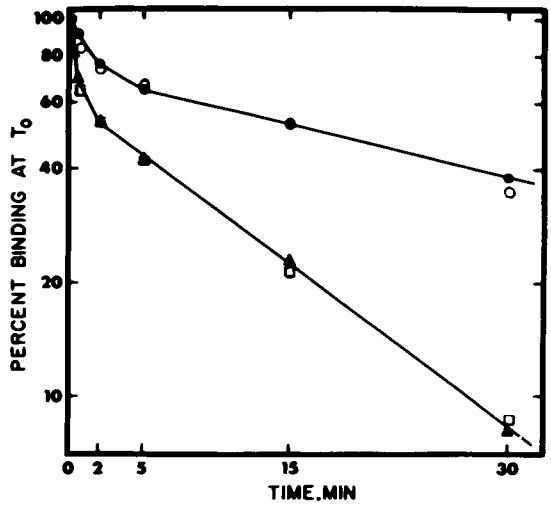


Fig. 3. Negative cooperativity in the dissociation of nerve growth factor (NGF) from receptors in heart and brain. The time course of dissociation of specifically bound $^{125}$I-nerve growth factor ($3 \times 10^{-9}$ M, equilibrated at 24°C for 2 hr) from heart and brain tissue suspensions at 24°C is followed. One set of samples (4) was diluted with 10 ml of Hanks' salt solution with 1 mg/ml of bovine serum albumin (heart [●], brain [○]), and another set was diluted in the same way except that the diluent contained $8 \times 10^{-8}$ M native NGF (heart [▲], brain [□]). At the times indicated, the samples were transferred to 0.8–μm Nuclepore filters under reduced pressure and washed twice with 5 ml of ice-cold Hanks' salt solution with 1 mg/ml of bovine serum albumin. The process required 15 sec. Nonspecific binding was determined under the conditions of the experiment in quadruplicate and subtracted to produce the data points shown. Data from W. A. Frazier and co-workers (61), used with permission.

thus widespread, although not universal (receptors for growth hormone, gonadotropins, and calcitonin, for example, do not appear to produce such interactions; vasopressin receptors in kidney exhibit positive cooperativity [49, 50]). In the systems which display negative cooperativity, the effect is present in both whole cell and purified plasma membrane preparations. It will be of interest to perform such studies with isolated receptors when the technology of receptor solubilization has been sufficiently improved.

## PHYSICOCHEMICAL CHARACTERISTICS OF THE NEGATIVE COOPERATIVITY

Detailed studies have thus far been reported only in the case of insulin receptors (33). The dissociation of $^{125}$I-insulin from the receptors, and the acceleration of dissociation by unlabeled insulin, are modulated by various factors such as pH, temperature, ionic strength, and urea. These studies, reported in detail elsewhere, (33) suggest that the insulin receptor sites exist under at least two reversible conformations: a "high affinity" conformation, present at low occupancy of the receptors by insulin, from which insulin dissociates slowly, and a "low affinity" conformation, at high occupancy of the receptors from which insulin dissociates fast. The proportion of sites in each state is regulated by the factors described above as summarized in Table II.

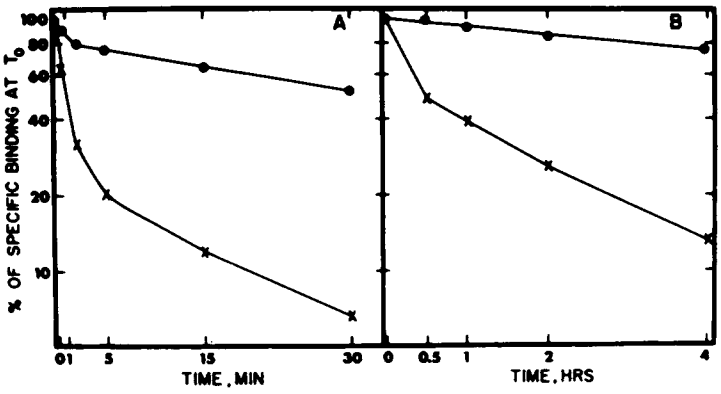


Fig. 4. Negative cooperativity in the dissociation of NGF from receptors in sympathetic ganglia at 24°C (A) and 0°C (B). A, time course of dissociation of specifically bound $^{125}$I-NGF ($2 \times 10^{-9}$ M, equilibrated at 24°C for 2 hr) from sympathetic tissue suspensions (2.4 μg/300 μl) at 24°C. One set of samples (4) was diluted with 33 vol of Hanks' salt solution with 1 mg of albumin per ml at 24°C (•) and another set was diluted in the same way, except that the diluent contained $2 \times 10^{-7}$ M native NGF (X). At the times indicated, the samples were transferred to 0.8-μm Nuclepore filters under reduced pressure and washed twice with 5 ml of ice-cold Hanks' salt solution with 1 mg of albumin per ml. The process required 12 sec. Nonspecific binding was determined under the conditions of the experiment in quadruplicate and subtracted from each data point. B, time course of dissociation of dissociation of specifically bound $^{125}$I-NGF ($2 \times 10^{-9}$ M) from sympathetic tissue suspensions at 0°C. The experimental design was identical to that of the experiment shown in A, except that the diluent was at 0°C and the tubes were maintained in an ice bath for the times indicated. Data from W. A. Frazier and co-workers (55), used with permission.

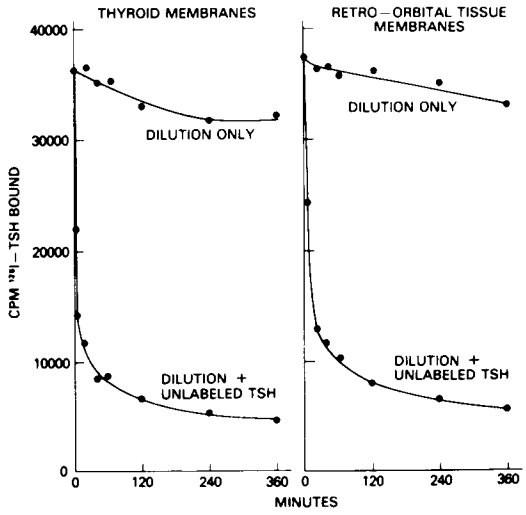


Fig. 5. Negative cooperativity in the dissociation of TSH from receptors in thyroid membranes (left) and retro-orbital tissue membranes (right). Experimental design similar to Figs. 2–4; for specific details, see Lee, Winand, and Kohn, Biochem. Biophys. Res. Commun., (1975), in press. Unpublished data kindly communicated by Dr. L. Kohn.

Table II. "Two-State" Model for Insulin Receptors

| "Slow-dissociating state" favored by: | "Fast-dissociating state" favored by: |
|---|---|
| Low fractional saturation with insulin | Increased fractional saturation with insulin |
| Alkaline pH (8–9) | Acid pH (5–6) |
| Low temperature (4°C) | High temperature (37°C) |
| High concentration of $Ca^{++}$–$Mg^{++}$ | Low concentration of $Ca^{++}$–$Mg^{++}$ |
| Concanavalin A (20 μg/ml) | Urea |

## STRUCTURAL REQUIREMENTS FOR NEGATIVE COOPERATIVITY

Binding to the receptor is an obvious condition for the hormone to induce site-site interactions among the receptor sites; however, if this condition is necessary, it is not sufficient, as the following studies demonstrate.

In the case of insulin, a myriad of analogs (animal insulins, insulin digested by enzymes, or chemically modified insulins) are available for study of structure-activity relationships. Extensive studies (51,52) have demonstrated that the affinity for binding of the various analogs to the receptor parallels the potency of these analogs in exerting the biological effects of insulin. In a study of 26 such analogs (36), we have found that the analogs induce site-site interactions in direct proportion to their occupancy of the receptors with two exceptions. Desalanine-desaparagine insulin (DAA) and desoctapeptide insulin (DOP) are totally unable to induce the site-site interactions, even at enormous concentrations at which they saturate the insulin receptor sites and exert maximal biological effects. A third compound, despentapeptide insulin, shows an impaired, but not absent ability to induce the site-site interactions.

Thus, the negative cooperativity and the biological effects are exerted through the binding of separate areas of insulin and its receptor: both contain a "cooperative" and a "bioactive" site. Study of the localization of the residues deleted in DAA and DOP insulin has allowed a tentative mapping of the cooperative site of insulin (manuscript in preparation). These residues, localized in a limited area at the surface of the insulin monomer, are also covered in the dimerization of insulin, consistent with the loss of cooperativity at high insulin concentrations (32, 36).

## INHIBITION OF THE NEGATIVE COOPERATIVITY OF INSULIN RECEPTORS BY CONCANAVALIN A

The plant lectin, concanavalin A (Con A), which has numerous biological effects on cells through binding to surface glycoproteins, markedly inhibits the negative cooperativity induced by insulin (53). In human cultured lymphocytes, Con A (20 μg/ml) markedly reduces the accelerated dissociation of the $^{125}$I-insulin-receptor complex in the presence of an excess unlabeled insulin. The effect of Con A is prevented by simple sugars that bind to Con A. In contrast, Con A even when present before insulin, fails to alter the initial rate of association of $^{125}$I-insulin, indicating that Con A does not occupy the "bioactive site" of the receptor. These conclusions were consistent with studies at steady state: in the presence of Con A, the graphical deviations due to negative cooperativity

were markedly reduced in the Scatchard, double-reciprocal, and other plots; the Hill coefficient was 0.81 instead of 0.53, again indicating that Con A interferes with the cooperative interactions (Fig. 6).

## QUANTITATIVE DESCRIPTION OF THE BINDING ISOTHERM IN THE PRESENCE OF COOPERATIVITY

We have recently presented a more precise quantitative analysis of the site-site interactions and described a plausible mechanism (54). As stressed above, kinetic data indicate that with increasing occupancy, insulin receptor sites are switched reversibly from a "high affinity state" in which insulin dissociates slowly ("empty sites" conformation) to a "low affinity," fast-dissociating state ("filled sites" conformation). The proportion of sites in each state for a given occupancy depends on pH, temperature, and ionic strength. In dealing with such site-site interactions, classical methods of steady-state analysis do not yield physically meaningful parameters. In the Scatchard plot of bound/free (B/F) vs. bound (B), only the abcissa intercept keeps its physicochemical meaning of binding capacity or $R_0$. We have proposed a new parameter, $\bar{K}$, for describing site-site interactions, where $\bar{K} = (B/F)/(R_0\text{-}B)$ and is the "average affinity" of the sites at a given fractional saturation. Plotting $\bar{K}$ against the fractional saturation ($B/R_0$) reveals that: (a) at very low occupancy, a limiting high $\bar{K}$ is obtained ("empty sites" conformation); (b) $\bar{K}$ begins to fall at an occupancy as low as 1–5% ("threshold"); (c) above 25% occupancy, a limiting low $\bar{K}$ is obtained ("filled sites" conformation). From this analysis, it is apparent that most of the reduction in binding of $^{125}$I-insulin at low concentrations of unlabeled insulin reflects negative cooperativity and not competition for occupancy of sites. Furthermore, the difference between the two extreme values of $\bar{K}$ in this "two state" model is much smaller than the difference between the "high" and "low" affinities of a

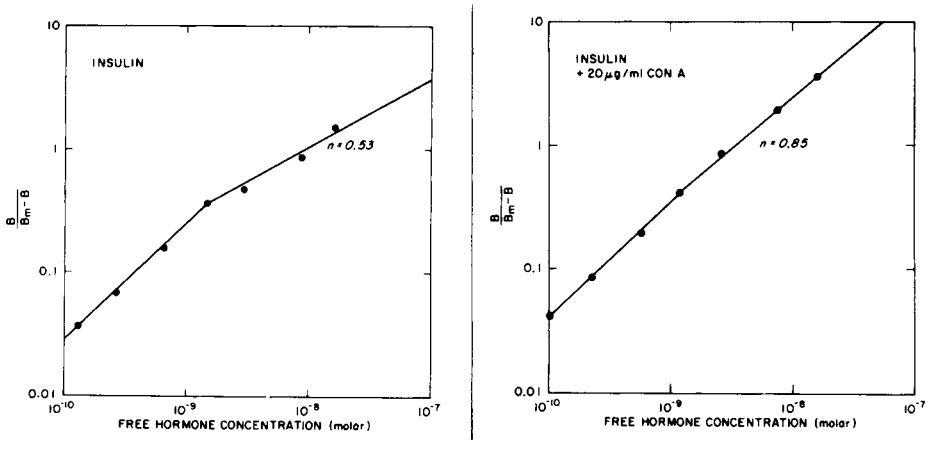


Fig. 6. Inhibition of the negative cooperativity of insulin receptors by concanavalin A. Left: Hill plot of insulin binding to receptors in human cultured lymphocytes. Notice the break in the line and the slope <1 at half saturation, corresponding to a Hill coefficient of 0.53. Right: 20 μg/ml of concanavalin A decreases the degree of nonlinearity and the Hill coefficient raises to 0.85.

model with two discrete classes of binding sites with differing affinities. Thus, the same range of sensitivities which would require the cell to synthesize two structurally distinct receptor molecules can be achieved with one molecular species endowed with the ability to undergo induced-fit conformational changes.

## NEGATIVE COOPERATIVITY AND MOLECULAR EVOLUTION OF RECEPTORS

The study of insulin receptors has given some clues, although indirect, that the cooperative properties of hormone receptors may have evolved very early in the natural history of the hormone (55). Human and other mammalian insulins are very similar in amino acid sequence and equipotent biologically. The exceptions are insulins of guinea pig and other histricomorphs, which differ by 33% from the human sequence and are biologically much less potent. In contrast, insulins of birds, which differ 14% from the human sequence, are 2–3 times more potent than human insulin. For all insulins studied, the biological potencies correlate with their affinities for a mammalian receptor. In recent studies we examined the insulin receptor of man, rat, mouse, guinea pig, chicken, and turkey in their reaction with insulins from man, pig, fish, guinea pig, and chicken sources. Irrespective of the species or tissue tested, the insulin receptors were identical in all respects, including specificity and negative cooperativity. All species bound chicken insulin > pork > fish >>guinea pig. Bird insulin was recognized much better than the other insulins by the receptor of all species, whereas the guinea pig recognized its own insulin much more poorly than the other insulins. Thus, despite marked evolutionary changes in insulin, the receptor has remained remarkably constant in its physicochemical properties and recognition of hormone structure. Further, conservation of the negative cooperativity indicates the importance of this mechanism in the action of insulin.

## CONCLUSION: NEW MODELS OF HORMONE BINDING AND ACTION

The binding of hormone to receptors appears to be a complex mechanism. Cooperative models, which are progressively replacing old concepts of drug action in pharmacology (56), are likely to play an increasing role in our understanding of hormone action. We have presented the first direct evidence that such models apply to hormonal receptors. The discovery of cooperativity in hormone binding implies that the receptor undergoes ligand-induced changes in conformation, but does not allow one, in the absence of independent information, to make any assumption as to the precise molecular nature of the conformational changes. The bulk of available data is consistent with a model in which the insulin receptor sites are switched from a state in which insulin dissociates slowly to a fast-dissociating state when occupancy by insulin increases; a purely "phenomenological" illustration of such a model is presented in Fig. 7, as the mirror image of the positive cooperativity in hemoglobin. Several possible mechanisms are currently investigated (33), including conformational changes in the tertiary or quaternary structure of oligomeric receptors (the most extreme being reversible association $\rightleftarrows$ dissociation), "clustering" of receptors through translational movements in the fluid membrane (17, 57), (which might explain the inhibition of site-site interactions of insulin

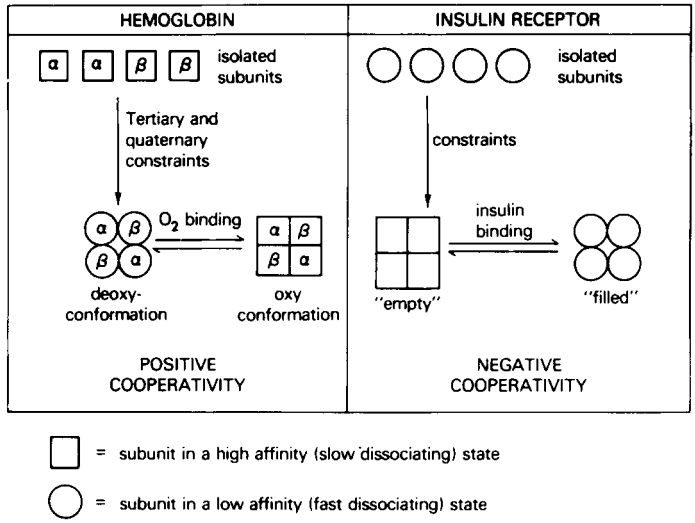


Fig. 7. A plausible model for the negative cooperativity in insulin receptors. This model was designed by analogy with the structure and function of hemoglobin. In hemoglobin, isolated chains ($\alpha$ and $\beta$) exhibit Michaelian saturation curves and high affinity for oxygen; the constitution of the tetramer involves constraints which bring the subunits into a state of lower affinity for oxygen. The binding of oxygen is concomitant with a release of the constraints, which improves the affinity of neighboring subunits for oxygen (positive cooperativity). The rationale for this mechanism, in which affinity for oxygen is low at low partial pressure of oxygen, is that hemoglobin is functionally a carrier molecule and must release oxygen more easily when its tissue concentration is low. For a receptor, which must bind the hormone at low concentrations, high affinity at low concentrations is favorable, which is the case in a negative cooperative binding. The negative cooperativity buffers the system against high hormone concentrations. The model on the right, built as the "mirror image" of hemoglobin, is one among many plausible ones. It is not implied that all "subunits" change shape simultaneously; intermediate steps (sequential model) have not been illustrated. From De Meyts et al., to be published (33).

receptors by Concanavalin A). Recent electron microscope studies of biologically active ferritin-labeled insulin by L. Jarett and co–workers in St. Louis, Mo. (58) and L. Orci and co-workers in Geneva (59), have directly demonstrated the occurrence of both dispersed and "clustered" distribution of insulin receptors on the membrane of fat cells (Fig. 8) and liver (Fig. 9, 10); if it is confirmed to be "physiological," this might be a major breakthrough in understanding the mechanism of the negative cooperativity. Conformational changes in receptors amplified through a clustering mechanism (57, 60), as recently emphasized by Levitzki (Fig. 10), could be the cause of the negative cooperativity in binding while "triggering" the membrane and inducing the biological effects. Such a mechanism constitutes, thus, the potential basis for a coherent theory of hormone action, including a role for excess or "spare" receptors.

Finally, by bringing together the technological progress made in the recent past in the direct study of hormone-receptors interactions, the refined theoretical framework of allosteric regulation, and the most recent concepts of membrane structure and function, it should be possible in the near future to derive a more unified picture of the delicate mechanisms by which hormones turn on their target cells.

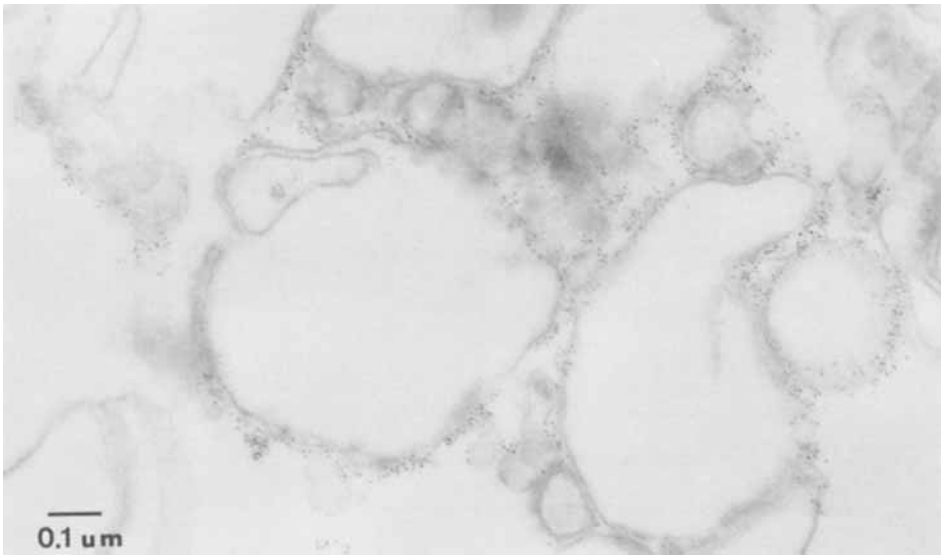


Fig. 8. Electron micrograph demonstrating binding of ferritin-insulin to adipocyte plasma membranes. Plasma membrane protein (1 mg) was incubated for 30 min at 30°C in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with 500 $\mu$U of ferritin-insulin. The incubation mixtures were diluted and processed for thin section electron microscopy. Fig. 8 demonstrates the irregular binding of the ferritin-insulin molecule to the outside of the membrane vesicles. Clumps of two to four ferritin molecules are seen, as well as individual ferritin cores. Ferritin-insulin is bound to about 75% of the vesicles. × 63,700. From L. Jarett and R. M. Smith (64), used with permission.

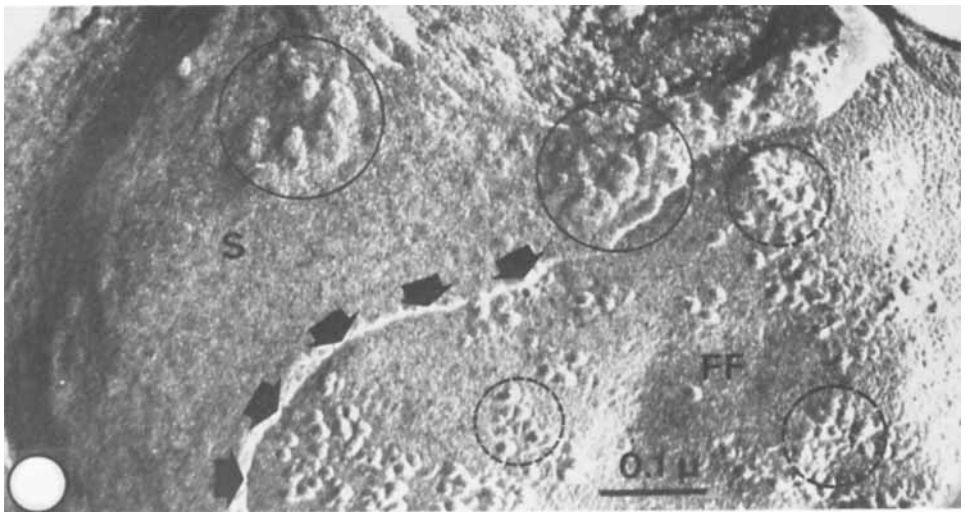


Fig. 9. Freeze-fractured and deep-etched purified liver plasma membrane incubated with insulin-ferritin complex. Freeze-fracturing has split the membrane and exposed its inner matrix, containing intramembranous particles (fracture face, FF). Subsequent etching has exposed the true (outer) surface of the membrane (S). The fracture face and the true surface are separated by a ridge (arrows): the fracture face contains clusters of intramembranous particles (dotted circles); the true surface of the membrane shows clusters of ferritin molecules (continuous circles). × 90,750. Courtesy of L. Orci, and co-workers (59), used with permission.

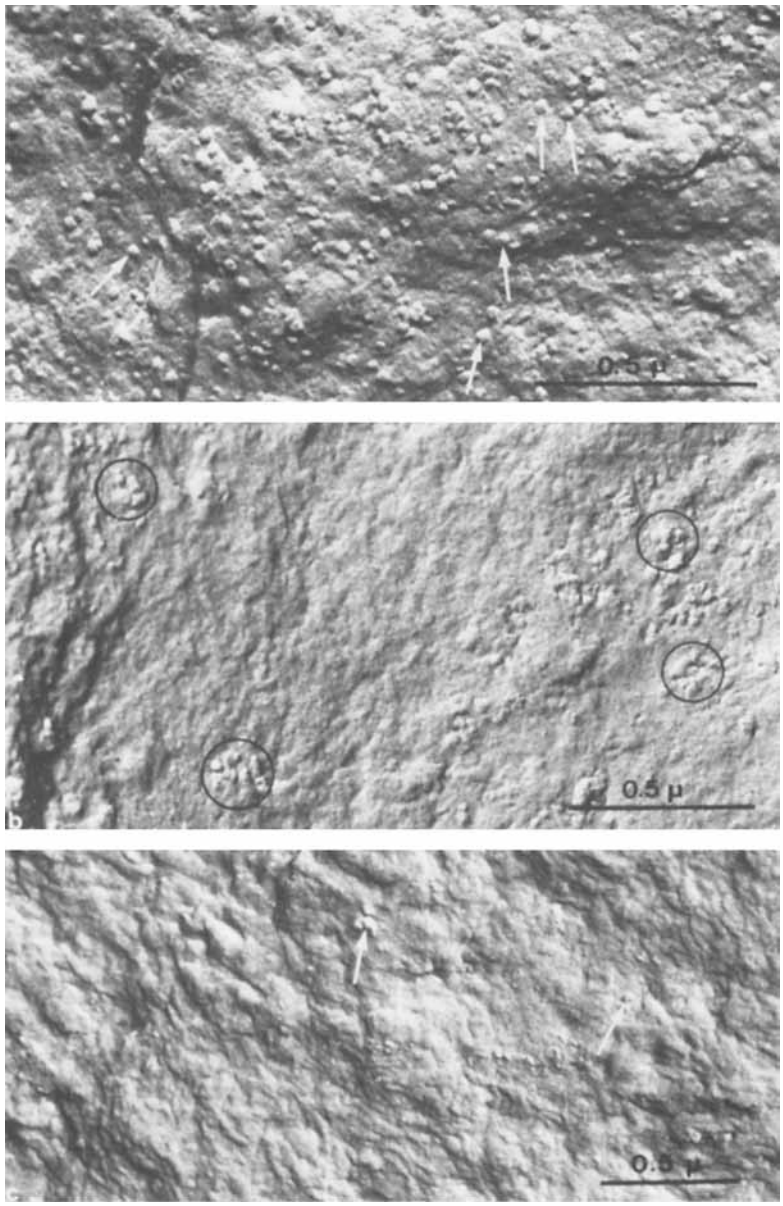


Fig. 10. Replicas (shadow casting) of purified liver plasma membranes incubated directly with insulin-ferritin complex (a and b) or after exposure to native insulin (c). (a) Shows a membrane surface with a predominantly diffuse pattern of ferritin molecules, (b) a cluster distribution of ferritin molecules, and (c) a membrane suface free of ferritin, except in some areas indicated by arrows. (a) × 74,000; (b) × 63,000; (c) × 46,000. Courtesy of L. Orci and co-workers (59), used with permission.

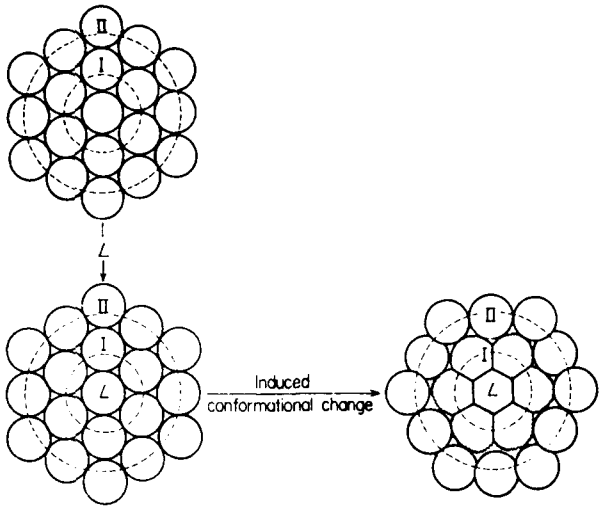


Fig. 11 A model for the negative cooperativity associated with receptor clustering. When one ligand molecule binds to 1 of the 19 available sites shown in the drawing, the receptor undergoes a structural transition. This transition propagates outwards towards layer I (six receptor molecules). In this case, the number of receptor molecules undergoing conformational change without binding of ligand is 86%. A modified model can also be offered where the receptor molecules on the membrane are not clustered a priori, and can move freely in the fluid membrane. Upon ligand binding, the receptor molecule undergoes a conformational change enabling it to interact with unbound receptor molecules and form a cluster; the unbound receptor molecules then undergo a conformational transition, converting them into a nonreceptive state. This conformational change could propagate further than shown above. Both models bring about negative cooperativity among receptors and explain "spare receptors." From A. Levitzki (57), with permission of Academic Press, Inc., New York.

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