

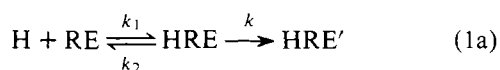
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## Mode of Coupling between the $\beta$ -Adrenergic Receptor and Adenylate Cyclase in Turkey Erythrocytes<sup>†</sup>

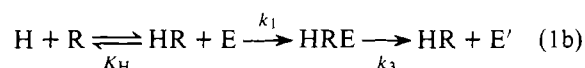
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**ABSTRACT:** The mode of coupling of the  $\beta$ -adrenergic receptor to the enzyme adenylate cyclase in turkey erythrocyte membranes was analyzed in detail. A number of experimental techniques have been used: (1) measurement of the kinetics of cyclase activation to its permanently active state in the presence of guanylyl imidodiphosphate, as a function of hormone concentration; (2) measurement of antagonist and agonist binding to the  $\beta$ -adrenergic receptor prior and subsequent to the enzyme activation by hormone and guanylyl imidodiphosphate. On the basis of these two approaches, all the models of receptor to enzyme coupling which involve an equilibrium between the enzyme and the receptor can be rejected. The binding and the kinetic data, however, can be fitted by two diametrically opposed models of receptor to enzyme coupling: (a) the precoupled enzyme-receptor model where activation of the enzyme occurs, according to the following scheme:



where H is the hormone, RE is the precoupled receptor-enzyme complex,  $k_1$  and  $k_2$  are the rate constants describing hormone binding, and  $k$  is the rate constant characterizing the formation of HRE' from the intermediate HRE. According to this model, the activated complex is composed of all of the interacting species. (b) The other model is the collision coupling mechanism:

pling mechanism:



where  $K_H$  is the hormone-receptor dissociation constant,  $k_1$  is the bimolecular rate constant governing the formation of HRE, and  $k_3$  the rate constant governing the activation of the enzyme. In this case the intermediate never accumulates and constitutes only a small fraction of the total receptor and adenylate cyclase concentrations. In order to establish which of the two mechanisms governs the mode of adenylate cyclase activation by its receptor, a diagnostic experiment was performed: Progressive inactivation of the  $\beta$  receptor by a specific affinity label was found to cause a decrease in the maximal binding capacity of the receptor and a proportional decrease in the rate of activation, but no change in the maximum level of activity was attained. Progressive inactivation of the enzyme by *p*-hydroxymercuribenzoate was found not to change the rate of activation nor the capacity of the receptor to bind hormone. Only the maximal level of activation was found to be decreased. These results are not compatible with the precoupled model of receptor and cyclase nor with floating receptor models in which an intermediate of hormone, receptor, and cyclase is in equilibrium with its reactants. The data strongly suggest that the collision coupling is the mode of coupling between the  $\beta$  receptor and cyclase coupling in turkey erythrocyte membranes.

**I**n the Appendix we have described five different molecular models for receptor to enzyme coupling. In this manuscript we present an attempt to analyze completely the mechanism of receptor to adenylate cyclase coupling in turkey erythrocyte membranes.

Turkey erythrocyte membranes possess an adenylate cyclase coupled to  $\beta$ -adrenergic receptors capable of being stimulated by 1-catecholamines (Davoren and Sutherland, 1963). One of the major problems concerning the cyclase-receptor complex is the mechanism by which hormone binding to the re-

ceptor induces the activation of adenylate cyclase. An intriguing aspect of cyclase activation by hormones concerns the permanently active enzyme which is formed in the presence of hormone and GppNHp<sup>1</sup> (Schramm and Rodbell, 1975; Pfeuffer and Helmreich, 1975; Sevilla et al., 1976; Levitzki et al., 1976; Spiegel et al., 1976). Although the highly active state is induced by hormone and therefore must be formed through interaction with the receptor, once formed this state

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<sup>1</sup> Abbreviations used are: PEP, phosphoenolpyruvate; PPL, propranolol; PHMB, *p*-hydroxymercuribenzoate; NHNP-NBE, *N*-[2-hydroxy-3-(1-naphthoxy)propyl-*N'*-bromoacetyl]ethylenediamine; TME, 50 mM Tris, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 7.4, buffer; HYP, hydroxybenzylpindolol; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; GppNHp, guanylyl imidodiphosphate.

is a permanent property of the enzyme and no longer involves receptor intervention. Thus propranolol, a specific  $\beta$ -adrenergic blocker, can no longer reduce in any way the activity of the highly active cyclase already formed but can block further activation of a partially active enzyme (Levitzki et al., 1976; Sevilla et al., 1976; Spiegel et al., 1976). A detailed description of this phenomenon has already been presented (Sevilla et al., 1976).

One of the means to reveal information about the state of coupling between the receptor and the enzyme is to perform a kinetic analysis on the rate of appearance of the permanently active adenylate cyclase as a function of hormone concentration. Because of the irreversibility of this process, the relative simplicity, and the slow appearance of the permanently active state (Sevilla et al., 1976), we have chosen this system to probe the state of receptor to enzyme coupling. Three basic experimental approaches were used in this study: (1) Examination of the kinetics of the process of enzyme activation induced by hormone as a function of hormone concentration; (2) examination of the kinetics of activation as a function of receptor concentration and cyclase concentration; and (3) measurement of 1-epinephrine and [ $^{125}$ I]HYP binding when the enzyme is either in its inactive state or in its permanently active state. These three lines of experimentation were proven to be extremely powerful in delineating the mode of receptor to enzyme coupling.

#### Materials and Methods

(a) *Materials.* [ $\alpha$ - $^{32}$ P]ATP and Na $^{125}$ I were obtained from the Radiochemical Centre (Amersham, England). GppNHp was obtained from Boehringer (Mannheim, West Germany). All other chemicals and biochemicals were of the highest purity available. All the solutions were prepared in Corning doubly distilled water.

(b) *Preparation of Turkey Erythrocyte Membranes.* Turkey erythrocyte membranes were prepared as previously described (Steer and Levitzki, 1975). The membranes were stored in a solution containing 25% sucrose, 0.1 mM DIFP, 50 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl $_2$ , and 1 mM EDTA, in liquid nitrogen.

(c) *Measurement of cAMP.* The amount of cAMP formed was measured by the method of Salomon et al. (1974). All ingredients were weighed out freshly before use. Protein concentration was measured by the method of Lowry et al. (1951).

(d) *The Kinetic Measurement of Cyclase Activation.* The membranes, stored in liquid nitrogen, were thawed out quickly and were washed twice in a Sorvall rotor at 23 000g for 15 min with a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM MgCl $_2$ , and 1 mM EDTA (TME). The hormones were weighed out just prior to use and dissolved in TME and were mixed to the desired concentrations with GppNHp which was also dissolved in TME. Final GppNHp concentration in the activation medium in all experiments was 0.1 mM. The enzyme preparation and the mixture of hormone and GppNHp were preincubated for 5 min at 37 °C separately to preequilibrate. At zero time a portion of the membrane preparation was added to the hormone plus GppNHp solution to a final concentration of 1.2–1.4 mg/mL. At the times desired, the activation reaction was stopped by removing 70- $\mu$ L portions into test tubes containing 40  $\mu$ L of 3.75  $\times$  10 $^{-5}$  M *dl*-propranolol kept at 0 °C. After completion of activation experiments, 40  $\mu$ L of a mixture of [ $\alpha$ - $^{32}$ P]ATP and a regenerating system containing theophylline, as described, were added to start the adenylate cyclase assay at 37 °C (Steer and Levitzki, 1975). The reaction was stopped at the desired time by 100  $\mu$ L of a mixture of c[ $^3$ H]-

AMP which contained about 24 000 cpm and 2% NaDod-SO $_4$ .

(e) *The Binding of [ $^{125}$ I]HYP and Its Displacement by 1-Epinephrine.* The membranes were thawed and prepared as described in the section on kinetic measurements and were brought to a concentration of 0.1 mg of protein/mL. The binding assay mixture consisted of 50 mM Tris-HCl (pH 7.4) and 2 mM MgCl $_2$  to which the appropriate amount of [ $^{125}$ I]HYP was added. The final volume of the binding assay was 0.5 mL. Five micrograms of the membrane preparation was added last, and the assay systems were incubated for 25 min at 37 °C. The nonspecific binding was determined by the addition of 0.8  $\times$  10 $^{-5}$  M nonradioactive HYP or 1.0  $\times$  10 $^{-5}$  M *dl*-propranolol (final concentrations). Fifty-microliter samples of the binding medium were taken out of the complete assay medium to determine the total radioactivity in the assay mixture, and the rest was poured onto glass-fiber filters, as previously described (Spiegel et al., 1976). The tubes were then washed twice and poured onto the Millipore with 10 mM Tris-HCl buffer (pH 7.4) previously equilibrated at 20 °C, and two additional washes of the Millipore were performed. The Millipores were counted in Packard auto gamma scintillation spectrometer. The displacement experiments were performed as the usual binding assay, but the appropriate amount of 1-epinephrine was added prior to the addition of the membranes. It was found that high concentrations of 1-epinephrine (1.0  $\times$  10 $^{-2}$  M) caused the same amount of displacement as 1.0  $\times$  10 $^{-5}$  M *dl*-propranolol.

(f) *Treatment of Membranes with the Affinity Label for the  $\beta$ -Adrenergic Receptor.* The irreversible  $\beta$ -adrenergic blocker *N*-[2-hydroxy-3-(1-naphthylloxy)propyl]-*N'*-bromoacetylenediamine (NHNP-NBE) (Atlas and Levitzki, 1976; Atlas et al., 1976) was weighed freshly before treatment and dissolved in double-distilled water to a final concentration of 5  $\times$  10 $^{-4}$  M. Various concentrations of NHNP-NBE (from 0.17 to 2.3  $\times$  10 $^{-4}$  M) were incubated with 1.48 mg/mL of membrane protein in the presence of 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 2 mM MgCl $_2$ , for 10 min at 20 °C in a final volume of 1.5 mL. After incubation, the membranes were diluted into a final volume of 12.0 mL and centrifuged at 23 000g for 10 min. The precipitate was resuspended in 12.0 mL of the Tris-MgCl $_2$ -EDTA buffer and re-centrifuged. This procedure was repeated four times. The precipitate was resuspended in the Tris-MgCl $_2$ -EDTA buffer to a final concentration of 2.0 mg/mL. Native membranes and membranes previously treated with NHNP-NBE were diluted to a final concentration of 0.2 mg/mL. Untreated membranes were incubated for 10 min at 20 °C and washed four times at 20 °C along with the affinity label-treated membranes. Fifty microliters of membranes was then diluted into 0.5 mL final volume of buffer containing 50 mM Tris-HCl (pH 7.4) and 2 mM MgCl $_2$  containing increasing concentrations of [ $^{125}$ I]-HYP. Nonspecific [ $^{125}$ I]HYP binding was determined in the presence of 1.0  $\times$  10 $^{-5}$  M *dl*-propranolol.

A sample of 50  $\mu$ L from each incubation mixture was counted in order to determine the total [ $^{125}$ I]HYP concentration. From the amount of [ $^{125}$ I]HYP bound and the total [ $^{125}$ I]HYP concentration, the concentration of free [ $^{125}$ I]HYP was calculated. The kinetics of adenylate cyclase activation by GppNHp and 1-epinephrine in the affinity labeled membranes was examined as described before (see Materials and Methods, section d).

(g) *Treatment of Membranes with PHMB.* PHMB was weighed out freshly before the experiment and dissolved to a concentration of 1.0 mM in Tris-MgCl $_2$ -EDTA buffer. Membranes at a final concentration of 1.0 mg/mL were in-

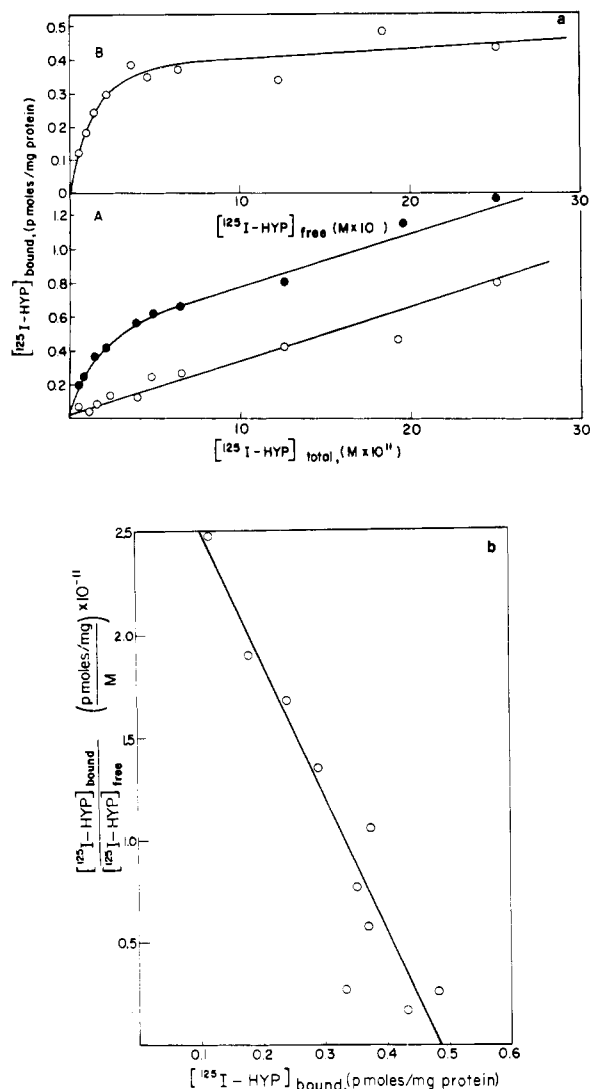


FIGURE 1: (a) Binding of  $[^{125}\text{I}]\text{HYP}$  to turkey erythrocyte membranes. The lower panel (A) represents the linear binding of  $[^{125}\text{I}]\text{HYP}$  in the presence of  $0.8 \times 10^{-6}$  M noniodinated HYP (O) and the total binding of  $[^{125}\text{I}]\text{HYP}$  (●). The upper panel (B) represents the specific binding of  $[^{125}\text{I}]\text{HYP}$ . (b) A Scatchard plot of the specific binding of  $[^{125}\text{I}]\text{HYP}$ . Data of Figure 1a (B) was replotted according to Scatchard.

cubated in the presence of  $1.0 \times 10^{-6}$  to  $1.0 \times 10^{-4}$  M PHMB for 10 min in an ice water bath ( $0^\circ\text{C}$ ) in a final volume of 1.0 mL. The incubation mixture was then diluted into 12.0 mL of Tris-MgCl<sub>2</sub>-EDTA buffer and centrifuged at 23 000g and washed once with Tris-MgCl<sub>2</sub>-EDTA buffer. The precipitate was then resuspended in Tris-MgCl<sub>2</sub>-EDTA buffer to a final concentration of 1.4 mg/mL. Adenylate cyclase activity was measured in the presence of 0.1 mM 1-epinephrine or 10 mM NaF or in the presence of  $2.7 \times 10^{-5}$  M *dl*-propranolol (basal activity).  $[^{125}\text{I}]\text{HYP}$  binding to PHMB-treated membranes was measured as described above (section d). The rate of activation of the PHMB-treated membranes by GppNHp and 1-epinephrine was determined as described in the legend to Figure 8.

(h) *Analysis of the Kinetic Results.* A primary inspection of the results was performed using a linearization of the kinetic function describing the assumed mechanism. Thus, initial estimates of the parameters characterizing the kinetic behavior of the activation were derived. Using these estimated parameters, the data were processed using an iterative nonlinear curve-fitting procedure NONLSQ of The Hebrew University

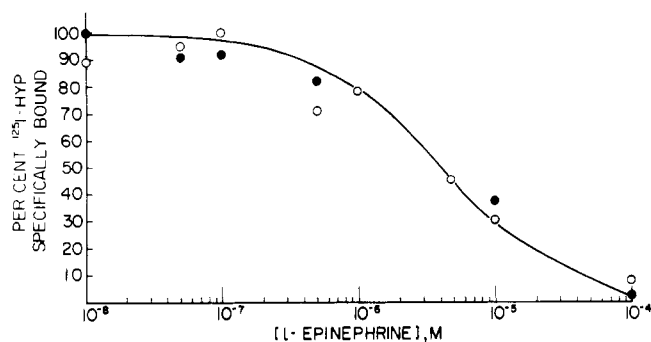


FIGURE 2: Displacement of  $[^{125}\text{I}]\text{HYP}$  by 1-epinephrine in permanently activated membranes. Membranes were preactivated with  $1.0 \times 10^{-4}$  M 1-epinephrine and  $1.0 \times 10^{-4}$  M GppNHp and washed four times. Control membranes were also washed four times. Binding was performed as described under Materials and Methods, in the presence of various concentrations of 1-epinephrine. The concentration of  $[^{125}\text{I}]\text{HYP}$  was  $1.3 \times 10^{-10}$  M, 6.5 times the dissociation constant (87% occupancy). The specific adenylate cyclase activity of the treated membranes was  $830 \text{ pmol of cAMP mg}^{-1} \text{ min}^{-1}$ : (O) untreated membranes; (●) preactivated membranes. The line constructed through the points is theoretical, according to eq 3 of the text.  $k_s = 5.5 \times 10^{-7}$  M.

Computation Center. This procedure uses the maximum-neighborhood method which is based on the algorithm of D. S. Marquardt (1963) and which combines the method of steepest descent with the method of the linear approach to the minimum. Usually, the fit was complete within eight iterations.

## Results

As summarized in Tables IV and V of the Appendix, one can distinguish different modes of enzyme to receptor coupling by examining: (1) the binding pattern of hormone; (2) the kinetic pattern of the appearance of the permanently active state of the enzyme  $E'$ , as well as (3) the dependence of the activation rate and maximum extent of the permanently active enzyme attainable on total receptor and total enzyme concentrations, modified individually.

(a) *Binding of Epinephrine to Native GppNHp and to GppNHp Preactivated Membranes.* Since direct epinephrine binding is not possible (Levitzki et al., 1974, 1975), we first examined carefully the binding of  $[^{125}\text{I}]\text{HYP}$  (Figure 1). It can be seen that the binding is completely noncooperative.

The displacement pattern of  $[^{125}\text{I}]\text{HYP}$  by 1-epinephrine was now compared between nonactivated and preactivated membranes (Figure 2). The binding pattern of  $[^{125}\text{I}]\text{HYP}$  was unaltered in membranes pretreated for 1 h with GppNHp alone (0.1 mM) or with 0.1 mM epinephrine and 0.1 mM GppNHp for 1 h followed by four washes (Figure 2).

At the  $[^{125}\text{I}]\text{HYP}$  concentration used for the displacement experiment, 87% occupancy of the receptor is achieved. The displacement pattern is presented in Figure 2 (0% displacement or 100% binding represents 87% absolute occupancy by  $[^{125}\text{I}]\text{HYP}$ ). The curve drawn through the experimental points is theoretical. First,  $K_s$  was derived by the following equation:

$$K_s = \frac{[S_{0.5}]K_1}{K_1 + [I]} \quad (2)$$

where  $K_s$  is the dissociation constant for 1-epinephrine;  $K_1$  is the dissociation constant for  $[^{125}\text{I}]\text{HYP}$ ;  $[I] = [[^{125}\text{I}]\text{HYP}]_{\text{free}}$ ; and  $[S_{0.5}]$  is the concentration of epinephrine where 50% displacement is found. This relationship was already derived earlier (Levitzki et al., 1975). Next, the curve was constructed

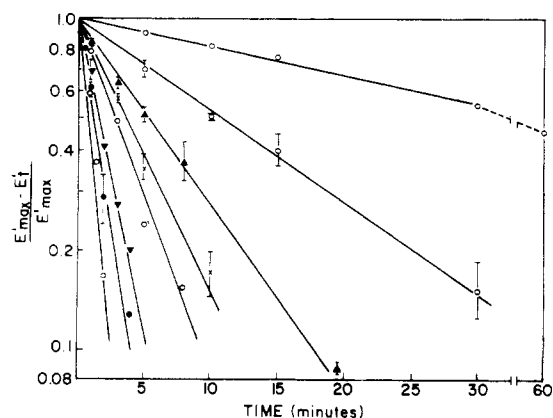


FIGURE 3: Time course of adenylate cyclase activation as a function of 1-epinephrine concentrations—a pseudo-first-order representation. Membranes were incubated at 37 °C with  $1.0 \times 10^{-4}$  M GppNHp and the indicated concentrations of 1-epinephrine. The final protein concentration in the preincubation media was 1.51 mg/mL. At the times indicated by the points, 70- $\mu$ L aliquots were taken out into 40  $\mu$ L containing  $3.75 \times 10^{-5}$  M propranolol which were kept at 0 °C. After completion of preincubation, assay of adenylate cyclase activity was performed for 20 min at 37 °C, as described under Materials and Methods. The graph represents results of three experiments. The bars designate the standard deviation of the mean of coinciding hormone concentrations and times of sampling. The following list of hormone concentrations corresponds to lines from the top abscissa to left-hand ordinate: (○)  $1.0 \times 10^{-8}$  M; (○)  $1.0 \times 10^{-7}$  M; (▲)  $4.0 \times 10^{-7}$  M; (X)  $8.0 \times 10^{-7}$  M; (○)  $1.0 \times 10^{-6}$  M; (▼)  $4.0 \times 10^{-6}$  M; (●)  $8.0 \times 10^{-6}$  M; (○)  $2.0 \times 10^{-5}$  M.

according to eq 3:

$$100 \times \frac{[RI]}{[R_T]} = \frac{[I]}{[I] + K_I \left[ 1 + \frac{[S]}{K_S} \right]} \quad (3)$$

which describes Michaelian competition by two ligands where [S], [I],  $K_S$ , and  $K_I$  have the previous meaning and  $[RI]/[R_T]$  is the fraction of labeled receptor.

The fit of the experimental points to eq 3 indicates that there is no cooperativity in 1-epinephrine binding either to the nonactivated membranes or to the activated membranes.

(b) *The Steady-State Assumption for the Formation of the Activated Cyclase.* Turkey erythrocyte membranes do not exhibit basal activity and an extremely low activity is induced by GppNHp alone (Sevilla et al., 1976). The process of activation was stopped at desired times by adding propranolol. Thus, the only species monitored is E'. It follows also that the formation of cAMP as a function of time should be linear at any level of E' formation, if E' is the only active enzyme species. Indeed, it was found that at every level of enzyme activation the production of cAMP is linear with time. Therefore, the only rate-limiting step appears to be the appearance of the permanently active enzyme E'.

(c) *The Kinetics of E' Formation.* The next question is whether E' is in equilibrium with its inactive form E or whether the conversion of E to E' is essentially irreversible. From the data in Figure 3 it is apparent that the formation of E' is irreversible in the presence of GppNHp, at all hormone concentrations. Even  $1.0 \times 10^{-7}$  M 1-epinephrine, which occupies about 1% of the total receptors (Levitzki et al., 1974, 1975), can bring about a maximal activation level. The reversal of the superactive state of the enzyme to its inactive form can be induced only by the combined action of GTP and 1-epinephrine or ATP and 1-epinephrine (Sevilla et al., 1976; Sevilla and Levitzki, 1977).

Since the formation of the permanently active-state E' is virtually irreversible and quite slow, the rate law governing this

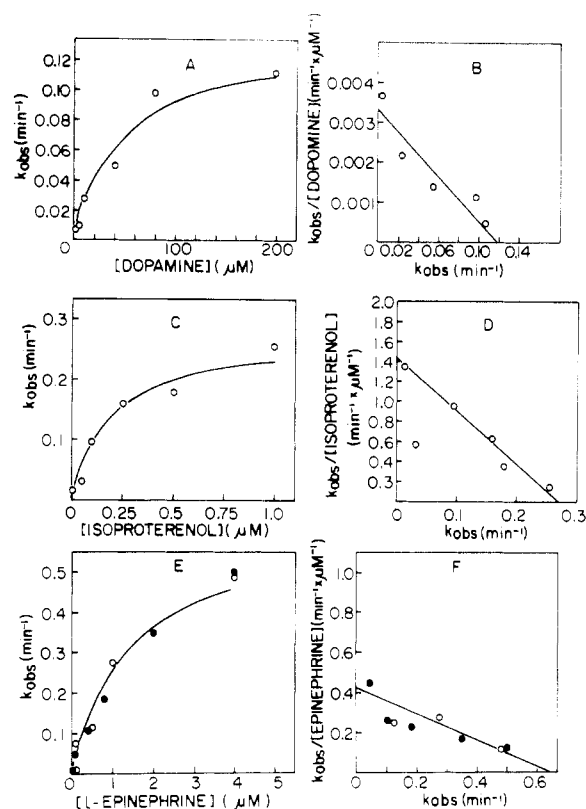


FIGURE 4: The dependence of the rate of E' formation on hormone concentration. Data presented in Figure 4 were similarly collected for dopamine and isoproterenol. For each hormone concentration, the slope  $k_{obsd}$  (eq 4 of the text) was derived. In the left-hand panels (A, C, E) of Figure 4, the direct dependence of  $k_{obsd}$  on hormone concentration is presented. In the right-hand panels (B, D, F) a linearization of the data is examined according to eq 5 of the text. The data of dopamine directly curve fitted according to eq 6, and the values of the parameters which govern the curve were found to be:  $k = 0.12 \pm 0.01 \text{ min}^{-1}$  and  $K_H = 34 \pm 7 \mu\text{M}$ . The parameters derived for isoproterenol in the right-hand panel (D) were used to build the curve in the left-hand panel (C):  $k = 0.273 \text{ min}^{-1}$  and  $K_H = 0.19 \mu\text{M}$ . The parameters for 1-epinephrine in panel F were used to build the curve in panel E:  $k = 0.64 \text{ min}^{-1}$  and  $K_H = 0.66 \mu\text{M}$ .

process could be examined in detail. First, we tested whether the transition was first order.

The hormone concentration is much higher than the total receptor concentration ( $[R_T] = 1.0 \times 10^{-9}$  M), even at the lowest hormone concentration used ( $1.0 \times 10^{-7}$  M). One can therefore follow the rate of E' formation as a pseudo-first-order process at each hormone concentration. The activation process can be described by the following equation:

$$\ln \frac{[E']_{max} - [E']_t}{[E']_{max}} = -k_{obsd}t \quad (4)$$

where  $[E']_{max}$  is the maximal activity obtained at infinite time and  $[E']_t$  is the activity attained at any given time  $t$ . The time dependence of accumulation of E' as a function of increasing hormone concentration according to eq 4 is presented in Figure 3. From Figure 3 one can see that at all hormone concentrations the appearance of E' follows a first-order process up to 90% total activation. The fact that at all hormone concentrations the intercept on the ordinate is at 100% validates the assumption that the formation of E' is the only irreversible process occurring during the process of activation.

A plot of  $k_{obsd}$  as a function of hormone concentration is presented in Figure 4. It can be seen that the dependence of  $k_{obsd}$  on hormone concentration is a saturable phenomenon and Michaelian in nature for the three different agonists (Figure 4).

TABLE I: Binding and Kinetic Parameters for the Different  $\beta$ -Agonists.

$\beta$ -agonist	dissoc constant to the $\beta$ -receptor			intrins rate of enz converts from inact. to act. state, $k$ ( $\text{min}^{-1}$ )
	from kinetics <sup>a</sup> of enz activat	from dose-resp of adenylate cyclase activat (M)	from direct-bind. measur <sup>b</sup>	
1-epinephrine	$6.6 \times 10^{-7}$	$6.0 \times 10^{-6}$	$2.0 \times 10^{-6}$	0.64
1-isoproterenol,	$1.9 \times 10^{-7}$	$5.0 \times 10^{-7}$	$2.1 \times 10^{-7}$	0.273
dopamine	$(3.7 \pm 0.7) \times 10^{-5}$	$2.2 \times 10^{-5}$	$2.4 \times 10^{-5}$	$0.12 \pm 0.01$

<sup>a</sup> From Figure 4. <sup>b</sup> From Levitzki et al. (1975). Except for 1-epinephrine, the dissociation constants of the values obtained are similar in all three methods.

(d) *Hormone Dependence of Activation.* In order to test whether the activation process has a noncooperative dependence on hormone concentration, the results presented in Figure 4 (right panel) were linearized according to the following equation:

$$k_{\text{obsd}} = \frac{k[\text{H}]}{K_{\text{H}} + [\text{H}]} \quad (5a)$$

which yields

$$\frac{k_{\text{obsd}}}{[\text{H}]} = \frac{k}{K_{\text{H}}} - \frac{k_{\text{obsd}}}{K_{\text{H}}} \quad (5b)$$

which is an Eadie-type representation where  $k$  is the intrinsic rate constant of the activation process and  $K_{\text{H}}$  the dissociation constant for hormone binding. It is apparent that the dependence of  $k_{\text{obsd}}$  on agonist concentration, for three different agonists, is noncooperative. The parameters  $k$  and  $K_{\text{H}}$  for the different agonists are summarized in Table I.

(e) *The Integrated Rate of cAMP Accumulation.* Since the activation of adenylate cyclase is a slow process, the accumulation of cAMP was measured in the presence of saturating GppNHp levels, and various levels of hormone should possess a definite lag time proportional to the time it takes the  $E'$  form to reach its maximal level. The accumulation of  $E'$  is given by eq 4 which can be rewritten as:

$$[E']_t = [E']_{\text{max}} \left\{ 1 - \exp \left[ -k \left( \frac{[\text{H}]}{K_{\text{H}} + [\text{H}]} \right) t \right] \right\} \quad (6)$$

where

$$k_{\text{obsd}} = k \left( \frac{[\text{H}]}{K_{\text{H}} + [\text{H}]} \right)$$

Therefore, the accumulation of cAMP as a function of time is given by the integration of eq 6:

$$\begin{aligned} \text{cAMP} &= k_3'[E_{\text{T}}]t \\ &+ \left( \frac{k_3'[E_{\text{T}}](K_{\text{H}} + [\text{H}])}{k'[\text{H}]} \right) \left\{ \exp \left[ -k \left( \frac{[\text{H}]}{K_{\text{H}} + [\text{H}]} \right) t \right] - 1 \right\} \end{aligned} \quad (7)$$

where  $k$  is the intrinsic rate constant and  $k_3'[E_{\text{T}}]$  the maximal specific activity.

Data collected from such an experiment conducted at different epinephrine levels at 25 °C—in order to generate a slow rate of hormone activation—were fitted according to eq 7 in a nonlinear curve-fitting procedure (Figure 5).

The fact that the process of enzyme activation is first order rules out models II, III, and V of receptor to enzyme coupling which predict deviations from first-order kinetics (see Appendix and Table V).

The only two models which can accommodate noncooperative hormone binding to the receptor with first-order kinetics of enzyme activation are model I, the precoupled receptor to enzyme model, and model IV, the collision coupling mode of coupling. Models II and III were rejected also on the basis of

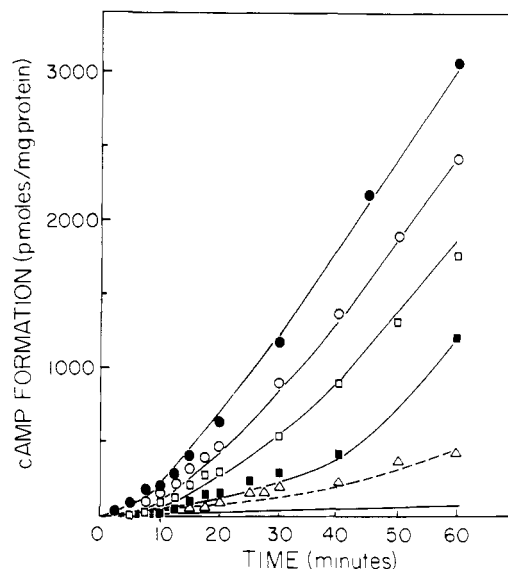


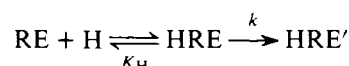
FIGURE 5: The integrated rate of cAMP accumulation. The accumulation of cAMP was measured by incubating membranes (1.43 mg/mL) in the presence of  $1.0 \times 10^{-4}$  M GppNHp and various concentrations of 1-epinephrine as well as in the presence of all the components of the adenylate cyclase assay. The experiment was performed at 25 °C, in order to slow down the reaction and obtain more experimental points in the lag period. At the various times indicated by the experimental points, 70- $\mu$ L portions were removed in 2% NaDodSO<sub>4</sub> containing c[<sup>3</sup>H]AMP to stop the reaction. The amount of cAMP formed was assayed using the Salomon method (see Materials and Methods). The data were processed according to a curve-fitting procedure according to eq 7 of the text, and the following parameters were derived:  $k_3'[E_{\text{T}}] = 61 \pm 4$  pmol  $\text{mg}^{-1} \text{min}^{-1}$ ,  $k' = 0.34 \pm 0.026$   $\text{min}^{-1}$ , and  $K_{\text{H}} = 37 \pm 28$   $\mu\text{M}$ . The symbols designate the following 1-epinephrine concentrations: ( $\Delta$ )  $5.0 \times 10^{-7}$  M; ( $\blacksquare$ )  $1.0 \times 10^{-6}$  M; ( $\square$ )  $3.0 \times 10^{-6}$  M; ( $\circ$ )  $6.0 \times 10^{-6}$  M; ( $\bullet$ )  $1.5 \times 10^{-5}$  M. The lines describe the fit according to the parameters derived from the curve-fitting procedure.

the binding experiments (see Results, section a).

Examination of the models reveals that the precoupled model (model I; eq 8) predicts that the apparent rate constant,  $k_{\text{obsd}}$ , is independent of the total receptor concentration, whereas the final concentration of attainable activated enzyme is proportional to the latter. The collision coupling model (model IV; eq 9), in contrast to model I, predicts that the apparent rate constant,  $k_{\text{obsd}}$ , is linearly dependent upon the total receptor concentration  $[R_{\text{T}}]$ , whereas the final concentration of attainable activated enzyme is independent of this quantity. These predictions are formulated in eq 8 and 9 which describe the accumulation of the active enzyme species.

$$[\text{HRE}'] = [\text{RE}_{\text{T}}] \left\{ 1 - \exp \left[ -k \left( \frac{[\text{H}]}{K_{\text{H}} + [\text{H}]} \right) t \right] \right\} \quad (8)$$

where the scheme for enzyme activation is:



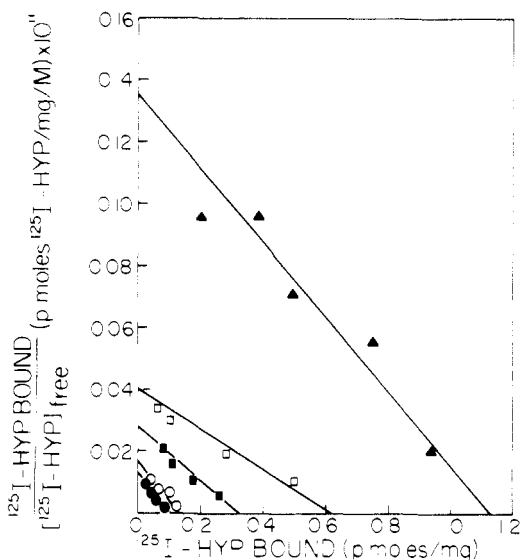
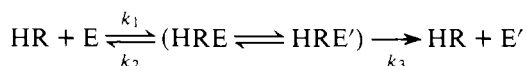


FIGURE 6: The binding of [ $^{125}\text{I}$ ]HYP to membranes treated with affinity label. Affinity labeling of the turkey erythrocyte membranes and the measurement of [ $^{125}\text{I}$ ]HYP binding to these membranes were measured as described under Materials and Methods. The [ $^{125}\text{I}$ ]HYP binding data are presented in the form of a Scatchard plot: ( $\blacktriangle$ ) untreated membranes; ( $\square$ )  $1.67 \times 10^{-5}$  M affinity label; ( $\blacksquare$ )  $4.34 \times 10^{-5}$  M affinity label; ( $\circ$ )  $1.0 \times 10^{-4}$  M affinity label; ( $\bullet$ )  $2.3 \times 10^{-4}$  M affinity label.

(see Appendix).

$$[E'] = [E_T] \left\{ 1 - \exp \left( -k_1 [R_T] \frac{[H]}{K_H + [H]} t \right) \right\} \quad (9)$$

where the scheme for enzyme activation is:



(see Appendix).

A diagnostic experiment was therefore designed in order to distinguish between the two possibilities.

The diagnostic experiment involves the following: affinity labeling of the receptor to different extents using a specific irreversible  $\beta$ -adrenergic blocker and then following the rate of accumulation of the active state of the enzyme in the presence of saturating concentrations of hormone and GppNHp. Different degrees of receptor modification will cause a decrease in the rate of E' formation, but the maximal level of E' attainable will remain unchanged if the receptor and the enzyme are uncoupled to each other (model IV). That this will be the result can be deduced from eq 9. If, however, the enzyme and the receptor are permanently coupled to each other, increasing the degree of receptor modification would result in a proportional decrease in the level of activated enzyme attainable, whereas the apparent rate constant  $k_{\text{obsd}}$  (eq 8) describing its formation will remain unchanged. Both models, however, predict that an irreversible inactivation of the catalytic unit will result in no change in the rate of accumulation of the active state of the enzyme. Both models also predict that in the latter case the maximal extent of activated enzyme attainable will be directly proportional to the degree of enzyme modification.

Furthermore, an irreversible inactivation of the catalytic unit would not have any effect on the binding properties of the receptor according to model IV. However, in the precoupled system (model I) such an effect would be likely to occur due to the close association of the receptor and the enzyme.

Models II and III both predict that the rate of activation will be governed by both receptor concentration and enzyme concentration. Model V, by contrast, predicts, similarly to model

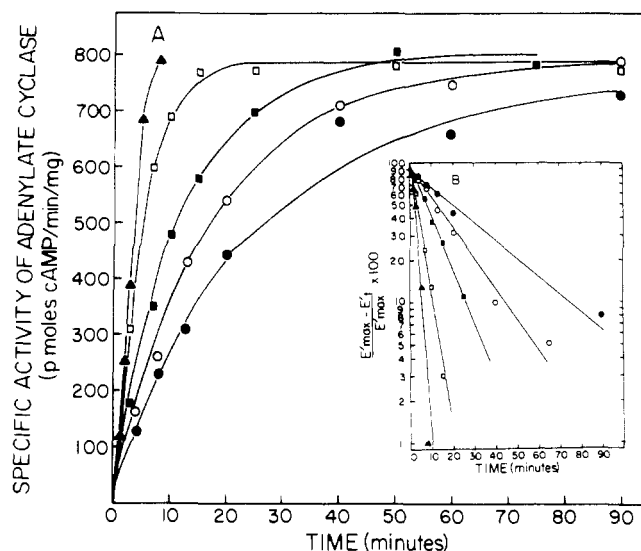


FIGURE 7: Time course of adenylate cyclase activation by saturating GppNHp and 1-epinephrine subsequent to affinity labeling treatment. The membranes were treated with the affinity label as described under Materials and Methods. The rate of enzyme activation to its permanently active state was measured in the presence of  $1.0 \times 10^{-4}$  M 1-epinephrine and  $1.0 \times 10^{-4}$  M GppNHp (saturating concentrations). (A) Progress curves for the accumulation of the active enzyme.  $E_{\text{max}}$ ' is the maximal specific activity attained which was equal in this particular preparation to  $790 \text{ pmol of cAMP mg}^{-1} \text{ min}^{-1}$ . (B) Data in A plotted on a semilogarithmic plot to demonstrate that the process of enzyme activation is first order.  $E_{\text{max}}$ ' is the maximal specific activity attained and  $E_t$ ' the specific activity in each of the time points: ( $\blacktriangle$ ) untreated membranes; ( $\square$ )  $1.67 \times 10^{-5}$  M affinity label; ( $\blacksquare$ )  $4.34 \times 10^{-5}$  M affinity label; ( $\circ$ )  $1.0 \times 10^{-4}$  M affinity label; ( $\bullet$ )  $2.3 \times 10^{-4}$  M affinity label.

IV, that the rate of activation depends solely on receptor concentration but in a saturable manner.

(f) *Variation of Receptor Concentration Using Affinity Label.* The amount of receptor in the turkey erythrocyte membrane was reduced progressively by treatment with increasing concentrations of the  $\beta$ -adrenergic blocker NHNP-NBE, as described in Figure 6. The extent of modification was monitored directly by the loss of [ $^{125}\text{I}$ ]HYP binding sites. The rate of formation of the permanently active state E' and its maximal concentration attainable were measured in the presence of saturating concentrations of 1-epinephrine and of GppNHp (Figure 7).

The maximal adenylate cyclase activity in the presence of saturating concentrations of 1-epinephrine and GppNHp was found to be identical for all degrees of receptor modification (Figure 8A). The rate at which this maximal activity is achieved, however, decreases as a function of increasing receptor inactivation (Figure 8). At all levels of receptor modification, the process of enzyme activation in the presence of saturating concentrations of 1-epinephrine and GppNHp is first order. At saturating concentrations of hormone and GppNHp, eq 9 obtains the simple form:

$$[E'] = [E_t] \{ 1 - \exp(-k_1 [R_T] t) \} \quad (10)$$

The values for  $k_1 [R_T]$  were derived from the slopes of the semilogarithmic plot (Figure 8B).

In a parallel experiment the extent of receptor modification was measured both by loss of [ $^{125}\text{I}$ ]HYP binding sites as well as by the loss of 1-epinephrine-dependent cyclase activity and compared with the decrease in the rate of accumulation of the permanently active state in the presence of saturating concentrations of 1-epinephrine and GppNHp (Table II).

Different membrane preparations vary slightly in their

TABLE II: Effect of Affinity Label on the  $\beta$ -Receptor-Adenylate Cyclase System.

concn of affn label appl (M)	[ <sup>125</sup> I]HYP binding			rate of enz activat. by GppNHp and 1-epinephrine		1-epinephrine dependent adenylate cyclase act.	
	extent <sup>a</sup> (pmol/mg)	dissoc constant (M)	% of max	$k_1[R_T]$ (min <sup>-1</sup> )	% of max	pmol of cAMP mg <sup>-1</sup> min <sup>-1</sup>	% of max
expt I							
0	1.12	$8.25 \times 10^{-11}$	100	0.374	100		
$1.67 \times 10^{-5}$	0.64	$1.77 \times 10^{-10}$	58	0.217	58		
$4.34 \times 10^{-5}$	0.34	$1.34 \times 10^{-10}$	30	0.178	47.6		
$1.0 \times 10^{-4}$	0.15	$1.03 \times 10^{-10}$	13.3	0.051	13.7		
$2.3 \times 10^{-4}$	0.105	$8.10 \times 10^{-11}$	9.3	0.030	8.1		
expt II							
0	0.45	$1.2 \times 10^{-11}$	100	0.190	100	$48 \pm 1$	100
$1.0 \times 10^{-5}$	0.34	$4.0 \times 10^{-11}$	75	0.152	76	$35.1 \pm 1.5$	74
$5.0 \times 10^{-5}$	0.27	$5.0 \times 10^{-11}$	60	0.120	63	$23.5 \pm 1.5$	49

<sup>a</sup> A consistent finding in our studies on turkey erythrocyte membranes was that whenever a high level of [<sup>125</sup>I]HYP binding was found (about 1.0 pmol/mg) the dissociation constant obtained was in the range of 0.8 to  $1.0 \times 10^{-10}$  M, and whenever a low level of [<sup>125</sup>I]HYP binding was found (about 0.45 pmol/mg) the dissociation constant obtained was in the range of 0.12 to  $0.5 \times 10^{-10}$  M (compare also with Figures 1-2).

absolute [<sup>125</sup>I]HYP-binding capacity and the characteristic rate constant ( $k_1[R_T]$ ) describing the accumulation of the permanently active state. Still, the extent of the receptor modification is found to be identical when monitored by three independent criteria: [<sup>125</sup>I]HYP binding, 1-epinephrine-dependent cyclase activity, and the characteristic rate constant  $k_1[R_T]$  (Table II). These experiments reject the precoupled model I and indicate a collision-coupling mechanism of receptor to enzyme coupling (model IV) is the one operating.

(g) *Variation of Adenylate Cyclase Concentration.* Subsequent to PHMB treatment, both the content of  $\beta$ -adrenergic receptors as well as adenylate cyclase activity were measured. The receptor content was measured by [<sup>125</sup>I]HYP binding, and the specific activity of the enzyme was monitored using either saturating concentrations of 1-epinephrine or of NaF (Table III). From Figure 8 and Table III it is apparent that PHMB modification results exclusively in the inactivation of the enzyme without affecting the  $\beta$  receptor.

cAMP formation as a function of time at different levels of PHMB modification and at saturating concentrations of 1-epinephrine and of GppNHp is presented in Figure 8.

The accumulation of cAMP as a function of time in the presence of saturating 1-epinephrine and GppNHp concentration represents the integrated rate equation of the activation process (eq 9), namely:

$$\text{cAMP} = k_3'[E_T]t + \frac{k_3'[E_T]}{k_1[R_T]} \{ \exp(-k_1[R_T]t) - 1 \} \quad (11)$$

when  $[H] \gg K_H$ ;  $k_3'[E_T]$  is the rate of cAMP production at the maximal activation level of the enzyme and is found to be 400 pmol of cAMP min<sup>-1</sup> mg<sup>-1</sup> (maximal specific activity). The value of  $k_3'[E_T]$  was determined from the limiting maximal slope of the progress curve for cAMP formation in the absence of PHMB modification (Figure 8).

The experimental cAMP accumulation is given by the points. The lines are the result of theoretical progress curves generated from eq 11 and inserting  $k_1[R_T] = 1.0 \text{ min}^{-1}$  and  $[E_T]$  values according to the degree of enzyme inactivation (Table III) by PHMB

(h) *Activation by GTP and Hormone.* Finally, we wanted to test whether the level of activity of the adenylate cyclase generated in the presence of GTP is a product of a steady state between the appearance and the disappearance of the activated state E', predicted to be the only active species of the adenylate

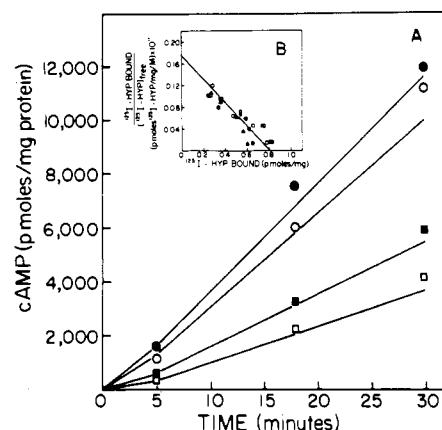
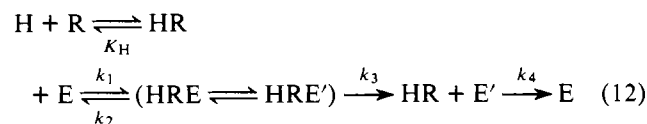


FIGURE 8: Time course of adenylate cyclase activation by saturating GppNHp and 1-epinephrine subsequent to PHMB treatment. (A) Membranes were treated with PHMB as described under Materials and Methods. The treated membranes were incubated at 37 °C in the presence of  $1.0 \times 10^{-4}$  M 1-epinephrine and  $1.0 \times 10^{-4}$  M GppNHp and all the components of the adenylate cyclase assay. At specified times, as indicated in this figure, samples were removed into 2% sodium dodecyl sulfate containing c[<sup>3</sup>H]AMP to stop the reaction. The amount of cAMP formed was assayed using the Salomon method (see Materials and Methods). The experimental points construct a progress curve for cAMP formation as a function of time at different degrees of PHMB modification. The data points were fitted according to eq 7 (see text). The values for the relative concentrations of  $[E_T]$  used for the fitting were: 100% (●), 87% (○), 50% (■), 32% (□), as determined experimentally. The lines were constructed according to eq 7 in the text, with  $k_1[R_T] = 1 \text{ min}^{-1}$  and  $k_3' = 400 \text{ pmol of cAMP/mg of protein}$ . (B) Binding of [<sup>125</sup>I]HYP to PHMB-treated membranes. Experimental details are given in the text. The data are presented in the form of a Scatchard plot: (▲) untreated membranes; (□) after treatment with  $4.0 \times 10^{-6}$  M PHMB; (■) after treatment with  $1.0 \times 10^{-5}$  M PHMB; (○) after treatment with  $5.0 \times 10^{-5}$  M PHMB; (●) after treatment with  $1.0 \times 10^{-4}$  M PHMB.

cyclase. When GTP occupies the regulatory site rather than GppNHp, the collision coupling model for receptor to enzyme coupling is described by the following sequence:



The rate of accumulation of E' at saturating hormone con-

TABLE III: Effect of PHMB on the  $\beta$ -Receptor-Adenylate Cyclase System.<sup>a</sup>

PHMB concn (M)	[ <sup>125</sup> I]HYP bind. extent (pmol/mg)	adenylate cyclase act.			
		l-epinephrine sp act. (pmol of cAMP mg <sup>-1</sup> min <sup>-1</sup> )	% of max	fluoride sp act. (pmol of cAMP mg <sup>-1</sup> min <sup>-1</sup> )	% of max.
0	0.66	55 ± 1	100	492 ± 12	100
1.0 × 10 <sup>-6</sup>	ND <sup>b</sup>	47 ± 1	85	443 ± 9	90
5.0 × 10 <sup>-6</sup>	0.78	26 ± 0.5	47	264 ± 7	53
1.0 × 10 <sup>-5</sup>	0.82	15.5 ± 0.5	28	177 ± 3	36
5.0 × 10 <sup>-5</sup>	0.80	0	0	0	0
1.0 × 10 <sup>-4</sup>	0.67	0	0	0	0

<sup>a</sup> Experimental details are given in the text. <sup>b</sup> ND, not determined.

centrations is given by:

$$[E'] = \frac{[E_T]}{1 + (k_4/k_1[R_T])} \{1 - \exp(-(k_1[R_T] + k_4)t)\} \quad (13)$$

After a steady-state level of E' is established, when *t* is large, PPL is introduced. After antagonist addition, the steady-state level of E' cannot now be maintained, since no HRE is formed. We can thus follow the process of E' decay to E, which is given by:

$$[E']_t = [E']_0 \exp(-k_4t) \quad (14)$$

where [E']<sub>*t*</sub> is the level of E' at time *t* and [E']<sub>0</sub> is the steady-state level of E' which equals the concentration of E' at the moment PPL is added—at zero time of the decay process.

When at zero time of the decay process one adds PPL together with [ $\alpha$ -<sup>32</sup>P]ATP, one can monitor the decay of the E' form to the E form by following the rate of accumulation of cAMP. This line of experimentation is entirely analogous to the experiment described in Figure 5 where the process of cAMP accumulation is followed as the integrated rate of accumulation of E' from E. The accumulation of cAMP is given by:

$$\frac{d[\text{cAMP}]}{dt} = k[E']_0 \exp(-k_4t) \quad (15)$$

Upon integration and considering that [cAMP]<sub>*t*</sub> = 0 when *t* = 0, one obtains:

$$\text{cAMP}|_0^t = -\left(\frac{k[E']_0}{k_4}\right) \exp(-k_4t) + C \quad (16)$$

when *t* = 0, [cAMP]<sub>*t*</sub>=0, and C = (k[E']<sub>0</sub>)/k<sub>4</sub>.

Equation 16 therefore obtains the form:

$$[\text{cAMP}]_t = \frac{k[E']_0}{k_4} [1 - \exp(-k_4t)] \quad (17)$$

When *t* → ∞, eq 17 obtains the form:

$$[\text{cAMP}]_\infty = \frac{k[E']_0}{k_4} \quad (18)$$

If one examines the ratio [cAMP]<sub>*t*</sub>/[cAMP]<sub>∞</sub>, one obtains:

$$[\text{cAMP}]_t/[\text{cAMP}]_\infty = [1 - \exp(-k_4t)] \quad (19)$$

When *k*<sub>4</sub>*t* ≪ 1, namely, when *k*<sub>4</sub> is much smaller than 1.0 and/or at short times *e*<sup>-*k*<sub>4</sub>*t*</sup> ≫ 1, eq 19 can be simplified to:

$$\ln \frac{[\text{cAMP}]_t}{[\text{cAMP}]_\infty} = -k_4t \quad (20)$$

where [cAMP]<sub>*t*</sub> represents the amount of cAMP measured at time *t* and [cAMP]<sub>∞</sub> represents the total amount of cAMP produced by E'. The value of *k*<sub>4</sub> derived from the slope of the

semilogarithmic plot can be reinserted into eq 19 until further iteration does not induce a change in *k*<sub>4</sub>. *k*<sub>4</sub> can also be derived from a direct curve fitting of the data of cAMP accumulation, according to eq 16.

An experiment was designed to test eq 17 and 20. The results are presented in Figure 9. The detailed experimental procedure is given in the legend to Figure 9. The curve through the points was built according to eq 16 using the value of *k*<sub>4</sub> = 0.0822 s<sup>-1</sup> (or 4.9 min<sup>-1</sup>) which was derived from panel B according to eq 20.

#### Discussion

*General Comments.* The understanding of events occurring subsequent to the binding of a hormone or a neurotransmitter to a cell-surface receptor, which eventually lead to the activation of a primary biochemical signal, has become a key problem in membrane biology. One of the most studied cases is the activation of the membrane-bound adenylate cyclase by specific hormones. In this case, the first step is the binding of the hormone to a specific receptor facing the outside of the cell. The last step is the activation of the enzyme facing the inside of the cell. Since both the enzyme and the receptor are membrane bound, it is generally accepted that the membrane or specific membrane components may play a role in the transmission of the message from the receptor to the enzyme. The events which occur in between the hormone-binding step and enzyme activation are too fast to be picked up by the experimental tools thus far accessible to study such systems. A turning point in the field was provided by Rodbell and his associates (Rodbell et al., 1971; Londos et al., 1974) who discovered that GTP and, more so, its nonhydrolyzable analogue GppNHp play a key role as allosteric activators of adenylate cyclase. It was found that when GppNHp replaces GTP as the auxiliary regulatory ligand, the process of enzyme activation is simplified, since GppNHp is not hydrolyzed as is GTP. Therefore, all molecules are activated to their maximal activity in an irreversible fashion. Since the process of enzyme activation by GppNHp in some systems such as the turkey erythrocyte  $\beta$ -receptor-dependent adenylate cyclase is totally dependent on hormone and is rather slow (Levitzki et al., 1976; Sevilla et al., 1976), it offers a unique experimental model to study the events occurring subsequent to the step of hormone binding to its receptor.

In the Appendix we have derived five general molecular models which describe different modes of coupling between the receptor and the adenylate cyclase. These models make specific predictions as to the mode of hormone binding, response to hormone, and the dependence of the hormonal response on receptor concentration and adenylate cyclase concentration. The predictions of each model differ and, therefore, we have conducted a detailed study of hormone binding, a ki-



netic analysis of the process of enzyme activation, and its dependence on hormone concentration, receptor concentration, and on cyclase concentration in the turkey erythrocyte  $\beta$ -receptor-dependent adenylate cyclase.

**Binding Studies.** The binding studies (Figures 1 and 2) reveal a noncooperative mode of binding (Michaelian binding) of 1-epinephrine as well as of [ $^{125}$ I]HYP to the receptor in the absence of GppNHp as well as subsequent to complete activation by hormone and GppNHp. It has been shown earlier (Levitzki et al., 1975) that [ $^3$ H]propranolol binding is unaltered subsequent to activation by hormone and GppNHp. Spiegel et al. (1976) have also shown that the binding of [ $^{125}$ I]HYP to turkey erythrocyte membranes is unaltered subsequent to enzyme activation. On the other hand, Maguire et al. (1976) and Lefkowitz et al. (1976) have both shown altered affinities of the receptor toward  $\beta$ -agonists in two clonal cell lines and in frog erythrocytes, respectively, when binding was performed in the presence of GppNHp. The affinity to the  $\beta$ -antagonist was, however, in their case too, unaltered. In contrast to these results, we present evidence that the hormone binding to the receptor of turkey erythrocytes is unaltered subsequent to GppNHp activation.

The basic finding that the binding pattern is noncooperative (Michaelian) is direct evidence that the classical equilibrium floating receptor-enzyme model (Boeynaems and Dumont, 1975) and the equilibrium dissociation model (models II and III in the Appendix) cannot account for the process of coupling between receptor and enzyme, unless the receptor is present in a large excess relative to the amount of enzyme. The precoupled collision-coupling model (models I and IV in the Appendix) predict simple Michaelian binding. In order to establish which of these two models applies to the  $\beta$ -receptor cyclase system of turkey erythrocytes, kinetic studies were performed.

**Kinetic Studies. Activation as a Function of Hormone Concentration.** Figures 3 through 5 demonstrate that the process of the GppNHp-bound enzyme activation by 1-epinephrine occurring therein is effectively irreversible. Furthermore, the conversion of the inactive enzyme to its active form is the only irreversible slow step in the overall scheme of interaction between the receptor, hormone, enzyme, and GppNHp.

From Figure 3, we deduce that the activation mechanism is strictly first order. This indicates once again that the equilibrium floating and the dissociation modes of receptor to enzyme coupling (models II and III in the Appendix) can be rejected, since both models predict deviations from first-order kinetics at all hormone concentrations.

Model V, the precoupled dissociation model, also predicts deviations from first-order kinetics, especially apparent at low hormone concentrations.

Thus, only the precoupled receptor-enzyme complex or the collision coupled mode of interaction (models I and IV) can account for both noncooperative binding as well as strict adherence to first-order kinetics of enzyme activation.

Figure 4 demonstrates the pattern of enzyme activation by three  $\beta$ -agonists: dopamine, a partial agonist; isoproterenol, a synthetic agonist; and epinephrine. Each agonist has a characteristic intrinsic rate constant governing the conversion of E form to E' form (Table I). The rate constant for the dopamine-induced conversion is slowest. The activation by metanephrine (not shown) is even slower than that induced by dopamine. Activation by isoproterenol is also slower than that of epinephrine. By testing the rate of conversion against the potency of the  $\beta$ -agonists in a systematic fashion, it will be possible to gain more insight into the nature of partial agonism

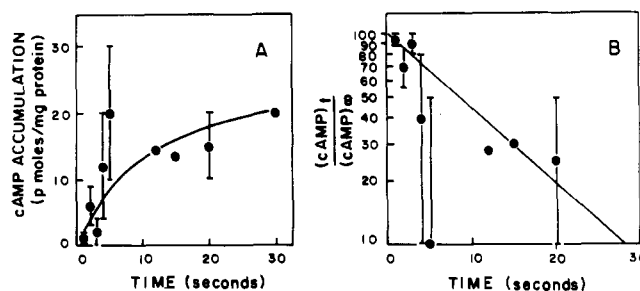


FIGURE 9: The rate of decay of the active species E' induced by PPL. The experiment was conducted as follows: for each test tube, 70  $\mu$ L of membranes, 0.785 mg/mL, were incubated for 5 min at 37  $^{\circ}$ C with 20  $\mu$ L,  $1.0 \times 10^{-3}$  M epinephrine and 50  $\mu$ L of the following cocktail: (final concentrations) ATP, 2.3 mM; MgCl<sub>2</sub>, 4.6 mM; theophylline, 2.5 mM; and a regenerating system which contained pyruvate kinase in saturating (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3.5 units/assay; PEP, 5.8 mM; and KCl, 11.5 mM. Following a 5-min incubation, 20  $\mu$ L of  $1.0 \times 10^{-3}$  M *d,l*-PPL, which contained 1 mCi/mL [ $\alpha$ - $^{32}$ P]ATP, was added. At various times, 100  $\mu$ L of 2% Na-DodSO<sub>4</sub>, which included c [ $^3$ H]AMP, was added to stop the reaction in each test tube. Quadruplicates were run for each time. The control consisted of repeating the experiment in the absence of 1-epinephrine. The results in panel A are the net accumulation of cAMP as a function of time. The results are plotted on a semilogarithmic scale to derive the rate of deactivation. The accumulation of cAMP in the absence of epinephrine was linear and was subtracted from each value. The curve through the points in panel A was built according to eq 16 of the text from the value of  $k_4 = 0.0822/s$  (0.493/min), which was derived according to eq 20 in the text.

(Arad and Levitzki, 1978, and in preparation).

In Figure 5 the accumulation of cAMP is presented as a function of time. The accumulation of cAMP follows the exact integral of the accumulation of the active enzyme E'. A lag time in cAMP accumulation is apparent. Such an apparent lag time in the accumulation of cAMP has been described also for the glucagon-receptor-adenylate cyclase system in liver cells by Salomon et al. (1975). This lag time can be fully accounted for by the rate constant of the transition between the inactive enzyme form E to the active form E', as generally described in scheme 1 by Salomon et al. (1975), but the transition is not via an equilibrium step as suggested therein—rather an irreversible process as shown in Figure 3.

(a) *Consequences of Affinity Label Treatment of the Receptor: the Validity of the Collision-Coupling Mechanism.* The irreversible blocking of the  $\beta$ -adrenergic receptor by the affinity label NHNP-NBE results in receptor inactivation. This inactivation was monitored using three criteria which were found to correspond to each other very closely: (a) treatment of the turkey erythrocyte membranes with increasing concentrations of NHNP-NBE causes a progressive loss of [ $^{125}$ I]HYP binding sites where the affinity of the unlabeled sites remains unchanged (Figure 6, Table II). (b) The maximal level of epinephrine-dependent adenylate cyclase activity in the absence of GppNHp was found to be proportional to the fraction of intact receptor remaining subsequent to affinity labeling (Table II). (c) The maximal rate of activation of adenylate cyclase by 1-epinephrine and GppNHp is reduced proportionately to the fractional inactivation of the receptor (Figure 7, Table II). This finding constitutes a direct proof that the apparent first-order rate constant describing the activation process is a linear function of receptor concentration (eq 10). Furthermore, this finding indicates that the intrinsic bimolecular rate constant ( $k_1$  in eq 10) remains unaltered subsequent to affinity labeling. Since this bimolecular rate constant is probably also a function of membrane structure, one may additionally deduce that the integrity of the membrane is unaltered subsequent to the affinity-label treatment.

The results of the experiments using the affinity label strongly indicate that the collision coupling mechanism is the one responsible for the activation of adenylate cyclase by the  $\beta$ -receptor.

(b) *Consequences of the PHMB Treatment.* PHMB treatment of the turkey erythrocyte membranes (Figure 8) results in the exclusive destruction of the cyclase activity without damage to the  $\beta$ -receptor and no change in its binding affinity toward [ $^{125}$ I]HYP (Figure 8). Thus, it can be seen from Figure 9 that the level of enzyme activation remaining after PHMB treatment decreases as a function of PHMB modification, but the rate constant characterizing the conversion of the inactive enzyme to its activated form remains unchanged. These findings further support the assertion that the receptor and the enzyme are separate entities.

*The Collision-Coupling Mechanism for Receptor to Enzyme Interaction.* The mechanism proposed for the activation of adenylate cyclase by 1-epinephrine and GppNHp in turkey erythrocytes is novel and is somewhat dissimilar from the classical occupancy theory. According to the collision-coupling model, the activated state of the enzyme is not in the form of a complex with its receptor. The precursor of the activated form of the enzyme is formed during the transient encounter between the hormone-receptor complex and the enzyme. During the encounter, the enzyme is rapidly activated, such that upon the rapid dissociation of HRE, a modified form of the enzyme is obtained whereby HR is regenerated with no change in the receptor. Indeed, the affinity (Figure 2) to the hormone remains unchanged subsequent to the activation by hormone and GppNHp. Thus, the rate-limiting step in enzyme activation is not the conversion to the permanently active state but, rather, the rate of encounter between the receptor-hormone complex and the enzyme.

According to the collision coupling, the species HRE never accumulates to any significant concentration, as compared to the total enzyme concentration or the total receptor concentration. This is due to the fact that once an encounter is formed all subsequent steps occur very fast. All that is necessary for enzyme activation is for the hormone-receptor complex and the enzyme to diffuse in the membrane so that a collision between the two components can occur. Indeed, at low temperatures, such as 5 °C, the hormone activation is completely abolished (Rimon, Hanski and Levitzki, in preparation). This finding may indicate that the receptor and the enzyme are unable to reach each other, even at local encounters. This may also explain why the temperature dependence of adenylate cyclase activity demonstrates a break in the Arrhenius plot only for the hormone-dependent activity in the absence of GppNHp, while this break is completely absent in the permanently active form of the enzyme achieved by 1-isoproterenol and GppNHp (Orly and Schramm, 1975). Indeed, the activity of the permanently active state does not require repeated collisions with the receptor according to the collision-coupling mechanism, but the steady-state level of the active enzyme in the absence of GppNHp will be maintained only through repeated encounters between the receptor and the enzyme.

*The Role of GppNHp and GTP.* When GTP and not GppNHp is used, the GTP is hydrolyzed at the regulatory site of the activated form of the enzyme concomitantly with the regeneration of the inactive form of the enzyme (Cassel and Selinger, 1976). Therefore, repeated encounters are necessary to maintain a steady-state concentration of the active enzyme. A guanyl regulatory subunit probably resides on the catalytic moiety as was demonstrated for the pigeon erythrocyte  $\beta$ -receptor-dependent adenylate cyclase (Pfeuffer and Helmreich, 1975; Pfeuffer, 1977). If one incorporates the step of

GTP hydrolysis into the scheme of enzyme activation, the equation describing the process will obtain the form of eq 12 (Levitzki, 1977) and eq 13.

In Figure 9 a typical experiment testing eq 12 is described. The experiment is difficult to conduct, as reflected in the high-error range, since both high membrane concentrations and short reaction times are required. The rate constant  $k_4$  of about 5 min<sup>-1</sup> is in fact the turnover number of the GTPase step. Since  $k_4$  is much higher (5 min<sup>-1</sup>) than  $k_3$ , a maximal steady-state concentration of E' is obtained within 0.3 to 0.5 min after exposure of the enzyme at saturating concentrations of hormone and GTP. Therefore, cAMP accumulation is effectively linear as a function of time in the absence of GppNHp (Steer and Levitzki, 1975). In the presence of GppNHp, the rate of appearance of E' is governed solely by the term  $k_1[R_T]$  which equals 0.4 to 1.0 min<sup>-1</sup>, depending on the particular membrane preparation (see Results), and, since this process is rather slow, cAMP accumulation as a function of time exhibits a lag period (Figure 5).

From eq 13 it follows that the maximal steady-state concentration of the activated state of the enzyme attainable, in the absence of GppNHp, is given by:

$$[E'] = \frac{[E_T]}{1 + \frac{k_4}{k_1[R_T]}} \quad (21)$$

Since  $k_1[R_T] < k_4$ , both treatment of the receptor with the affinity label and of the enzyme with PHMB will reduce the maximal level of active enzyme in the presence of GTP, as demonstrated in Tables II and III.

Since the ratio  $k_4/k_1[R_T]$  is about 7 to 10, only 10 to 14% of the total enzyme concentration is active at the steady state in the presence of GTP. When GppNHp is used, all the enzyme attains the active state, as  $k_4 = 0$ . Therefore, the specific activity of adenylate cyclase is seven- to tenfold higher in the presence of GppNHp than in the presence of GTP (Levitzki, 1977). This latter argument holds also for a situation in which the receptor and the enzyme are permanently coupled to each other (see Appendix and Levitzki, 1977).

Since the active enzyme species is most likely the same in the presence of either GppNHp and GTP, it follows that the GppNHp-activated enzyme should exhibit kinetic properties identical to those exhibited by the enzyme in the presence of GTP. Indeed, the  $S_{0.5}$  for Mg<sup>2+</sup>, the Hill coefficient with respect to Mg<sup>2+</sup>, the kinetic parameters, and the Hill coefficient for Ca<sup>2+</sup> inhibition are identical in the two situations (Levitzki et al., 1975, 1976; Sevilla et al., 1976; Hanski et al., 1977).

*General Significance.* The finding that the receptor and the adenylate cyclase are functionally uncoupled but form a short-lived complex may be of general significance. Other receptors may function by a similar mechanism, where the catalytic moiety is activated by a receptor only during the transient encounter between the two components which move into the membrane. It should be pointed out, however, that the other modes of receptor to enzyme coupling (see Appendix) may be operating in other cases. For example, the activation of turkey erythrocyte adenylate cyclase by adenosine occurs by the precoupled mode of activation (Tolkovsky and Levitzki, 1978, accompanying manuscript; Braun and Levitzki, 1978).

#### Note Added in Proof

Recently we have demonstrated that the rate constant ( $k_{obsd}$ ) which describes the rate of enzyme activation by the hormone-bound receptor is linearly dependent on membrane

fluidity for the *l*-catecholamine mode of cyclase activation and independent of membrane fluidity for the adenosine mode of activation. These findings are in agreement with our findings that the mode of catecholamine activation is by the collision coupling mechanism (model IV) and that the adenosine receptor is permanently coupled to the enzyme.

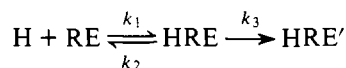
#### Acknowledgments

We acknowledge Dr. D. Atlas for providing us with the  $\beta$ -receptor affinity label and for having given us invaluable advice.

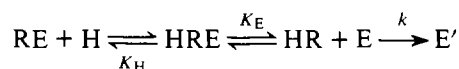
#### Appendix

*Models for Receptor to Adenylate Cyclase Coupling: Theory.* Five molecular models describing different modes of receptor to adenylate cyclase coupling are described:

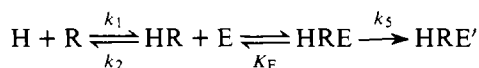
(I) The precoupled receptor-enzyme model:



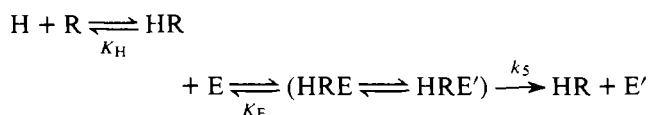
(II) The dissociation receptor-cyclase model:



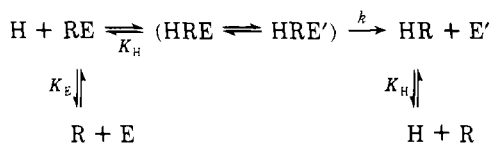
(III) The floating receptor-cyclase equilibrium model:



(IV) The collision coupling model:



(V) The precoupled-dissociation model:



where R = receptor, E = enzyme, E' = the activated form of the enzyme,  $K_H$  = the hormone-receptor dissociation constant, and  $K_E$  the receptor enzyme dissociation constant. Using these five models, the patterns of hormone binding and the kinetic features of enzyme activation are derived. Using these derivations, diagnostic tests which can be used to analyze real experimental situations are formulated.

In order to elucidate the way by which the interaction of receptor and adenylate cyclase is dependent on hormone, a complete description of this system in terms of hormone-receptor-cyclase interactions must be analyzed. Boeynaems and Dumont (1975) have listed several possible models for receptor-enzyme interactions and their dependence on hormone concentration in general terms.

The purpose of this theoretical section is to interrelate the tertiary system hormone-receptor-cyclase in kinetic terms in order to describe the adenylate cyclase in turkey red blood cells. It should be emphasized that an analysis of some of the models has been published by Boeynaems and Dumont (1975) and by Jacobs and Cuatrecasas (1976). However, in these treatments the kinetic consequences were not discussed and a complete solution of some of the equations has not been presented.

The following analysis will be divided into five parts. A general derivation of each model in terms of active cyclase will

be given along with the experimental predictions associated with each model. In all models discussed below, two major assumptions have been applied:

(1) The binding of hormone to the receptor is rapid and reversible. Such rapid equilibrium is well established for the  $\beta$ -adrenergic receptors of turkey erythrocytes (Levitzki et al., 1974, 1975) analyzed in the present study.

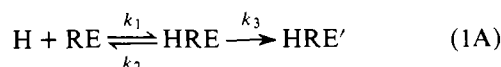
(2) During the process of activation, the rate of formation of the hormone-receptor-enzyme complex (HRE) is either fast compared to the rate of transition of the permanently active form, or the transition step is fast compared to the rate of formation of the hormone-receptor-enzyme complex (HRE).

This second assumption is justified on the basis of two sets of observations: One is that the accumulation of cAMP in the adenylate cyclase assay, in the presence of GTP, is linear with time. The other is that the appearance of the permanently active state of the enzyme in the presence of GppNHp is a purely exponential process without a lag period. These assumptions are valid in the turkey erythrocyte system. If the species HRE were to accumulate to a significant fraction of the total enzyme concentration at a rate comparable to the rate of transition to the permanently active form, the appearance of the permanent state would occur nonexponentially with a lag time equal to the time it takes for HRE to reach a steady-state concentration.

Thus, it can be presumed that either the concentration of HRE is always small compared to  $E_T$  due to the fast transition rate or that an equilibrium level of HRE is achieved fast compared to the step of enzyme activation. The different models for receptor-enzyme interactions are listed below.

#### (I) The Precoupled Receptor-Cyclase Complex

This model is described by the equation



where H is hormone, RE the receptor enzyme complex,  $K_H$  the hormone receptor dissociation constant ( $K_H = k_2/k_1$ ), and  $k_3$  the rate constant characterizing the conversion of the enzyme from the inactive state HRE to the stable active state HRE'.

In the absence of a guanyl nucleotide, HRE accumulates and thus represents the *bound* complex of hormone to the receptor. In the presence of nucleotide, HRE' is also formed. If the nucleotide is nonhydrolyzable, like Gpp(NH)p, HRE' accumulates until no more free receptor is available. In the presence of a hydrolyzable nucleotide, like GTP, HRE' is rapidly converted back to HRE.

*Hormone Binding.* Using eq 1A one can obtain an expression for the dependence of [HRE] on [H].

The conversion equation for RE is:

$$[RE]_T = [RE] + [HRE] \quad (2A)$$

Since  $[H] \gg [RE]_T$ ,  $[H] = [H]_T$ , therefore

$$[HRE] = \frac{k_1[H][RE]_f}{k_2} \quad (3A)$$

From eq 2A and 3A it follows that:

$$[HRE] = \frac{k_1[H][RE]_T}{k_2[1 + (k_1[H]/k_2)]}$$

$$= \frac{[H][RE]_T}{k_2/k_1 + [H]} = \frac{[H][RE]_T}{K_H + [H]} \quad (4A)$$

The ratio  $k_2/k_1$  is, in fact, the hormone-receptor dissociation

TABLE IV: Mode of Hormone Binding as Predicted by Different Models or Receptor to Enzyme Coupling.

model	formulation	mode of binding
(I) precoupled	$RE + H \xrightleftharpoons{K_H} HRE$	noncoop
(II) dissoc	$RE + H \xrightleftharpoons{K_H} HRE \xrightleftharpoons{K_E} RH + E$	neg coop <sup>a</sup>
(III) equilib. float.	$H + R \xrightleftharpoons{K_H} HR + E \xrightleftharpoons{K_E} HRE$	neg coop
(IV) collis coupling	$H + R \xrightleftharpoons{K_H} HR$	noncoop
(V) precoupled dissoc	$H + RE \xrightleftharpoons{K_H} HRE$ $H + R + E \xrightleftharpoons{K_H} HR$	noncoop

<sup>a</sup> Cooperativity increases as  $K_E$  decreases relative to  $[R_T]$  or  $[E_T]$ .

constant and can be defined as:

$$K_H = k_2/k_1 \quad (5A)$$

Thus the binding of hormone is noncooperative (Figure 10).

*The Formation of Permanently Active Enzyme.* Using eq 1A one can derive an expression for the rate of accumulation of the activated species of the enzyme HRE', in the presence of GppNHp.

At any time point, the relation

$$[RE]_T = [RE] + [HRE] + [HRE'] \quad (6A)$$

must hold. Applying the quasi-steady-state assumption for HRE, we obtain:

$$[HRE] = \frac{k_1[H]([RE]_T - [HRE] - [HRE'])}{k_2 + k_3} \quad (7A)$$

or:

$$[HRE] = \frac{[H]}{(k_2 + k_3)/k_1 + [H]} ([RE]_T - [HRE']) \quad (8A)$$

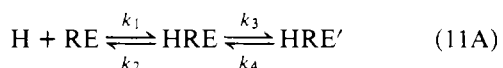
The rate of accumulation of the active species HRE' is given by

$$\frac{d[HRE']}{dt} = k_3[HRE] = \frac{k_3[H]([RE]_T - [HRE'])}{(k_2 + k_3)/k_1 + [H]} \quad (9A)$$

Upon integration and considering that  $[HRE'] = 0$  at  $t = 0$ , one obtains:

$$[HRE'] = [RE]_T \left\{ 1 - \exp \left[ - \frac{k_3[H]}{(k_2 + k_3)/k_1 + [H]} t \right] \right\} \quad (10A)$$

*The Steady-State Level of Active Enzyme in the Presence of GTP.* In the presence of GTP the scheme presented by eq 1A must be modified:



where  $k_3$  is the rate constant of enzyme activation and  $k_4$  the rate constant describing the conversion of the active enzyme to its inactive state, concomitantly with the hydrolysis of GTP.

Applying once again the steady-state assumption for HRE:

$$\frac{d[HRE]}{dt} = 0 = k_1[RE][H] + k_4[HRE'] - (k_2 + k_3)[HRE] \quad (12A)$$

namely:

$$[HRE] = \frac{k_1[RE][H] + k_4[HRE']}{k_2 + k_3} \quad (13A)$$

thus:

$$\frac{d[HRE']}{dt} = \frac{k_3k_1[RE]_T}{k_2 + k_3 + k_1[H]} - [HRE'] \frac{k_1[H](k_3 + k_4) + k_2k_4}{k_2 + k_3 + k_1[H]} \quad (14A)$$

Integrating eq 14A and applying the boundary condition  $[HRE] = 0$  when  $t = 0$ , one obtains:

$$[HRE'] = \frac{\frac{k_3}{k_3 + k_4} [H][RE]_T}{\frac{k_2k_4}{k_1(k_3 + k_4)} + [H]} \times \left\{ 1 - \exp \left[ \frac{\frac{k_2k_4}{k_1} + (k_3 + k_4)[H]}{\frac{k_2 + k_3}{k_1} + [H]} t \right] \right\} \quad (15A)$$

When steady state is achieved ( $t$  is large) eq 15A is transformed to:

$$[HRE'] = \frac{1}{1 + k_4/k_3} \frac{[RE]_T[H]}{\frac{k_2}{k_1(1 + k_3/k_4)} + [H]} \quad (16A)$$

If  $k_4 \gg k_3$  (as in the case of turkey erythrocyte adenylate cyclase, Levitzki, 1977), the affinity toward hormone, measured in the presence of GTP, is identical to that measured directly by binding, namely,  $k_2/k_1 = K_H$ . The maximal activity of the enzyme obtained in the presence of high hormone is given by:

$$[HRE']_{\max} = \frac{[RE]_T}{1 + k_4/k_3} \quad (17A)$$

which indicates that  $[HRE']_{\max} \ll [RE]_T$  when  $k_4 \gg k_3$ . This expression is similar to the one obtained by Levitzki (1977). A somewhat modified model which builds a steady-state level of  $[HRE']$  in the presence of GTP:



yields an equation for  $[HRE']$  which is identical to eq 5A.

The rate governing the reversal process may be experimentally derived after achieving a steady-state level of  $HRE'$  by the concomitant addition of the  $\beta$ -adrenergic antagonist PPL and labeled ATP.  $HRE'$  will decay rapidly to the ground state by a first-order process and cAMP will accumulate exponentially. Such an experiment is presented in the text. Such an experiment can be performed for other receptor cyclase systems as well.

This model predicts (a) no basal cyclase activity in the absence of hormone; (b) the rate at which the enzyme is activated depends on hormone concentration and is saturable according to a Michaelian function; (c) the affinity of the hormone to the

TABLE V: Dependence of the Rate of Enzyme Activation on Hormone Concentration as Predicted by the Different Models of Receptor to Enzyme Coupling.

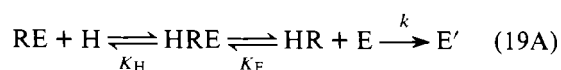
model	overall kinetic features		type of depend. of app 1st-ord rate const on hormone concn	effect of reducing [E <sub>T</sub> ]	effect of reducing [R <sub>T</sub> ]
	rapid equilib prior to activat	rapid activat compared to equilib			
(I) precoupled	1st ord	1st ord	noncoop	reduct of max act. attain. but no change in rate of activat	(1) reduction in max bind. and in max act. attain. (2) no change in rate of activat
(II) dissoc	deviat from 1st ord	deviat from 1st ord	<i>a</i>	reduct of max act. attain. and in activat. rate as in model II	reduct in max bind. and in max act. attain. as in model II
(III) equilib floating	deviat from 1st ord	2nd ord	<i>a</i>		
(IV) collis coupling	model excludes this condit.	1st ord	noncoop	reduct in max extent of enz activat with no change in rate const for activat	reduct in the rate const of enz activat with no change in max extent of enz activat
(V) precoupled dissoc	not exam.	deviat from 1st ord at low hormone concn	<i>a</i>	reduct extent of enz activat	reduction in rate const of enz activat with no change in max extent of enz activat

<sup>a</sup> Due to deviations from first-order kinetics, no dependence at the rate constant of enzyme activation on hormone concentration can be derived.

receptor as measured kinetically should be identical or higher to that measured directly by binding experiments; (d) the decrease of receptor concentration or of enzyme concentration by irreversible inactivation should not affect the *rate* at which the activated species [HRE'] appears but should decrease the maximal level of HRE' attainable either in the presence of GppNHp or in the presence of GTP (Tables IV and V).

#### (II) The Dissociation Receptor-Cyclase Model

The model is described by the equation:



This model postulates that E' is the active species of the cyclase. As in model I, the receptor is obligatorily coupled either to E or to R.

**Hormone Binding.** Both R and RE bind hormone. In the absence of guanyl nucleotides, E' is not formed and one can write:

$$[HRE] = \frac{[RE][H]}{K_H} \quad (20A)$$

and

$$[HR] = \frac{K_E[HRE]}{[E]} = \frac{K_E[RE][H]}{K_H[E]} \quad (21A)$$

Since the law of conservation holds:

$$[E_T] = [RE] + [HRE] + [E] \quad (22A)$$

and

$$[R_T] = [RE] + [HRE] + [HR] \quad (23A)$$

one obtains:

$$[E] = [E_T] - [RE] - [HRE] = [E_T] - [RE] \left(1 + \frac{[H]}{K_H}\right) = [E_T] - [RE] \left(\frac{K_H + [H]}{K_H}\right) \quad (24A)$$

The bound form of the receptor includes the species HR and HRE. We can therefore define

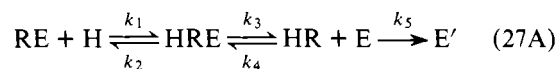
$$B = [HR] + [HRE] \quad (25A)$$

where B is the concentration of the bound hormone. When one derives the expression for B one obtains

$$B^2 + \frac{B}{K_H + [H]} \left[ K_H \left( [E_T] + \frac{K_E[H]}{K_H + [H]} \right) - [R_T](2[H] + K_H) \right] + \frac{[R_T]^2[H](K_H + [H]) - [R_T][H]([E_T] + K_E)K_H}{(K_H + [H])^2} = 0 \quad (26A)$$

Simulation of eq 28A reveals that the binding is *negatively cooperative* (Table IV, Figure 10). The extent of negative cooperativity becomes more pronounced as K<sub>E</sub> is reduced relative to [E<sub>T</sub>]. Reducing K<sub>E</sub> results in the relative increase of HRE, the low-affinity form, relative to HR, the high-affinity species.

**The Rate of Formation of the Permanently Active Enzyme.** The process of enzyme activation can be described as:



If one derives the rate of E' formation, one obtains:

$$\frac{d[E']}{dt} = \frac{[E_T] - [E']}{\left\{ k_5 + \frac{k_4(K_H + [H])}{k_5 k_3 [H]} \right\} [R_T] - \frac{k_4(K_H + [H])}{k_5 k_3 [H]} ([E_T] - [E'])} \quad (28A)$$

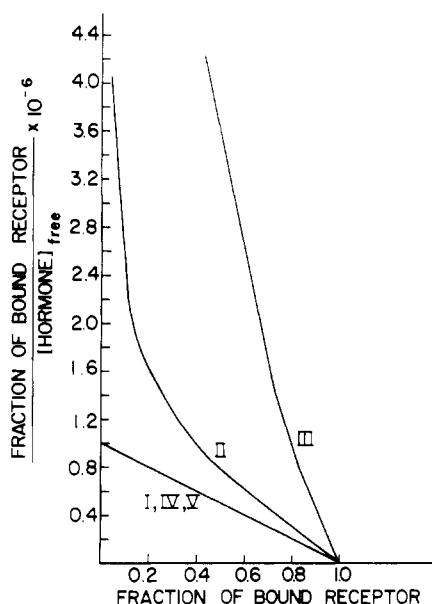


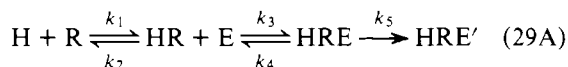
FIGURE 10: Simulation of hormone binding according to the different models of receptor to enzyme coupling. Simulation conditions presented in the figure are:  $[R_T] = [E_T] = 10K_E$ ,  $K_H = 1.0 \times 10^{-6}$  M. Simulation was conducted with hormone concentrations from  $1.0 \times 10^{-8}$  to  $1.0 \times 10^{-5}$  M. Changing the simulation conditions does not change the general characteristics of the curve. The Roman numerals signify the model according to which the simulation was conducted.

From the full derivation (Tolkovsky and Levitzki, 1979) of eq 28A it is apparent that the kinetics of accumulation of  $E'$  deviates strongly from first-order kinetics. It can also be seen that reducing  $[R_T]$  or  $[E_T]$  by irreversible inactivation results in both the reduction of the maximal level of  $E'$  attainable and a decrease in its rate of formation (Table V).

To summarize, the predictions of the dissociation model are: (1) In the absence of hormone there might be basal activity, if not all enzyme units are coupled to receptor units. (2) The binding of hormone will be negatively cooperative, although one intrinsic binding constant governs the hormone binding. This is due to the fact that there are two hormone-bound species being present in parallel: HR and HRE. (3) The kinetics which govern the accumulation of  $E'$  is not first order. (4) The effect of irreversibly reducing either  $[R_T]$  or  $[E_T]$  will slow down the rate of accumulation of  $E'$  and decrease the maximal level of  $E'$  attained.

### (III) The Floating Receptor-Enzyme Equilibrium Model

In this model it is assumed that equilibrium exists between the hormone-receptor complex and the enzyme:



where  $K_H = k_2/k_1$  and  $K_E = k_4/k_3$ .

This type of model belongs to the class of floating receptor models which allow the receptor to float in the membrane (Jacobs and Cuatrecasas, 1976).

**Binding of Hormone.** In the absence of enzyme activation, the hormone binding species are R and RE. Thus:

$$[HRE] = \frac{[HR][E]}{K_E} = \frac{[H][R][E]}{K_H K_E} \quad (30A)$$

$$[HR] = \frac{[H][R]}{K_H} \quad (31A)$$

Consequently, the total amount of bound hormone B is given

by:

$$B \equiv [HR] + [HRE] = [R] \left[ \frac{[H]}{K_H} + \frac{[E][H]}{K_E K_H} \right] \quad (32A)$$

When one derives the expression for B one obtains:

$$B^2 - B \frac{K_E K_H [H] + 2[H]^2 [R_T] + K_H^2 K_E + ([H][R_T] + [H][E_T] K_H)}{[H](K_H + [H])} + \frac{[R_T][H]K_H K_E + [H][E_T][R_T]K_H + [H]^2 [R_T]^2}{[H](K_H + [H])} = 0 \quad (33A)$$

Simulation of eq 33A reveals negative cooperativity in binding (Figure 10, Table IV). The tighter the interaction between HR and E, the further away are the two apparent binding constants derived for H from the thermodynamic dissociation constant.

**The Rate of HRE Formation.** Using the scheme presented in eq 29A one can derive the rule of HRE' accumulation (in the presence of GppNHp) as a function of time:

$$d[HRE']/dt = k_5[HRE] \quad (34A)$$

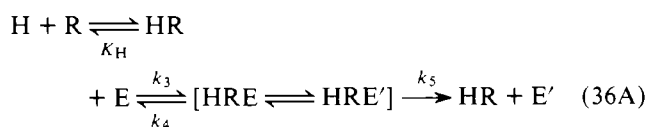
deriving (Tolkovsky and Levitzki, 1979) the expression for  $d[HRE']/dt$  one obtains:

$$\frac{d[HRE']}{dt} = k_5 \left( \frac{-[HRE']}{2} + C - \sqrt{\frac{[HRE']^2}{4} - [HRE']([R_T] + C) + C^2 - [E_T][R_T]} \right) \quad (35A)$$

It can be seen that the rate law which governs the formation of the active species HRE' deviates strongly from first-order kinetics.

### (IV) The Collision Coupling Model

In this model the mode of enzyme activation is described by the equation:



where  $K_H = k_2/k_1$ . The collision coupling model is in fact a special case of the floating equilibrium model, model III. In its formulation a few assumptions are made: (1)  $k_4$  is very large, so that the affinity of HR for E is small and thus  $k_3$  becomes rate limiting; (2)  $k_5$  is also very rapid compared to  $k_3$  and is of the same order of magnitude as  $k_4$ ,  $k_5 \geq k_4$ ; (3) HRE never accumulates. In this case the irreversible step of activation (in the presence of GppNHp) is no longer between HRE and HRE' but describes the collision between HR and E, since  $k_5 \geq k_4$ , and both are extremely fast. In analogy with the interaction of an enzyme with substrate, E is the substrate and R is the catalytic entity which is regenerated concomitantly with the formation of the "product"  $E'$ .

**Hormone Binding.** The binding pattern of hormone in this case is simple, since the only hormone-bound species which exists in significant amounts is HR. Therefore:

$$[HR] = \frac{[R_T][H]}{K_H + [H]} \quad (37A)$$

and the binding of hormone is noncooperative (Table IV, Figure 10).

*The Kinetics of Appearance of the Permanently Activated State.* For the model presented in eq 36A one can write:

$$[E_T] = [E] + ([HRE] \rightleftharpoons [HRE']) + [E'] \quad (38A)$$

$$[R_T] = [R] + ([HRE] \rightleftharpoons [HRE']) + [HR] \quad (39A)$$

Also

$$([HRE] + [HRE']) \ll [RE] + [E] + [E'], [HR] + [RE] + [R] \quad (40A)$$

One can write:

$$d[E']/dt = k_5([HRE] \rightleftharpoons [HRE']) \quad (41A)$$

namely:

$$\frac{d([HRE] \rightleftharpoons [HRE'])}{dt} = k_3[HR][E] - (k_4 + k_5)([HRE] \rightleftharpoons [HRE']) \quad (42A)$$

At the steady state which is rapidly established

$$([HRE] \rightleftharpoons [HRE']) = \frac{k_3[HR][E]}{k_4 + k_5} \quad (43A)$$

since eq 37A holds and:

$$[E] = [E_T] - [E'] \quad (44A)$$

one can rewrite eq 42A:

$$\frac{d[E']}{dt} = \frac{k_5 k_3 [R_T][H][E_T]}{(k_4 + k_5)(K_H + [H])} - \frac{k_5 k_3 [R_T][H][E']}{(k_4 + k_5)(K_H + [H])} \quad (45A)$$

and

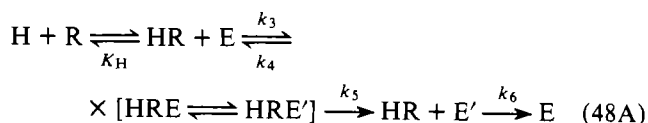
$$\frac{d[E']}{[E_T] - [E']} = \left( \frac{k_5 k_3 [R_T][H]}{(k_4 + k_5)(K_H + [H])} \right) dt \quad (46A)$$

Integrating eq 46A one obtains:

$$[E'] = [E_T] \left[ 1 - \exp \left( - \frac{k_3 k_5 [R_T][H]}{(k_4 + k_5)(K_H + [H])} t \right) \right] \quad (47A)$$

In contrast to model III, the rate constant governing the appearance of the activated state depends solely on the total receptor concentration and not on both the total receptor and enzyme concentrations. The maximal level of activated enzyme attainable is solely dependent on the total enzyme concentration and is *independent* of the receptor concentration. The overall process of enzyme activation is first order and the apparent first-order rate constant is linearly dependent on total receptor concentration and increases as a function of hormone concentration in a noncooperative fashion.

*The Activation of the Enzyme in the Presence of GTP.* In the presence of GTP the activated form of the enzyme is reverted to its inactive form concomitantly with the hydrolysis of GTP at the regulatory site (Cassel and Selinger, 1976; Levitzki, 1977). Under these conditions, the collision coupling model assumes the scheme:



The equation governing the rate of appearance of E' is:

$$[E'] = \frac{[E_T] k_5 k_3 [R_T][H]}{(k_4 + k_5)(K_H + [H])} \frac{k_5 k_3 [R_T][H]}{(k_4 + k_5)(K_H + [H])} + k_6 \times \left[ 1 - \exp \left( - \frac{k_3 k_5 [H][R_T]}{(k_4 + k_5)(K_H + [H])} + k_6 \right) t \right] \quad (49A)$$

At high hormone concentrations and if  $K_5 \gg k_4$ , eq 49A obtains the form:

$$[E'] = \frac{[E_T]}{1 + \frac{k_6}{k_3 [R_T]}} [1 - \exp(-k_3 [R_T] + k_6)t] \quad (50A)$$

After steady-state conditions have been achieved ( $t$  is large), the concentration of enzyme in its active form is given by:

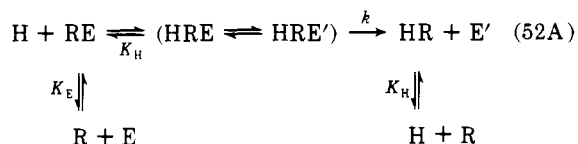
$$[E'] = \frac{[E_T]}{1 + \frac{k_6}{k_3 [R_T]}} \quad (51A)$$

which is identical with eq 21 in the text. As in model I it can be seen that only a fraction of the total enzyme is in its active form when the physiological regulator GTP is present. Also, as in model I when GppNHp is present as the allosteric activator, all the enzyme is converted to its active form after enough time has elapsed.

This model predicts: (a) noncooperative binding of the hormone; (b) first-order kinetics of activation of the enzyme in the presence of saturating GppNHp; (c) the apparent first-order rate constant describing the process of activation is linearly dependent on total receptor concentration and is independent of enzyme concentration. Thus, irreversible inactivation of the receptor will cause a proportional decrease in the rate of enzyme activation but not in the maximal activity attainable in the presence of GppNHp. Irreversible inactivation of the enzyme will cause a decrease in the maximal activity attainable but not affect the rate constant of activation in the presence of GppNHp; (d) the apparent first-order rate constant of activation of the enzyme in the presence of GppNHp increases as a function of hormone concentration in a saturable fashion according to a noncooperative dependence curve.

#### (V) Combination Models

One can combine the models discussed so far and construct combination models. One such combination model is presented in the following scheme (Tables IV and V):



This model can be termed precoupled-dissociation model.

It can be shown (Tolkovsky and Levitski, 1979) that this model predicts noncooperative hormone binding (Figure 10, Table IV). However, the time course of appearance of E' deviates strongly from first-order kinetics (derivations not shown).

#### Conclusions

Different modes of enzyme to receptor coupling within the membrane predict both different patterns of hormone binding and different mechanisms of activation of the receptor-dependent enzyme (Tables IV and V, Figure 10). The possibility

to generate conditions under which the process of hormone-dependent enzyme activation is essentially irreversible simplifies the mathematical analysis and sharpens the feasibility to design experiments to probe the mode of receptor to enzyme coupling. Thus, performing binding experiments in conjunction with detailed kinetic analysis of enzyme activation may allow us to decide which of the possible modes of coupling operates in a real situation. Such an analysis is presented in the adjoining communication dealing with the mode of coupling of  $\beta$ -adrenergic receptor to the adenylate cyclase in turkey erythrocyte membranes. Since numerous hormone-dependent adenylate cyclases have been shown to depend on GTP and to respond to GppNHp, such an analysis can be extended to other hormone-dependent adenylate cyclases.

In some of the models explored the response in the presence of GTP, not GppNHp, was also analyzed. Under such conditions, not all the enzyme is converted to its activated form, but a steady-state concentration of active enzyme is achieved. This latter situation is the one encountered under physiological conditions.

The full mathematical analysis of the models describing receptor to enzyme coupling is given elsewhere (Tolkovsky and Levitzki, 1979).

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