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Interconversion between Different States of Affinity of the Human Growth Hormone Receptor on Rat Hepatocytes: Effects of Fractional Site Occupancy on Receptor Availability[†]

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ABSTRACT: Isolated rat hepatocytes accumulate a slowly dissociable human growth hormone (hGH) binding fraction with incubation time. Slowly dissociable [^{125}I]hGH is receptor bound, intact and immunocompetent. Fifty-six percent of the bound hormone was slowly dissociable within 3 min of the initiation of hGH–hepatocyte incubation. Subsequently, the proportion of slowly dissociable [^{125}I]hGH increased at the expense of the rapidly dissociable fraction. This suggested that binding induced interconversion between different states of affinity of the hGH receptor. Preincubation with hGH diminished the capacity of hepatocytes to subsequently bind [^{125}I]hGH. Receptor occupancy resulting from accumulation of slowly dissociable hGH accounted for 37 and 62% of the decreased binding after preincubation with 0.79 and 7.9 nM hGH, respectively. Fractional receptor occupancy, among but distinguishable from other processes, may account for the inverse relationship between site number and applied hormone concentration. Addition of hGH to the medium of [^{125}I]-hGH–hepatocyte incubates increased the extent of loss of label from hepatocytes. The progressive retention of intact]^{125}I]-hGH by hepatocytes with site occupancy and invariant receptor affinity subsequent to fractional saturation was inconsistent with negative cooperativity. A mechanism in which hGH diminished reassociation of [^{125}I]hGH with available sites during dissociation was consistent with the available binding data. The interrelationship between peptide hormone in rapid and slow equilibrium with the medium is of fundamental importance in modulating receptor binding and availability.

Isolated rat hepatocytes retain a slowly dissociable human growth hormone (hGH)[1] binding component (Donner et al., 1978). The slowly dissociable fraction of bound hGH results from a receptor-mediated process (Donner et al., 1980) and is largely localized to the exterior surface of the hepatocyte. This hormone is intact and immunocompetent. It results from processes distinguishable from cellular events leading to internalization and degradation (Donner et al., 1980) of bound label.

Preincubation with unlabeled peptide hormones decreased the capacity of target cells to bind subsequently applied labeled hormone (Gavin et al., 1974; Roth et al., 1975). Decreased binding has been attributed to the internalization and degradation of hormone or hormone–receptor complexes (Terris & Steiner, 1975; Carpenter & Cohen, 1976). Another possible explanation for the inverse relationship between functional receptor number and the concentration of hormone to which cells were exposed prior to isolation is occupancy of such sites by slowly dissociable hormone. One goal of this study was to evaluate the effect of accumulation of slowly dissociable hormone on receptor availability and binding properties.

[1] Abbreviations used: hGH, human growth hormone; [^{125}I]hGH, iodine-125-labeled human growth hormone; HBSS, Hank's balanced salt solution; BSA, bovine serum albumin, fraction V.
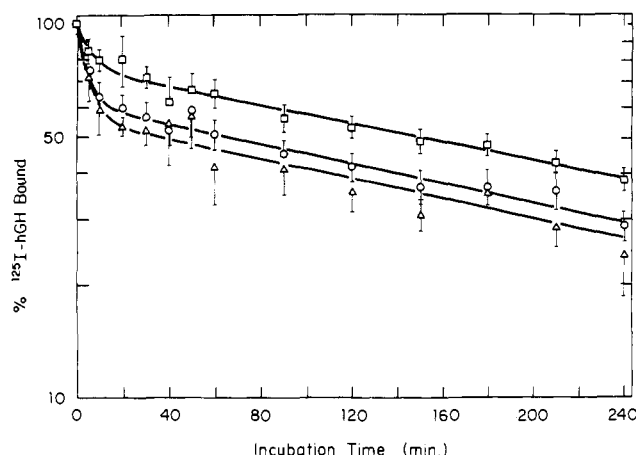
FIGURE 1: Dissociation of [^{125}I]hGH from hepatocytes after short incubation intervals. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (2.3 nM). In a parallel set of flasks, hGH (1.8 μM) was additionally present to compete for [^{125}I]hGH sites. The flasks were shaken for 3 ($\triangle$), 6 (O), and 10 ($\square$) min. The suspensions were centrifuged (30 s, 1500 g), and the supernatant containing unbound label was aspirated from above the resultant cell pellet. The hepatocytes were resuspended in fresh medium and added to beakers containing 1 L of HBSS–0.1% albumin. The solution was stirred to maintain a homogeneous distribution of cells in the dissociation medium. At timed intervals, 10-mL aliquots were drawn into disposable plastic hypodermic syringes through 14-gauge cannulas, placed in 16 × 100 mm disposable glass test tubes, and centrifuged (3 min, 1500g). The supernatants were removed from above the cell pellets were assayed for ^{125}I in a γ counter. Assay at the moment of cellular dilution was considered to represent the zero time point. Samples were also withdrawn for assay of binding at subsequent times. The amount of hormone was expressed as a percentage of the zero time point. Each point represents the mean of triplicate determinations.

In previous studies of hormone receptor regulation, binding site heterogeneity (Kahn, 1975) and negative cooperativity (De Meyts et al., 1973, 1976; Limbird & Lefkowitz, 1976) have been described. The progressive retention of intact, receptor-bound hGH suggested possible interconversion between hormone receptor states of lower to higher affinity. Mechanisms leading to ligand retention through site interconversion have found wide acceptance in explaining the binding and response of target tissues to neurotransmitters (Katz & Thesleff, 1957; Sugiyama et al., 1976; Maguire et al., 1977). A second goal of this study was to determine the applicability of such binding models to a peptide hormone receptor system.

Experimental Procedure

Materials were obtained and methods and data analysis were as described in the accompanying paper (Donner et al., 1980).

Results

[^{125}I]hGH was associated with hepatocytes for short time intervals (3, 6, and 10 min), after which unbound hormone was removed by centrifugation. The cells were then resuspended in a large excess of fresh hormone-free medium to promote dissociation (Figure 1). After each association interval, the subsequent course of dissociation was biphasic. Rate constants for the loss of rapidly and slowly dissociating [^{125}I]hGH were computer derived [eq 2 of Donner et al., (1980)]. The rate of loss of rapidly and slowly dissociating [^{125}I]hGH did not depend on the duration of association (Table I). The percentage composition of slowly dissociable [^{125}I]-hGH increased with longer association times and, consequently, increased receptor occupancy. This was accompanied

Table I: Dissociation of [^{125}I]hGH from Hepatocytes after Short Incubations[a]

| incubn time (min) | rate constant, k_{-1} (s^{-1}) | | % label remaining bound after 4 h | fraction (%) of rapidly dissociable label |
	rapid component (×10^3)	slow component (×10^5)		
3	3.2 ± 1.7	5.0 ± 0.8	24	44 ± 3
6	3.7 ± 0.04	4.9 ± 0.01	29	39 ± 0.5
10	2.4 ± 1.3	4.5 ± 0.6	38	24 ± 4

[a] The dissociation of [^{125}I]hGH was assayed as described in the legend to Figure 1. The dissociation curves were resolved into rapidly and slowly dissociating hormone components by nonlinear regression analysis.

Table II: Dissociation of [^{125}I]hGH from Hepatocytes after Varied Incubation Periods[a]

| incubn time (min) | rate constant, k_{-1} (s^{-1}) | |
	rapid component (×10^3)	slow component (×10^5)
5[d]	2.0 ± 0.9	3.6 ± 1.2
15	1.5 ± 0.8	3.5 ± 0.1
30	b	2.6 ± 4.1
75	b	1.0 ± 2.8
120	b, c	4.0 ± 1.3

[a] The dissociation of [^{125}I]hGH was as described in the legend to Figure 2. The dissociation curves were resolved into rapidly and slowly dissociating components by nonlinear regression analysis. [b] Due to the diminished fraction of rapidly dissociable label, the error involved in calculation of this rate was so large as to preclude its accurate definition. [c] After 2 h of incubation, 10% of the bound label was rapidly dissociable as suggested by the average of eight dissociation experiments. [d] Average of two experiments.
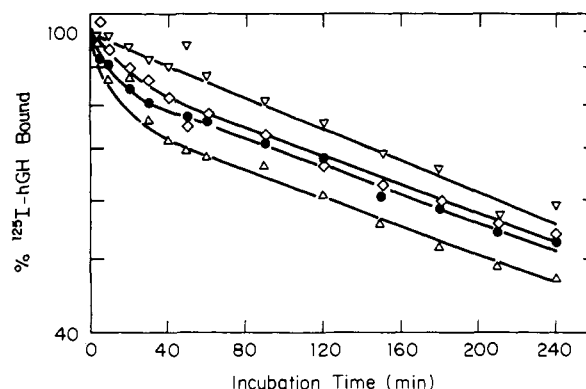

FIGURE 2: Dissociation of [^{125}I]hGH from hepatocytes after varied incubation periods. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (2.3 nM). In a parallel set of flasks, hGH (1.8 μM) was additionally present to compete for [^{125}I]hGH sites. The flasks were shaken for 5 ($\triangle$), 15 ($\bullet$), 75 ($\diamondsuit$), and 120 ($\triangledown$) min. Dissociation was then assayed as described in the legend to Figure 1.

by a decrease in the fraction of label in rapid equilibrium with the medium.

Dissociation of [^{125}I]hGH from hepatocytes was initiated after more extended association times. The fraction of slowly dissociable [^{125}I]hGH increased with incubation time (Figure 2, Table II). After 2 h of incubation only 10% of the bound label was rapidly dissociable and nearly monocomponent dissociation curves were observed. The rate constant for loss of slowly dissociable [^{125}I]hGH was invariant with association time. The error involved in computer resolution of the rate of loss of rapidly dissociating label increased as the proportion
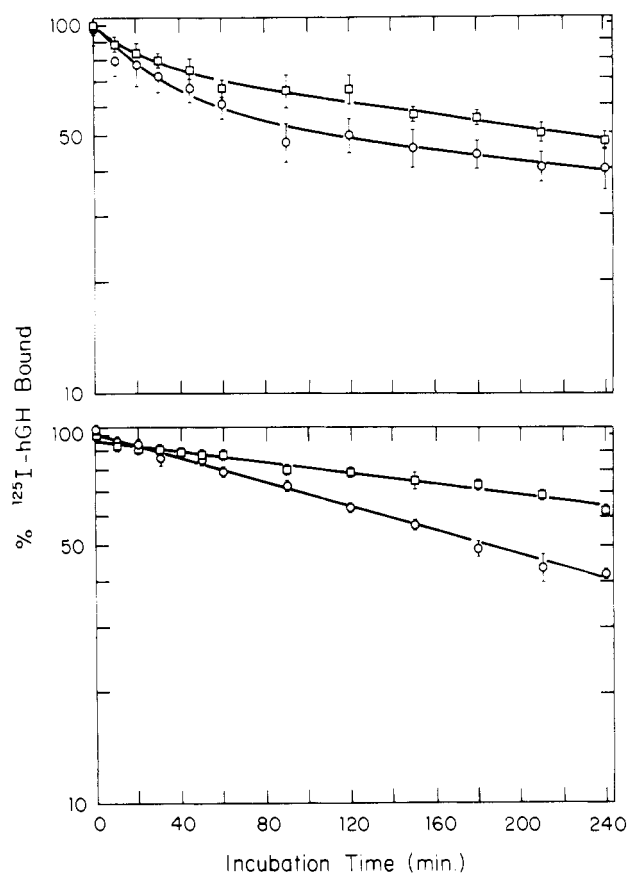
FIGURE 3: (Top panel) Dissociation of [^{125}I]hGH from hepatocytes in the presence and absence of hGH. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to two incubation flasks containing [^{125}I]hGH (3.0 nM) and medium. In a parallel set of flasks, hGH (1.8 μM) was additionally added to compete for [^{125}I]hGH binding sites. After 2 h of association, dissociation was initiated by dilution of the incubation mixtures into 1 L of balanced salt solution–0.1% albumin. In the second set of dissociation flasks, hGH (4.5 μM) also was added to the dissociation medium. Dissociation was then assayed as described in the legend to Figure 1. The results are expressed as the percentage of hormone remaining bound after the indicated incubation time. Curves obtained in the absence (□) and presence (○) of native hGH in the dissociation medium are shown. (Bottom panel) Dissociation of [^{125}I]hGH from hepatocytes in the presence and absence of hGH after removal of unbound label. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to two incubation flasks containing [^{125}I]hGH (3.0 nM) and medium. In a parallel set of flasks, hGH (1.8 μM) was additionally added to compete for [^{125}I]hGH binding sites. After 2 h of association, the suspensions were centrifuged (30 s, 1500g) and the supernatant containing unbound hormone was aspirated from the resultant cell pellet. The hepatocytes were resuspended in fresh, hormone-free medium and added to beakers containing 1 L of HBSS–0.1% albumin. In the parallel set of dissociation flasks, hGH (10 nM) was added to the dissociation medium. Dissociation was then assayed as described in the legend to Figure 1. The results are expressed as the percentage of hormone remaining bound after the indicated incubation time. Curves obtained in the absence (□) and presence (○) of native hGH in the dissociation medium are shown.

of such hormone diminished. For this reason, the most accurate values for loss of [^{125}I]hGH were those obtained after short incubations (as in Figure 1).

Addition of native hGH to the dissociation medium of [^{125}I]hGH–hepatocyte incubates increased the extent of loss of label whether unbound hormone was removed from the medium (Figure 3, bottom panel) or not (Figure 3, top panel). In systems containing unbound [^{125}I]hGH in the medium, the proportion of rapidly to slowly dissociating hormone was in-
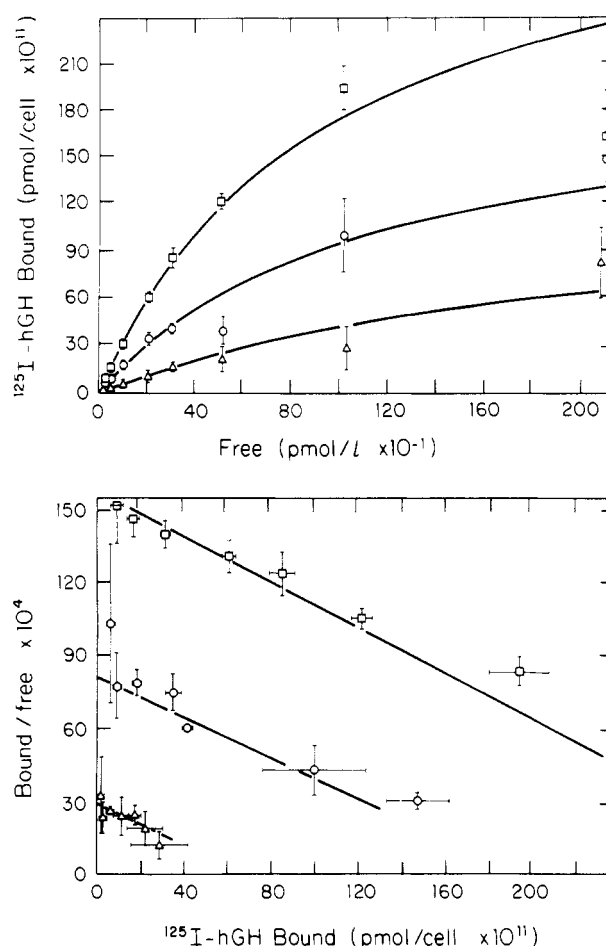

FIGURE 4: (Top panel) Saturation assay of hepatocyte suspensions preincubated and not preincubated with hGH. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. The suspension was divided into three equal portions. One part served as a control; the others were incubated with hGH (0.79 and 7.9 nM) for 30 min. The suspensions were centrifuged (50g, 2 min) and the supernatants aspirated from above the resultant cell pellets. The pellets were resuspended in fresh medium (5 mL), recentrifuged, and finally suspended in 6 mL of medium. Aliquots of each suspension were added to flasks containing varying concentrations of [^{125}I]hGH or [^{125}I]hGH + hGH (3.8 μM) and medium. The flasks were shaken for 2 h, and triplicate 100-μL aliquots were taken from each vessel for assay of binding. Aliquots of the incubation medium were assayed for the concentration of free hormone. Computer-fitted data are shown in which the concentration of [^{125}I]hGH bound per cell number is plotted against the concentration of free hormone. Specific binding curves for the control (□) and 0.79 (○) and 7.9 (△) nM hGH incubates are shown. (Bottom panel) Scatchard analysis of the saturation assay of preincubated and nonpreincubated cell suspensions. The data from the top panel were replotted according to Scatchard. Specific curves for the control (□) and 0.79 (○) and 7.9 (△) nM hGH incubates are shown.

creased by the presence of native hGH (Table III). Removal of unbound [^{125}I]hGH from the dissociation medium resulted in a lag time of ~50 min prior to the observation of an increased extent of dissociation in the system containing native hGH. Monocomponent dissociation curves were obtained as the small fraction of rapidly dissociable label was lost during the wash procedures required to remove unbound [^{125}I]hGH from about the cells.

The binding properties of hepatocytes exposed and not exposed to physiologic concentrations of hGH were compared in a saturation assay (Figure 4, top panel). Data were best fit to the presence of a single site and Scatchard plots were monocomponent (Figure 4, bottom panel). Preincubation with hGH decreased the subsequent uptake of [^{125}I]hGH by he-

Table III: Dissociation of [^{125}I]hGH from Hepatocytes in the Presence and Absence of hGH[a]

| | rate constant, k_{-1} (s^{-1}) | | | |
incubn condition	rapid component (×10^4)	slow component (×10^5)	% label bd after 4 h	fraction (%) of rapidly dissociable label
no hGH in dissn medium	7.9 ± 4.7	3.4 ± 0.7	47.8	21.5 ± 6.8
hGH in dissn medium	5.2 ± 3.0	2.8 ± 2.2	40.1	41.3 ± 14.9
no hGH in dissn medium, unbound label removed	b	2.7 ± 0.3	62.2	4.7 ± 2.5
hGH in dissn medium, label removed	b	5.9 ± 1.2	42.1	5.7 ± 23.0

[a] The dissociation of [^{125}I]hGH was as described in the legends to Figures 3 and 4. The dissociation curves were resolved into rapidly and slowly dissociating components by nonlinear regression analysis. [b] Insufficient data to permit resolution of a rate constant.

Table IV: Scatchard Analysis of the Saturation Assay of Preincubated and Nonpreincubated Cell Suspensions[a]

| | | | sp binding curves | |
incubn procedure	sites/cell	K_D (M)	% binding rel to control	K_D (M)
control	5329	(2.08 ± 0.23) × 10^{-9}	100	(0.94 ± 0.12) × 10^{-9}
7.9 × 10^{-10} M hGH	4173	(2.67 ± 0.39) × 10^{-9}	58	(1.06 ± 0.21) × 10^{-9}
7.9 × 10^{-9} M hGH	3734	(2.03 ± 1.97) × 10^{-9}	21	(1.05 ± 0.72) × 10^{-9}

[a] Nonlinear regression analysis was performed (Experimental Procedure) on the data within the Scatchard plots in Figure 4. The intercept on the x axis represented the concentration of hormone bound at saturation. The slope of each derived component of the curve represented the negative value of the disassociation constant ($-1/K_D$) for that class of sites. The data were fit to a single-site model (eq 1).

patocytes (Table IV). The binding capacity decreased as the hGH concentration to which cells were preexposed increased. Incubation with 0.79 and 7.9 nM hGH decreased the specific binding of [^{125}I]hGH by 42 and 79% relative to controls not preexposed to hGH. The decrease in the total binding systems was similar. This suggested that the decreased binding capacity was related to the high-affinity site. In both the total and specific systems, the decrease in binding capacity was not accompanied by any significant change in the affinity of the sites remaining available to [^{125}I]hGH.

One possible cause for apparent site loss could be occupancy of receptors by slowly dissociable hGH (Figures 1 and 2). For the purpose of testing this, occupancy was assumed to be that fraction of sites to which hGH was bound at any time, t. Site loss was assumed to be a decrease in cellular binding capacity not accountable for by occupation or requiring additional kinetic or cellular processes subsequent to binding. Occupancy was the amount of hormone bound during the 30-min preincubation with hGH (caption to Figure 4) minus the amount of hGH which dissociated during the 2-h time course of the saturation-assay. At 0.79 nM hGH, association data [Figure 1 of Donner et al., (1980)] suggested that after 30 min binding was 65% of the steady-state level. At 7.9 nM hGH, steady-state conditions were obtained within 30 min. The fraction of sites occupied was 0.65[H]/[H] + K_D and [H]/[H] + K_D at 0.79 and 7.9 nM hGH, respectively, after preincubation (where [H], is the applied hGH concentration and K_D, the dissociation constant, is 1 nM). Subsequent loss of hGH was calculated by estimating that after 30 min ~20% of the prebound hormone was rapidly dissociable and ~80% was slowly dissociable (Figures 1 and 2; Tables I and II). Then

$$[HR]_{t=120min}/[HR]_{t=0} = Ae^{-k_{-1}'t} + (1 - A)e^{-k_{-1}''t}$$

where [HR]$_{t=0}$ is the concentration of hGH bound after the 30-min preincubation, [HR]$_{t=120}$ is the concentration of hGH bound after the 2-h saturation assay, A is the fraction of sites (0.2) having a first-order rate of k_{-1}', $1 - A$ is the fraction of sites (0.8) having a first-order dissociation rate of k_{-1}'', k_{-1}' is 3.2 × 10^{-3} s^{-1} (Table I), and k_{-1}'' is 5.0 × 10^{-5} s^{-1}. Fifty-five percent of the hGH bound during preincubation remained so after the additional 2 h of saturation assay.

During the 30-min incubation, 28 and 89% of the sites were occupied at 0.79 and 7.9 nM hGH, respectively. At the end

of saturation assay, 15 and 49% (55% of the preincubation values) of the sites were occupied. At 0.79 nM hGH there was a 42% decrease in specific binding capacity. Thirty-seven percent of this loss was accounted for by occupancy. At 7.9 nM hGH there was a 79% decrease in binding capacity. Sixty-two percent of the site loss resulted from occupancy by unlabeled hormone.

Cell populations preincubated with [^{125}I]hGH (5.8 nM) for 20, 50, and 80 min prior to a saturation assay yielded binding data different from those of untreated suspensions. [^{125}I]hGH was employed during preincubations (in contrast with the procedure described above, Figure 4) so that the total concentration of cellularly bound label would be known at the end of the assay. In total binding systems (Figure 5, top panel), the concentration of [^{125}I]hGH accumulated with and without preincubation was identical at hormone concentrations approaching site saturation. In preincubated systems, the total uptake was increased at low concentrations due to the inability of prebound label to rapidly reequilibrate with the medium (as suggested by Figures 1 and 2). "Nonspecific" binding increased with preincubation, again resulting from the inability of the prebound slowly dissociable [^{125}I]hGH to reequilibrate with the high concentration of native hGH which followed it. A consequence of this was that Scatchard plots of specific curves (Figure 5, bottom panel) suggested a decrease in hGH binding capacity with preincubation. For clarity, the 50-min incubation alone is compared with the untreated control. There was no change in the affinity of sites remaining available for binding after preincubation as evidenced by the parallel binding isotherms.

The amount of prebound [^{125}I]hGH remaining cellularly associated in the nonspecific system to which [^{125}I]hGH + hGH (2.5 μM) was added was calculated. The number of sites occupied by slowly dissociable prebound [^{125}I]hGH increased initially and remained constant after the 50-min incubation time. Curve fitting defined 3566 sites in the control and 1925 sites in the treated suspension after 50 min (Figure 5). A total of 1641 sites were not available for binding. Preincubation resulted in the occupation of 1150 sites, which was 70% of the decrease in uptake.

Discussion

Isolated rat hepatocytes accumulate a slowly dissociable [^{125}I]hGH binding fraction (Donner et al., 1978). This label

is intact, immunocompetent [^{125}I]hGH (Donner et al., 1980). At physiological concentrations, slowly dissociable hormone accumulates on hepatocytes via a receptor-mediated process (Donner et al., 1980). Fifty-six percent of the bound growth hormone was slowly dissociable within 3 min of the initiation of incubation (Figure 1; Table I). Subsequently, the proportion of slowly dissociable [^{125}I]hGH increased as the fraction of rapidly dissociable label diminished. After 2 h when a steady state relating free and bound [^{125}I]hGH had been obtained, ~90% of the bound label was slowly dissociable (Figure 2; Table II). This suggested that significant concentrations of hormone could remain receptor bound for substantial times subsequent to initial site occupancy. For this reason, the contribution of hormone retention in a slowly dissociable state to previously proposed mechanisms of receptor regulation was considered.

Addition of native hGH to the dissociation medium of [^{125}I]hGH–hepatocyte incubates increased the extent of loss of label from cells (Figure 3; Table III), a phenomenon termed negative cooperativity (De Meyts et al., 1973, 1976). The concept of such negative cooperativity is based on the assumption that increased site occupancy in the presence of native hormone resulted in diminished receptor affinity (De Meyts et al., 1973, 1976). In considering the viability of such a mechanism, a number of additional observations should be considered. Interaction of [^{125}I]hGH with hepatocytes resulted in retention of hormone by the cell subsequent to the initial binding process. During the course of associations, increased receptor occupancy resulted in a progressive increase in the proportion of high-affinity receptor sites from which label dissociated slowly. As receptor occupancy increased, the proportion of slowly dissociable hormone increased and the overall extent of [^{125}I]hGH loss from hepatocytes diminished (Figure 2). Fractional receptor occupancy by native hGH was not accompanied by a change in the affinity of sites remaining available for binding with [^{125}I]hGH (Table IV). Each of these observations is inconsistent with the interpretation of an increased rate of loss of [^{125}I]hGH in the presence of native hGH on the basis of negative cooperativity.

In the growth hormone hepatocyte system, a rebinding mechanism (Silhavy et al., 1975; Donner et al., 1978) in which hGH diminished reassociation of label with available sites during dissociation is consistent with the equilibrium and kinetic data described above. Studies of the retention of labeled ligand by protein (Silhavy et al., 1975) have shown that sufficient binding site concentration or a diffusion barrier to completely free dissociation may explain changes in the course of hormone loss upon the addition of unlabeled ligand. An unstirred aqueous layer about the cell (Sha'afi et al., 1967) or compartmentalized (Kahn & Baird, 1978), internalized (Terris & Steiner, 1975), aggregated (Schlessinger et al., 1978), or cryptic sites (Cuatrecasas, 1971b) on cells may represent appropriate barriers to free diffusion within which rebinding could occur. Such a mechanism would predict that a decrease in the concentration of unbound [^{125}I]hGH about the cell would diminish rebinding. For the purpose of testing for rebinding directly, dissociation of [^{125}I]hGH from hepatocytes was assayed in systems containing free label [Figure 3 (top panel)] and those from which unbound [^{125}I]hGH had been removed [Figure 3 (bottom panel)]. In the system containing unbound [^{125}I]hGH, the course of dissociation is described by the equation

$$-d[HR]/dt = k_{-1}[HR] - k_1[H][R] \qquad (1)$$

The overall rate of loss of hormone results from dissociation of receptor-bound hormone (HR) and association (or reasso-

ciation) of free hormone (H) with available receptors (R). In the absence of free hormone, the time course of dissociation will be

$$-d[HR]/dt = k_{-1}[HR] \qquad (2)$$

As free hormone dissociates from the cell and accumulates in the medium, the state described by eq 1 will be approached. Application of native hormone to the system containing free label (eq 1) would prevent [^{125}I]hGH rebinding in direct relation to the extent of isotopic dilution of labeled by native hormone. The conditions described by eq 2 would then be approached. The lag prior to the effect of hGH on the time course of dissociation of [^{125}I]hGH from hepatocytes in the absence of unbound label was consistent with a time course during dissociation which was initially first order (eq 2). Accumulation of unbound label in the medium ultimately led to a higher order dissociation rate. Under such circumstances, native hGH was effective in altering the course of [^{125}I]hGH dissociation by maintaining conditions approximating a pseudo-first-order dissociation. The significance of rebinding or retention effects has been elegantly described elsewhere (Silhavy et al., 1975).

An inverse relationship between cellular responsiveness and the concentration of hormone to which target cells were exposed exists in a large number of peptide hormone systems (Lesniak & Roth, 1976; Hinkel & Tashjian, 1975; Gavin et al., 1974; Sharpe, 1976). Down regulation, decreased binding of labeled hormone, occurred subsequent to the incubation of receptor systems with native hormone. One mechanism through which receptor number may be regulated is by internalization of sites subsequent to hormonal exposure (Carpenter & Cohen, 1976; Catt et al., 1979). There is a rapid conversion of bound epidermal growth factor and insulin (Smith & McConnell, 1978; Schlessinger et al., 1978) from a mobile to a relatively immobile state. This is rapidly followed by internalization of bound ligand. As described in the accompanying paper (Donner et al., 1980), these studies were conducted under conditions (low temperatures and hormone concentrations and short incubation times) designed to minimize such internalization. The observation of accumulation of slowly dissociable [^{125}I]hGH in liver plasma membranes (not shown) lends assurance that a large portion of the hormone retention described resulted from agonist-mediated changes in receptor affinity.

Preincubation with hGH decreased the capacity of hepatocytes to subsequently bind [^{125}I]hGH. Receptor occupancy resulting from preincubation was calculated (Results). Occupancy of sites accounted for between 37 and 62% of the decrease in hepatocyte binding capacity. The fraction of diminished binding directly related to site occupancy increased as receptor saturation was approached. It is at such high hormone concentrations that desensitization of cellular responsiveness occurs (De Rubertis & Craven, 1976). It was of importance that cells had been obtained from animals devoid of hGH or rat growth hormone. Receptor sites were completely available at the initiation of these experiments. Other studies of peptide hormone receptor regulation have not allowed for the contribution of occupancy of sites by slowly dissociable endogenous hormone. $t_{1/2}$ values (~6.4 h) for loss of slowly dissociable hGH demonstrate that a wash period of ~25 h (4 half-lives of the slowly dissociable fraction) would be required to remove all cellularly bound hGH from the hepatocyte, e.g., from a nonhypophysectomized rat. Such an interval would permit comparison with cells not previously exposed to hGH. Extensive wash intervals would also be required to clear insulin (D. B. Donner, unpublished experi-
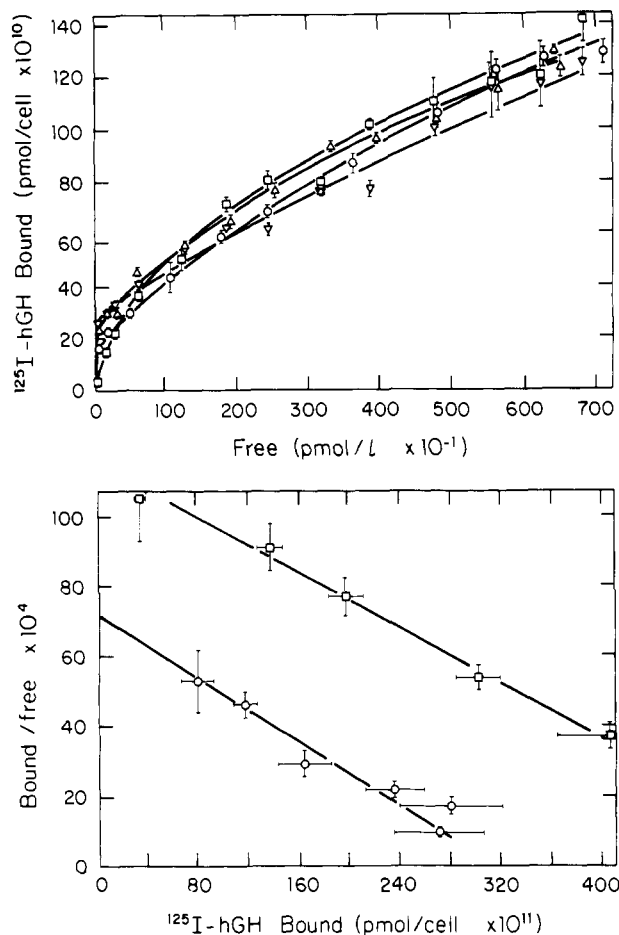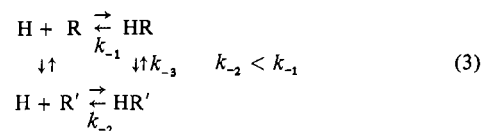
FIGURE 5: Saturation assay of hepatocyte suspensions preincubated and not preincubated with [^{125}I]hGH. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. The suspension was divided into four equal portions. One part served as a control; the others were incubated with [^{125}I]hGH (5.8 nM) for 20, 50, and 80 min, respectively. The suspensions were centrifuged (50g, 2 min) and the resultant cell pellets resuspended in fresh medium. Aliquots of each suspension were added to flasks containing varying concentrations of [^{125}I]hGH and medium. In a second set of flasks, hGH (2.5 μM) was added additionally. The flasks were shaken for 2 h, and triplicate 100-μL aliquots were taken for assay of binding. Aliquots of the incubation medium were assayed for the concentration of free hormone. A tracing of a computer-generated plot of the total [^{125}I]hGH bound per cell number vs. the free hormone concentration is shown (top panel) for the control (□) and 20- (O), 50- (△) and 80-min (▽) incubations. Nonspecific uptake was used to calculate specific binding data which are plotted according to Scatchard (bottom panel). The nonspecific binding used in this calculation represents radioactivity bound during the preincubation period and radioactivity bound in the presence of 2.5 μM hGH during the second binding period. The control (□) and 50-min (O) incubates are shown.

ments) and glucagon receptors on the isolated hepatocyte of endogenous hormone (Martin et al., 1978a,b). Fractional receptor occupancy may account for part of the inverse relationship between site number and applied hGH concentration.

Kinetic binding data suggested a number of mechanisms by which to account for the receptor-mediated retention of slowly dissociable [^{125}I]hGH by hepatocytes. Linear Scatchard plots obtained from growth hormone saturation experiments (Figure 4) were inconsistent with the presence of independent, heterogeneous sites. The increase in the proportion of slowly dissociable [^{125}I]hGH with time suggested that growth hormone binding sites interconvert between states of affinity. The proportion of rapidly dissociable label decreased as slowly dissociable hormone accumulated (Figure 2). A time-dependent increase in the proportion of receptors of higher af-

finity would result in hormonal retention by the cell. Increasing the association times (and receptor occupancy) (Figures 1 and 2) shifts the equilibrium initially favoring the lower affinity receptor state, R, to a condition favoring the higher affinity state, R' (eq 3).

$$
\begin{array}{c}
H + R \underset{k_{-1}}{\overset{\rightarrow}{\leftarrow}} HR \\
\downarrow\uparrow \qquad \downarrow\uparrow k_{-3} \qquad k_{-2} < k_{-1} \\
H + R' \underset{k_{-2}}{\overset{\rightarrow}{\leftarrow}} HR'
\end{array} \qquad (3)
$$

The linear Scatchard plots obtained from growth hormone saturation experiments do not suggest the presence of two sites in equilibrium with the medium. Since only ∼10% of the bound [^{125}I]hGH was rapidly dissociable after 2 h of incubation, the lower affinity receptor state could escape detection.

If dissociation of [^{125}I]hGH from the higher affinity receptor state is not favored relative to reversion to the occupied lower affinity state ($k_{-2} \ll k_{-3}$), a sequential model describes hormone binding

$$
H + R \underset{k_{-1}}{\rightleftharpoons} HR \underset{k_{-3}}{\rightleftharpoons} HR' \qquad (4)
$$
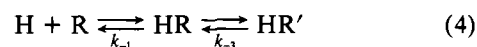
As only the lower affinity receptor state, R, is in equilibrium with the medium, a linear Scatchard plot is predicted by this model. Biphasic dissociation would occur if $k_{-1} \gg k_{-3}$. At the present time it is not possible to completely distinguish between the models described by eq 3 and 4, one being a limiting case of the other.

The sequential model (eq 4) explains an apparent inconsistency between the dissociation constants (K_D) obtained from equilibrium and kinetic measurements. The dissociation constant of the hGH receptor obtained from Scatchard analysis was ∼1 nM. The K_D for slowly dissociable [^{125}I]hGH was 1.3 × 10^{-10} M [k_1 = 3.89 × 10^5 M^{-1} s^{-1}, Figure 1 of accompanying paper; k_{-1} = 5.0 × 10^{-5} s^{-1}, Table I]. The K_D for rapidly dissociable label was 8.2 × 10^{-9} M (employing a dissociation rate of 3.2 × 10^{-3} s^{-1}, Table I). The apparent dissociation constant (K_{Dapp}) for [^{125}I]hGH is (assuming eq 4)

$$
K_{Dapp} = \frac{[H][R]}{[HR] + [HR']}
$$

K_{Dapp} is a summation of the dissociation constant (K_{D1}) for [^{125}I]hGH with its receptor, R, and an isomerization constant (K_{D2}) describing the equilibrium between HR and HR'. Then

$$
K_{D1} = k_{-1}/k_1 = [H][R]/[HR]
$$

$$
K_{D2} = k_{-3}/k_3 = [HR]/[HR']
$$

$$
K_{Dapp} = \frac{[H][R]}{[HR] + [HR]/K_{D2}} = \frac{K_{D1}K_{D2}}{1 + K_{D2}}
$$

At equilibrium ∼10% of the sites were in the lower affinity state from which label dissociated rapidly.

$$
K_{D2} = 0.11
$$

$$
K_{Dapp} = \frac{(8.2 \times 10^{-9}) \times 0.11}{1 + 0.11} = 8.1 \times 10^{-10} \text{ M}
$$

This value is in good agreement with the K_D obtained from Scatchard analysis (∼1 nM). Scatchard analysis presents an average affinity value, whereas kinetic analysis may be used to resolve a system into its component affinity states.

Correlations between cell surface binding and cellular activation (Birnbaumer & Rodbell, 1969; Cuatrecasas & Illiano, 1971; Cuatrecasas, 1971a,b, 1972; Rodbell, 1973; Gliemann et al., 1975) and desensitization (Shuman et al., 1976; Zor

al., 1976; De Rubertis & Craven, 1976; Bockaert et al., 1976) are numerous. Occupancy of sites accounts for part of the diminished binding of [^{125}I]hGH to hepatocytes from normal rats relative to hypophysectomized rats (D. B. Donner, unpublished results). Normal rats are less sensitive to hGH than are hypophysectomized animals in terms of the capacity of hormone to induce growth (Pecile & Muller, 1972). Receptor binding of glucagon mediates the activation of adenylate cyclase and cAMP accumulation in liver (Rodbell, 1973; Birnbaumer & Pohl, 1973; Birnbaumer et al., 1974; Sonne et al., 1978). The accumulation of slowly dissociable glucagon is related to the subsequent desensitization of adenylate cyclase to further hormonal stimulation (D. B. Donner, unpublished experiments). Acetylcholine (Weiland et al., 1977; Grunhagen et al., 1977; Quast et al., 1978), catecholamine (Ross et al., 1977; Lefkowitz & Williams, 1977), and other peptide hormone receptors (Ketelslegers et al., 1975) retain a slowly dissociable fraction of bound ligand subsequent to site occupancy. A two-state model (eq 3 or 4) in which one receptor class, is of lower affinity, and is active and a second receptor class is of higher affinity and is inactive has been viewed as functioning as a regulatory mechanism by which target cell response to neurotransmitters may be modulated (Katz & Thesleff, 1957; Rang & Ritter, 1970). The relationship between receptor-mediated changes in cellular sensitivity with processes related to hormonal internalization (Carpenter & Cohen, 1976), degradation (Terris & Steiner, 1975), and compartmentalization (Kahn & Baird, 1978) remains to be completely explored (Catt et al., 1979). However, in itself, the two-state model of peptide hormone action proposed represents an exquisitely sensitive mechanism through which target cell response may be modulated in inverse relation with endogenous hormone levels.

What has been demonstrated is that the interrelationship between peptide hormone in rapid and slow equilibrium with the medium is of fundamental importance in modulating receptor availability and binding properties. Thus, mechanisms regulating receptor binding and response to peptide hormones and neurotransmitters may be viewed as operating through similar molecular processes.

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