stoichiometry as a true one at the moment.

The rather complicated kinetic scheme required to simulate the 1:2 ratio with a true 1:1 stoichiometry will be fully justified only if we fail to observe the tetramer with any direct measurement in equilibrium conditions.

Acknowledgments

We thank the C.I.M.C.F. of the University of Naples for the access to the 270-MHz spectrometer. It is a pleasure to acknowledge the skillful technical assistance of M. R. Vaccaro and R. Turco of I.C.M.I.B.

References

Beato, M. (1976) *J. Steroid Biochem. 7*, 327–334.
Beato, M., & Baier, R. (1975) *Biochim. Biophys. Acta 392*, 346–356.
Beato, M., Arnemann, J., & Voss, H. J. (1977) *J. Steroid Biochem. 8*, 725–730.
Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Xavier, A. V. (1973) *J. Magn. Reson. 11*, 172.
Cohen, P., Griffin, J. H., Camier, M., Caizergues, M., Fromageot, P., & Cohen, J. S. (1972) *FEBS Lett. 25*, 282.
Crane-Robinson, C., Danby, S. E., Bradbury, E. M., Garel, A., Kovacs, A. M., Champagne, M., & Daune, M. (1976) *Eur. J. Biochem. 67*, 379.
De Marco, A., & Wüthrich, K. (1976) *J. Magn. Reson. 24*, 201.
Fridlansky, F., & Milgrom, E. (1976) *Endocrinology 99*, 1244–1251.
Handloser, C. S., Chakrabarty, M. R., & Moshev, M. W. (1973) *J. Chem. Educ. 50*, 510.
Markley, J. L. (1974) *Acc. Chem. Res. 8*, 70.
Markley, J. L., Williams, M. N., & Jardetzky, O. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 645.
Nieto, A., Ponstingl, H., & Beato, M. (1977) *Arch. Biochem. Biophys. 180*, 82–92.
Ponstingl, H., Nieto, A., & Beato, M. (1978) *Biochemistry 17*, 3908–3912.
Puigdomênech, P., & Beato, M. (1977) *FEBS Lett. 83*, 217–221.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Tanford, C. (1963) in *Physical Chemistry of Macromolecules*, p 528, Wiley, New York.

Characterization of the Slowly Dissociable Human Growth Hormone Binding Component of Isolated Rat Hepatocytes†

David B. Donner,* Jan Casadei, Louise Hartstein, Dwight Martin, and Martin Sonenberg

ABSTRACT: Human growth hormone (hGH) bound to specific sites on rat hepatocytes. The time course of hGH dissociation was comprised of more than one component. Dissociation was resolved into rapid ($t_{1/2}$ = 10.5 min) and slow ($t_{1/2}$ = 6.4 h) fractions. The amount of slowly dissociable hormone increased for the first 75 min during which time cells and [^{125}I]hGH associated. Subsequently, the amount of slowly dissociable hGH was constant. The time courses of hGH receptor binding and subsequent retention of slowly dissociable label were similar. The capacity of hepatocytes to accumulate slowly dissociable label was saturated by hGH over the same concentration range as the high-affinity binding site ($K_D \approx 2$ nM). This suggested that a receptor-mediated process was responsible for the accumulation of slowly dissociable hGH. Rapidly dissociable label was intact [^{125}I]hGH and fragments resulting from growth hormone degradation. Slowly dissociable hGH recovered from hepatocytes by acid extraction was intact and immunocompetent. There was a large increase in the extent of [^{125}I]hGH degradation between 23 and 37 °C. Over this temperature range, the proportion of hGH not in rapid equilibrium with the medium decreased. High concentrations of hGH decreased the amount of slowly dissociable [^{125}I]hGH retained by hepatocytes by competing for high-affinity sites. The interaction of [^{125}I]hGH with low-affinity degradative systems was favored by the presence of hGH. The temperature and concentration dependencies of hGH retention and degradation distinguished these processes.

Mechanistic descriptions of peptide hormone–receptor binding and subsequent cellular response have assumed that bound hormone was free to dissociate rapidly to the medium (Cuatrecasas, 1974; Kahn, 1976). Isolated rat hepatocytes accumulate a slowly dissociable human growth hormone (hGH)[1] binding fraction with increasing incubation time (Donner et al., 1978a). Some characteristics of the accumulation and retention of insulin (unpublished experiments) and glucagon (Martin et al., 1978a,b) are similar to those of hGH. This suggested that rapidly reversible equilibrium binding may not entirely describe peptide hormone–receptor interactions.

Retention of intact, receptor-bound epidermal growth factor (Schecter et al., 1978) and thyrotropin (De Rubertis et al., 1975) was required for persistent cellular response to hormone. Therefore, a first step toward evaluating the ultimate significance of cellularly retained hGH was to determine whether this fraction of bound label was intact and receptor bound. The slowly dissociable hGH binding fraction on hepatocytes

† From the Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York 10021. *Received September 19, 1979.* Supported in part by Grants AM 19846, AM 22121, AM 15773, and CA 08748 from the National Institutes of Health.

* Address correspondence to this author. D.B.D. is the recipient of a Research and Development Award from the American Diabetes Association.

[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; Cl_3AcOH, trichloroacetic acid.

0006-2960/80/0419-3293$01.00/0

from hypophysectomized rats was characterized. This permitted definition of the relationship between high-affinity receptor binding and hGH retention of slowly dissociable hormone in the absence of potential site occupancy by endogenous rat growth hormone. The integrity of bound label was assayed to determine whether intact hGH was retained. Equilibrium and kinetic measurements related saturable, high-affinity binding to accumulation of slowly dissociable hGH. The data obtained demonstrate that intact hGH is retained via a receptor-mediated process by hepatocytes for substantial time intervals and should be considered in mechanistic descriptions of hormone–receptor interactions.

Experimental Procedure

Materials

Hypophysectomized, male Sprague-Dawley rats (150–180 g, Charles River Laboratories) were fed Purina laboratory chow ad libitum. Rats were not used for at least 3 weeks after operation to ensure complete removal of the pituitary (based on lack of growth). After such times, blood was completely depleted of endogenous rat growth hormone. $Na^{125}I$ (carrier free) was purchased from New England Nuclear. Other materials and their sources were as follows: bovine serum albumin fraction V, fatty acid poor (Sigma); trichloroacetic acid (J. T. Baker); crude collagenase, Type I, from *Clostridium histolyticum* (125–200 units/mg) and hyaluronidase (300 USP units/mg, Worthington Biochemical Co.); Hank's balanced salt solution (HBSS), with and without calcium and magnesium, and trypan blue (Grand Island Biochemical Co.); chloramine-T (Matheson Coleman and Bell); Sephadex G-75 (Pharmacia).

Purified hGH was prepared by Dr. Brij Saxena (Cornell University Medical College) (Saxena & Henneman, 1966). hGH monomer was isolated by gel filtration and was used in all studies. This hormone possessed an activity of 1.0 IU/mg as determined by weight gain assay in the hypophysectomized rat (Donner et al., 1978b).

Methods

Human growth hormone was iodinated by an alteration of the method of Greenwood et al. (1963) and Lesniak et al. (1973). Hepatocytes were prepared by modification of the method of Berry & Friend (1969). Generally, (2–20) $\times 10^6$ cells were isolated per liver. As isolated in our laboratory, hepatocytes were greater than 90% viable as evaluated by trypan blue exclusion (Merchant et al., 1964), possessed a functional amino acid transport system, synthesized protein, and were hormonally responsive (Donner et al., 1978c). At the conclusion of association and saturation experiments hepatocytes were generally ~80% viable as assayed by trypan blue exclusion. The more extended time course of some dissociation experiments diminished viability to ~50%.

Binding Assay. The glassware used in all studies was siliconized to minimize cellular adhesion to glass surfaces. The standard binding assay (Rodbell et al., 1971) consisted of incubating hepatocytes at 23 °C in HBSS containing 1% albumin with [^{125}I]hGH. The incubation pH was 7.2 at the initiation and between 7.2 and 7.5 at the conclusion of assays. Hormone–hepatocyte incubates were shaken to ensure adequate interaction between the cells and the medium. Aliquots (100 μL) of the suspensions were removed from the incubation flask with an Eppendorf pipet and layered over 250 μL of a 5% sucrose solution in a 400 μL centrifuge tube (Beckman). This was centrifuged (1 min, 10000*g*) in a Beckman 152 microcentrifuge. The supernatant was aspirated from above the resultant cell pellet which was washed once with 250 μL of 5% sucrose solution. This was recentrifuged for 30 s to ensure that the cell pellet remained intact, and the solution was aspirated away. The tip of the microfuge tube containing the pelleted cells and labeled hormone was cut with a razor and placed in a γ vial for assay of ^{125}I. All samplings were conducted in triplicate.

Data generated employing radiolabeled hormone alone have been designated as "total" binding (Kahn, 1976). Such uptake contained receptor and nonreceptor cellular binding as well as adsorption to glass and plastic surfaces. In some instances, a high concentration ($\sim 10^{-6}$ M) of unlabeled hGH was additionally present. The component of radioactive ligand not displaced by high concentrations of native hormone was "nonspecifically" bound. Subtraction of nonspecifically bound hormone from the total radioactive uptake generated "specific" binding curves ($K_D \approx 10^{-10}$–10^{-9} M).

Dissociation of [^{125}I]hGH from Isolated Hepatocytes. Hepatocytes were incubated in HBSS containing 1% bovine serum albumin (23 °C, pH 7.4) with [^{125}I]hGH. The flasks were shaken for appropriate time intervals to permit hormone–hepatocyte interaction. In a number of instances unbound hGH was removed from the cell suspension prior to dilution to initiate dissociation, as described in the figure legends. The suspensions were added to beakers containing 1 L of HBSS and 0.1% albumin. This represented a 1:200 dilution of the reaction mixture. The solution was stirred to maintain a homogeneous distribution of cells in the dissociation medium. At timed intervals, 10 mL aliquots of dissociation medium were drawn into 10 mL disposable plastic hypodermic syringes through 14-gauge cannulas, placed in 16 × 100 mm disposable glass test tubes, and centrifuged (3 min, 1200*g*). The supernatants were removed from the cell pellets which 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 bound was expressed as a percentage of the zero time value.

Dissociation of [^{125}I]hGH from Hepatocytes after Various Association Times. Hepatocytes were incubated in HBSS containing 1% albumin with [^{125}I]hGH or [^{125}I]hGH + hGH. The flasks were shaken to ensure adequate mixing of cell suspension and medium. After various time intervals, 200 μL aliquots of the reaction suspension were removed with an Eppendorf pipet and placed in test tubes containing 5 mL of ice-cold medium. These were centrifuged, and the supernatants containing unbound hormone were aspirated from the cell pellets. The cells were resuspended in 10 mL of medium and transferred to reaction vessels that were shaken. Immediately after the resuspension, 400 μL aliquots of suspension were placed in microfuge tubes and centrifuged (10000*g*, 1 min). The supernatants were aspirated from the resultant cell pellets which were washed once with 5% sucrose and recentrifuged as described for the binding assay. Samples also were withdrawn for assay of binding at stated time intervals. Hormone binding was expressed as the concentration of [^{125}I]hGH bound per cell at any time. Each point represents the mean of triplicate determinations.

Trichloroacetic Acid Precipitability of Cellularly Bound [^{125}I]hGH. Hepatocytes were incubated in HBSS containing 1% albumin (pH 7.4) with [^{125}I]hGH. Binding was assayed as described above. The precipitability of cellularly associated label was determined by addition of 250 μL of a 10% solution of trichloroacetic acid to cell pellets isolated from the binding assay. The cell pellet was stirred with a fine wire rod until

it was well mixed with trichloroacetic acid. The microfuge tube was recentrifuged for 1 min, and the supernatant solution containing nonprecipitable label was siphoned off. The tip of each microfuge tube, containing the trichloroacetic acid precipitable label and cell pellet, was cut and placed in a glass tube for assay of ^{125}I.

Trichloroacetic Acid Precipitability of the Total Label. Aliquots of incubation medium (containing both free and cellularly bound label) were added to 1 mL of HBSS containing 1% albumin. One milliliter of trichloroacetic acid solution (10% w/v) was added to this to produce a protein precipitate. The solution was centrifuged (1100*g*, 3 min) in a desk top centrifuge. The supernatant was recovered from the resulting pellet of ^{125}I-labeled protein. The supernatant and precipitate were assayed for ^{125}I in a γ counter. The integrity of the protein was defined as the ratio of counts per minute in the precipitate divided by the total counts per minute in the precipitate and the supernatant.

Paper Electrophoresis. ^{125}I-Labeled hormone samples recovered from hepatocyte incubates were placed in the center of a strip of Whatman 3 MM chromatography paper (4 × 24 cm). The paper was moistened with 0.05 M phosphate buffer (pH 7.4) and placed in an electrophoresis chamber. Electrophoresis was carried out for 1.5 h at 250 V. The paper was cut into 1-cm sections and assayed for ^{125}I in a γ counter. Under the influence of the electric field, [^{125}I]hGH did not migrate whereas inorganic iodide did. The integrity of the peptide was estimated by the ratio of counts per minute remaining at the center of the strip divided by the total counts per minute applied to the strip.

Gel Filtration on Sephadex G-75. Label recovered from [^{125}I]hGH–hepatocyte incubates was applied to columns packed with Sephadex G-75 (0.5 × 26 cm). The columns were equilibrated and eluted with HBSS–1% BSA. Fractions of 0.5 mL were collected. Hormonal integrity was defined as the counts per minute eluting at the same volume as the [^{125}I]hGH standard divided by the total counts per minute eluted from the column.

Radioimmunoassay. Radioimmunoassay of hGH and [^{125}I]hGH was conducted by using antisera and a radioimmunoassay kit obtained from Schwarz/Mann.

Data Analysis. A PDP 11/70 computer (Digital Equipment Corp.) was used to fit hormone binding data to appropriate mathematical expressions by using nonlinear least-squares curve-fitting techniques (Bevington, 1969). Equilibrium data were fitted to a rearrangement of equations given by Tanford (1961) to describe states of multiple equilibria in which there exist noninteracting sites with different intrinsic affinities.

$$[HR] = \sum_{n=1}^{3} \frac{[H][R_0]_n}{[H] + K_n} \quad (1)$$

where $[R_0]_n$ = total concentration of receptor (occupied + unoccupied) belonging to binding site class n, K_n = equilibrium dissociation constant of receptors belonging to binding site class n, [H] = the concentration of free hormone in the system, and [HR] = the concentration of receptor-bound hormone. The above relationship allowed for a maximum of three independent, noninteracting classes of sites. The data were fitted to determine the floating parameters $[R_0]_n$ and K_n. Fitted data were plotted as [HR] vs. [H] and [HR]/[H] vs. [HR] according to Scatchard (1949). The least number of site classes which gave an acceptable fit to the experimental data was used in the final display of parameters and fitted curves.

Dissociation data were fitted to the equation

$$[HR]_{t=t}/[HR]_{t=0} = Ae^{-k_{-1}'t} + (1 - A)e^{-k_{-1}''t} \quad (2)$$

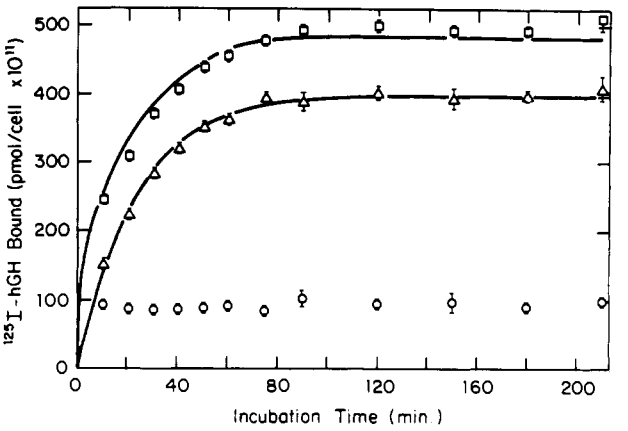

FIGURE 1: Association of [^{125}I]hGH with hepatocytes. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to flasks containing [^{125}I]hGH (0.79 nM) and medium. In a second flask, hGH (4.5 μM) was added additionally to compete for [^{125}I]hGH binding sites. The flasks were shaken and aliquots withdrawn at appropriate time intervals for assay of binding. In the computer-generated plot each point represents the mean of triplicate determinations. The total (□) and specific (△) binding curves are shown. Specific data were fit to eq 3 as described under Methods. The nonspecific points (○) were not connected as these data did not fit a bimolecular association function (eq 3, Methods).

where $[HR]_{t=0}$ = the concentration of hormone–receptor complex before the start of dissociation, $[HR]_{t=t}$ = the concentration of hormone–receptor complex at time = t of dissociation, A = fraction of receptor sites having a first-order dissociation rate constant of k_{-1}', and $1 - A$ = fraction of sites having a first-order dissociation rate constant of k_{-1}''. The program solved for the floating parameters A, k_{-1}', and k_{-1}''. While this relationship arbitarily resolved a two-site system, a one-site system was assumed to exist when k_{-1}' and k_{-1}'' overlapped within their standard deviations or when A or $1 - A$ was a very small fraction.

The association of [^{125}I]hGH with hepatocytes was fitted to the solution of the differential equation

$$d[HR]/dt = [H][R]k_1 - [HR]k_{-1} \quad (3)$$

where k_1 and k_{-1} are the association and dissociation rate constants, respectively. The solution of this equation is (Rodbard & Weiss, 1973)

$$[HR]_t = P(1 - e^{-Bk_1t})/[(P/Q) - e^{-Bk_1t}] \quad (4)$$

where $P = a + (a^2 - b^2)^{1/2}$, $Q = a - (a^2 - b^2)^{1/2}$, $B = P - Q = 2(a^2 - b^2)^{1/2}$, $a = (1/2)([H_0] + [R_0] + k_{-1}/k_1)$, and $b = [H_0][R_0]$. The limiting value of [HR] with increasing time was Q. The fitted parameters obtained were k_1, k_{-1}, and $[R_0]$.

Tracings of computer-generated plots of binding data are presented, as indicated in the figure legends. Error bars represent standard deviations of triplicate determinations.

Results

[^{125}I]hGH and native hGH compete for binding sites on hepatocytes (Donner et al., 1978b,c). Half-maximal binding of 1.34 nM [^{125}I]hGH was observed at an applied concentration of 1.8 nM hGH. This confirmed the near identity of the labeled and native hormone molecules and permitted derivation of specific binding curves. The association of [^{125}I]hGH (0.79 nM) with hepatocytes was initially rapid (Figure 1). An apparent steady state was obtained after ~75 min of incubation and was retained for at least 2 h. Greater than 80% of the total radioactive uptake was displaceable by high concentrations of native hGH and was therefore con-

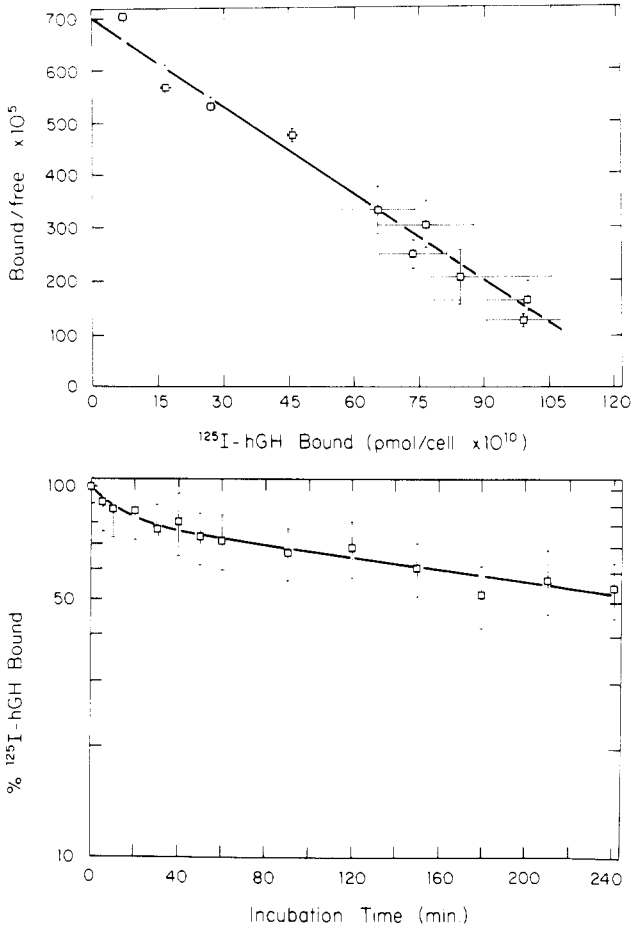

FIGURE 2: (Top panel) Binding of [^{125}I]hGH to hepatocytes as a function of growth hormone concentration. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (varied concentrations) and medium. In a parallel set of flasks, hGH (7.5 μM) was added additionally to compete for [^{125}I]hGH binding sites. The flasks were shaken for 2 h and aliquots taken for assay of binding. A computer-generated Scatchard plot of the specific binding is shown. Data were fitted to eq 1 as described under Methods. Each point represents the mean of triplicate determinations. (Bottom panel) Dissociation of [^{125}I]hGH from hepatocytes. Hepatocytes were equilibrated in Hank's balanced salt solution–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (1.3 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 45 min. The suspensions were centrifuged (30 s, 1500g), 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. Dissociation was then assayed as described under Methods. Each point represents the mean of triplicate determinations.

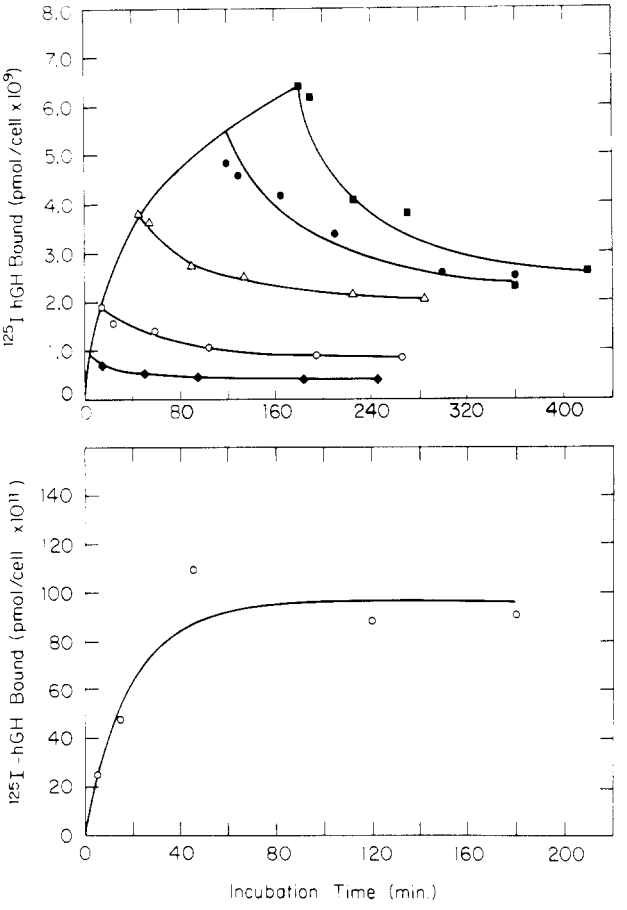

FIGURE 3: (Top panel) Dissociation of [^{125}I]hGH from hepatocytes after various periods of incubation. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 15 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (1.99 nM) and medium. In a second set of flasks, hGH (2.6 μM) was added additionally. The flasks were shaken, and after varied incubation times dissociation was assayed as described under Methods. The total dissociation curves after 5 (♦), 15 (○), 45 (△), 120 (●) and 180 (■) min of incubation are shown. For clarity, the zero time dissociation points were connected. Each point represents the mean of triplicate determinations. (Bottom panel) The course of the retention of hGH by hepatocytes. [^{125}I]hGH was permitted to dissociate (4 h) from hepatocytes after varied periods of association as described for the upper panel. The concentration of hormone remaining bound after dissociation was corrected for the loss of slowly dissociable label. The dissociation rate (3.0×10^{-5} s^{-1}) of the slowly dissociable component was used to determine the amount of hormone bound prior to dissociation. This method of analysis did not assume a constant ratio of rapidly to slowly dissociating hGH. The data obtained were fitted to a second-order rate equation (eq 3). In the computer-generated plot each point represents the accumulation of slowly dissociable hGH at any time, t, of association.

sidered specific. On the assumption of a bimolecular interaction between [^{125}I]hGH and the hepatocyte (eq 3), a rate constant of 3.89×10^5 M^{-1} s^{-1} was defined from the specific binding curve.

The specific binding of [^{125}I]hGH to hepatocytes was saturable with respect to growth hormone concentration. The data yielded linear Scatchard plots (Scatchard, 1949) (Figure 2, top panel) suggestive of a single class of sites in equilibrium with the medium. Nonlinear regression analysis of saturation experiments defined 7000–10 000 sites/cell with a K_D of 0.8–2.0 nM. The course of growth hormone dissociation from hepatocytes was fit (eq 2) to the presence of more than a single kinetic process (Figure 2, bottom panel). Dissociation curves were resolved into rapidly and slowly dissociating components by nonlinear regression analysis. The rate constants for the loss of rapidly and slowly dissociating label were $(1.1 \pm 2.0) \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 10.5 min) and $(3.0 \pm 1.7) \times 10^{-5}$ s^{-1} ($t_{1/2}$ = 6.4 h), respectively.

Dissociation of [^{125}I]hGH from hepatocytes was initiated after varied periods during which hormone and hepatocytes were permitted to associate (Figure 3, top panel). After each association interval, the subsequent course of dissociation was biphasic. Knowing the concentration of slowly dissociable [^{125}I]hGH remaining bound after 4 h of dissociation after any period of association and the rate of loss of slowly dissociable hormone, the concentration of slowly dissociable hGH bound prior to the onset of dissociation was calculated (legend to Figure 3, bottom panel). The amount of slowly dissociable hGH increased for the first 75 min of association. Subse-

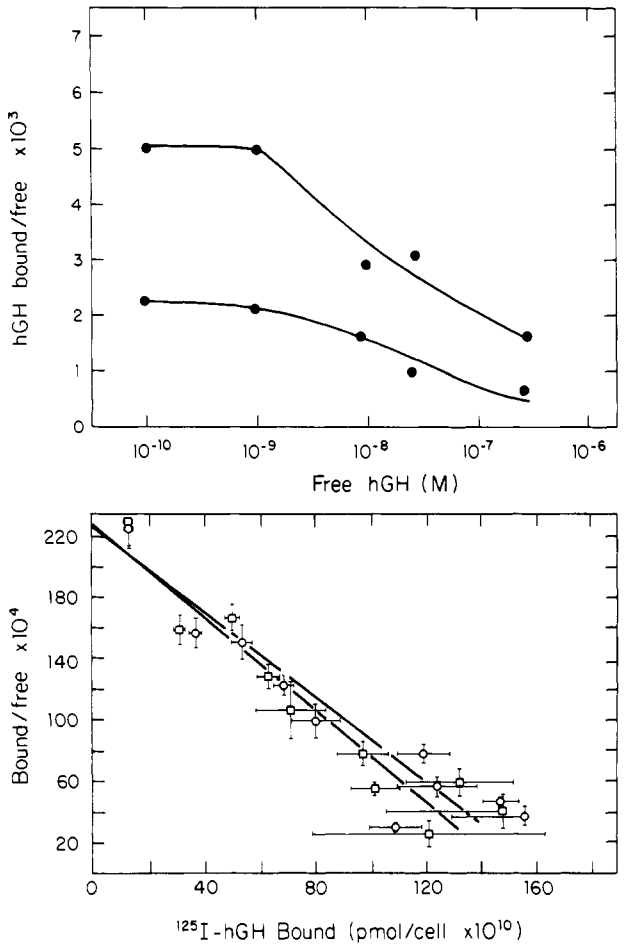

FIGURE 4: (Top panel) Retention of [^{125}I]hGH by hepatocytes as a function of hormone concentration. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 20 min at 10 and 30 °C. Aliquots of the cell suspension were added to incubation flasks containing increasing concentrations of growth hormone ([^{125}I]hGH + hGH). The suspensions were shaken (2.5 h, 10 °C; 30 min, 30 °C), aliquots removed and centrifuged (1100*g*, 3 min), and the resultant cell pellets resuspended in fresh medium. The hepatocytes were diluted 50-fold relative to their initial concentration to promote dissociation of bound hormone. Dissociation continued for 4 h, after which binding was assayed after separation of medium from the cells by centrifugation. The concentration of bound divided by free growth hormone is plotted against the free hormone concentration at 10 °C (upper curve) and 30 °C (lower curve). Each point represents the mean of triplicate determinations. (Bottom panel) Saturation of growth hormone hepatocyte binding at 10 and 30 °C. Hepatocytes were equilibrated in HBSS–1% albumin for 20 min at 10 and 30 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (varied concentrations) and medium. In a parallel set of flasks, hGH (7.5 μM) was additionally present to compete for [^{125}I]hGH binding sites. The flasks were shaken for a sufficient time to permit equilibration (2.5 h, 10 °C; 30 min, 30 °C) and aliquots taken for assay of binding. Scatchard plots of specific binding at 10 °C (○) and 30 °C (□) are shown. Data were fit to eq 1 as described under Methods. Each point represents the mean of triplicate determinations.

quently, the amount of slowly dissociable hormone was invariant. Slowly dissociable [^{125}I]hGH accumulated on hepatocytes with a time course similar to that of high-affinity binding of hormone (compare Figures 1 and 3).

Hepatocytes were exposed to increasing concentrations of growth hormone (hGH + [^{125}I]hGH) in a saturation assay at 10 and 30 °C. After equilibration, the cells were suspended in fresh, hormone-free medium to promote dissociation of bound label. The highest concentrations of applied hGH ($\sim 10^{-7}$ M) were sufficient to saturate high-affinity sites with the properties suggested by the Scatchard plot in Figure 2 ($K_D \approx 2$ nM). In Figure 4 (top panel) the growth hormone remaining bound after 4 h of dissociation (*B*) divided by the free hormone concentration (*F*) is plotted against the amount of hormone applied over a wide concentration range. At equilibrium, less slowly dissociable hormone was retained at 30 °C than at 10 °C. The number and affinity of [^{125}I]hGH binding sites did not vary over this temperature range (Figure 4, bottom panel). Elevated temperature favored rapid dissociation of growth hormone by increasing the proportion of sites in rapid equilibrium with the medium (not shown). This accounted for the diminished amount of slowly dissociable hormone observed at higher temperatures. At 10 and 30 °C, the capacity of hepatocytes to accumulate slowly dissociable label was saturated by hGH over the same concentration range as the high-affinity hormone binding site.

Table I: Recovery of [^{125}I]hGH from Hepatocytes[a]

incubn procedure	hormonal integrity (%) compared with unreacted [^{125}I]hGH assay procedure: Cl_3AcOH pptn	gel filtration	paper electrophoresis	radio immunoassay
[^{125}I]hGH + cells, 10 °C	82	83	76	78
[^{125}I]hGH + cells, 23 °C	84	69	72	90
[^{125}I]hGH + cells, 37 °C	41	29	66	33

[a] Hepatocytes were equilibrated to the indicated temperature in HBSS–1% bovine serum albumin for 20 min. Aliquots of cell suspensions were added to temperature-equilibrated flasks containing [^{125}I]hGH (6.8 nM) and medium. The flasks were shaken at 10, 23, and 37 °C for 2 h. The suspensions were centrifuged (100*g*, 2 min), and the cell pellets were resuspended in fresh medium and recentrifuged twice to remove unbound label. The cells were resuspended in medium containing hGH (15 μM) which prevented rebinding of label released during dissociation. The suspensions were incubated at their respective temperatures (2 h), after which label in the medium was recovered and assayed as described under Methods. Data are expressed as hormonal integrity as a percentage of the noncellularly treated [^{125}I]hGH controls.

[^{125}I]hGH recovered from hepatocytes by dissociation into hormone-free medium at 10, 23, and 37 °C was degraded (Table I). There was a large increase in the extent of growth hormone degradation between 23 and 37 °C. Label released to the medium rapidly was both intact and degraded [^{125}I]-hGH. At 37 °C, 50–70% of the label recovered from the medium of hepatocyte incubates was intact [^{125}I]hGH after 30 min. Subsequently, low concentrations of degraded hormone were released to the medium. After 4 h ~40% of the recovered label was [^{125}I]hGH (not shown). [^{125}I]hGH bound to hepatocytes was also recovered by dissociation into acidic medium (Table II). At 10 and 23 °C there was an ~10% loss of integrity relative to noncellularly exposed [^{125}I]hGH. At 37 °C, degradation was slightly more extensive, representing an ~14% loss relative to control.

The precipitability of bound label (total system) was assayed during dissociation (Figure 5). The precipitability of label remaining bound 2 min after the initiation of dissociation was less than that of [^{125}I]hGH not exposed to hepatocytes (indicated at the zero time point, Figure 5). Precipitability increased subsequently and ultimately was at least that of control [^{125}I]hGH. Label bound in incubates containing excess native hGH (nonspecific system) was less precipitable than that in those incubates containing much lower concentrations of [^{125}I]hGH alone. Subsequent to the 2-min time point, the precipitability of cellularly associated label increased. After

Table II: Recovery of [^{125}I]hGH from Hepatocytes by Dissociation into Acidic Medium[a]

incubn procedure	hormonal integrity (%) compared with unreacted [^{125}I]hGH assay procedure			
	Cl_3AcOH pptn	gel filtration	paper electrophoresis	radioimmunoassay
[^{125}I]hGH + cells, 10 °C	92	92	94	114
[^{125}I]hGH + cells, 23 °C	90	89	92	84
[^{125}I]hGH + cells, 37 °C	86	85	91	73

[a] The procedure was as described in footnote *a* of Table I with the exception that dissociation was effected by finally resuspending washed cell suspensions in isotonic 0.1 N HCl for 10 min at room temperature. Supernatant solutions were assayed as described under Methods. The data are expressed as hormonal integrity as a percentage of the noncellular treated [^{125}I]hGH controls.

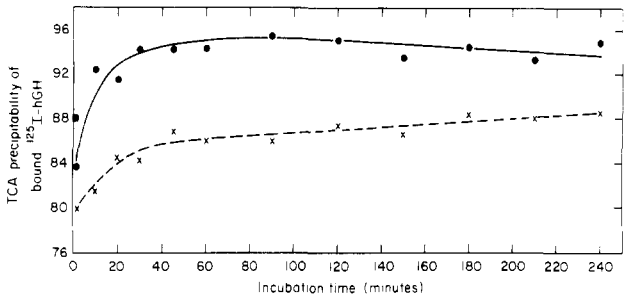

FIGURE 5: Trichloroacetic acid precipitation of bound [^{125}I]hGH during dissociation. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 20 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (2.3 nM) and medium. In a second flask, hGH (3.3 μM) was added additionally. The flasks were shaken for 2 h, the suspensions centrifuged (1000*g*, 2 min), and the resultant cell pellets suspended in ice-cold medium (10 mL) and recentrifuged. The pellets were finally suspended in medium (5 mL, 23 °C) and added to beakers containing HBSS–0.1% bovine serum albumin (1 L). Dissociation was then assayed as described under Methods. Trichloroacetic acid solution (1 mL, 10% w/v) was added to cell pellets recovered from the dissociation assay which were dispersed by agitation. The tubes were recentrifuged and the supernatants containing trichloroacetic acid soluble label were aspirated. The precipitability of incubates containing [^{125}I]hGH alone (●) and [^{125}I]hGH + hGH (×) was determined by dividing the counts per minute in the precipitated pellets by the counts per minute in pellets not exposed to trichloroacetic acid. The symbol at the zero time (■) point represents the precipitability of [^{125}I]hGH prior to incubation with hepatocytes. Each point is the mean of triplicate determinations.

4 h, nonspecifically bound [^{125}I]hGH was as precipitable as labeled hormone not exposed to hepatocytes.

The precipitability of bound and free label was assayed during dissociation of [^{125}I]hGH from hepatocytes (Figure 6). During association the precipitability of label in the medium gradually decreased. After 60 min, the precipitability of free label in an isotopically diluted system was greater than that of label in a flask containing [^{125}I]hGH alone. After 10 min, the precipitability of bound label was less than that of [^{125}I]hGH not exposed to hepatocytes. After 20 min, precipitability in the total system increased until it was at least equal to that of noncellularly exposed [^{125}I]hGH. The precipitability of bound label in isotopically diluted incubates was less than that when lower concentrations of [^{125}I]hGH alone were present. After 90 min, the precipitability of label in the isotopically diluted incubates approached that in the total system.

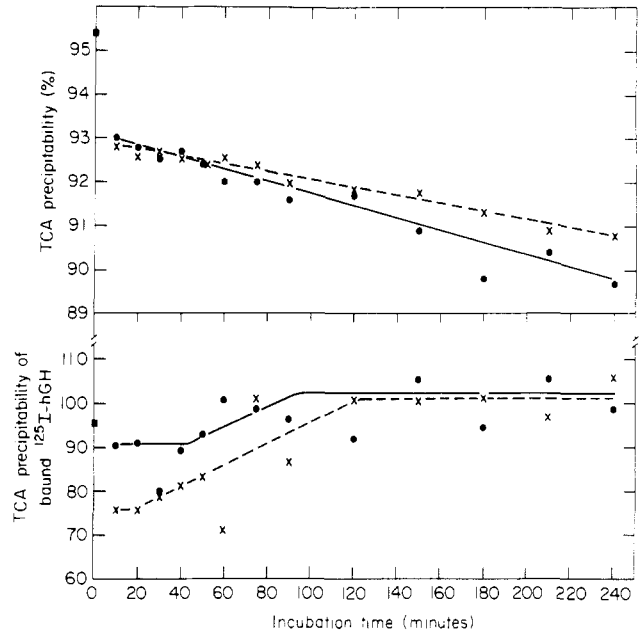

FIGURE 6: Precipitability of [^{125}I]hGH during association with hepatocytes. Hepatocytes were equilibrated in HBSS–1% bovine serum albumin for 20 min at 23 °C. Aliquots of the cell suspension were added to incubation flasks containing [^{125}I]hGH (1.8 nM) and medium. In a second flask, hGH (0.9 μM) was added additionally. Aliquots were taken at appropriate time intervals for assay of bound and free [^{125}I]hGH precipitability as described under Methods. Each point represents the mean of duplicate determinations. The precipitability of bound (lower panel) and free (upper panel) label in systems containing [^{125}I]hGH alone (●) and [^{125}I]hGH + hGH (×) is shown. The symbol at the zero time point (■) represents the precipitability of [^{125}I]hGH prior to incubation with hepatocytes.

Discussion

Hepatocytes contain a high-affinity binding site which displays the specificity (Donner et al., 1978b; Ranke et al., 1976), equilibrium, and kinetic binding properties expected of a growth hormone receptor (Donner & Sonenberg, 1975; Donner et al., 1978a). [^{125}I]hGH associated rapidly with the hepatocyte. The rate of [^{125}I]hGH binding (3.89×10^5 M^{-1} s^{-1}) was similar to that of other peptide hormones with a number of tissues (Kahn, 1976). The number and affinity of sites derived from Scatchard plots of saturation data [Figures 2 (top panel) and 4 (bottom panel)] were consistent with the characteristics of growth hormone binding to liver membranes (Herington et al., 1976), hepatocytes (Ranke et al., 1976), and a variety of tissues (Arrenbrecht, 1974; Lesniak et al., 1974).

Previous study of the hGH–hepatocyte interaction demonstrated that a slowly dissociable hormone binding component increased with incubation time (Donner et al., 1978a). Since slowly dissociable label comprised a significant portion of the cellularly associated hormone, the properties of this fraction of bound label were characterized. Slowly dissociable hGH accumulated on hepatocytes at physiological concentration (~2 nM, Figure 3). Initiation of dissociation after varied incubation times during which [^{125}I]hGH and hepatocytes associated resulted in courses of dissociation best fit to the presence of more than a single kinetic process. The similar time courses of growth hormone receptor binding (Figure 1) and accumulation of slowly dissociable hormone suggested that these processes were related to one another.

The slowly dissociable growth hormone binding fraction on hepatocytes was saturable with respect to hormone concentration (Figure 4). This was indicated by a decreasing ratio of slowly dissociable bound hormone divided by free hormone in incubates to which increasing growth hormone concentra-

tions were added. A limiting B/F ratio was approached between 10^{-8} and 10^{-7} M hGH. The high-affinity, low-capacity growth hormone binding site described above [Figures 2 (top panel) and 4 (bottom panel)] was 80–97.5% saturated over this concentration range. The capacity of hepatocytes to accumulate a slowly dissociating fraction of bound growth hormone was saturated over the same concentration range required to fully occupy the hGH receptor. Thus, the equilibrium and kinetic binding properties characterizing the interaction of [^{125}I]hGH with high-affinity sites and accumulation of slowly dissociable hormone suggest that the same receptor mediated both processes.

Label was recovered from acid extracts of hepatocyte incubates at 10, 23, and 37 °C (Table II). After 2 h of association, ~90% of the bound label dissociated slowly from hepatocytes. The wash to which hepatocyte suspensions were subjected prior to acid extraction removed most of the rapidly dissociable label. The acid extract was comprised almost completely of slowly dissociable hormone. The precipitability of bound label increased with the proportion of slowly dissociable label. The label ultimately retained was at least as precipitable as the [^{125}I]hGH applied to hepatocytes and rebound to fresh cell suspensions as well as noncellularly exposed hormone (not shown). The slowly dissociable binding fraction was intact, immunocompetent hGH.

The isolation of intact, immunoreactive, slowly dissociable [^{125}I]hGH suggested that processes leading to hormone retention and degradation were distinguishable. Growth hormone degradation increased with temperature. A significant component of hGH metabolism was probably mediated by internalization of bound label (Terris & Steiner, 1975) as some enzymes associated with peptide hormone degradation are intracytoplasmic (Hammond & Jarett, 1975; Brush, 1971; Ansorge et al., 1971, 1973), and the extent of endocytosis increases greatly between 23 and 37 °C in mammalian cells (Silverstein et al., 1977). Degradation observed at lower temperatures may have occurred at the cell membrane (Crofford et al., 1972; Varandani & Nafz, 1976). Whereas degradation increased with temperature, the proportion of slowly dissociable hGH decreased between 10 and 30 °C. The different temperature dependencies of hormone retention and degradation suggested that they were mediated through different cellular processes.

High concentrations of native hGH diminished the precipitability of bound label during association and dissociation of [^{125}I]hGH from hepatocytes. This may have been accomplished by minimizing the interaction of [^{125}I]hGH with high-affinity sites, thereby favoring uptake by lower-affinity degradative systems. The observation of [^{125}I]hGH degradation under conditions where high-affinity binding was competitively inhibited by isotopic dilution with hGH suggested that at least part of the hormone degradation was not mediated at the receptor. The accumulation of most slowly dissociable [^{125}I]hGH was saturable at low hormone concentrations (10^{-8} M). [^{125}I]hGH degradation occurred over a broad concentration range (10^{-9}–10^{-6} M). The concentration dependencies of the accumulation of slowly dissociable hormone and degradation distinguished these processes.

Plasma membrane proteins may be internalized at a greater rate than they are degraded (Tweto & Doyle, 1977). Intact, internalized proteins may therefore accumulate intracytoplasmically during the degradative process. Intact insulin has been localized to the cytoplasmic compartment of adipocytes (Kono & Suzuki, 1979; Kahn & Baird, 1978). Intracytoplasmic slowly dissociable growth hormone may therefore accumulate with a time course similar to that described in Figure 4 (top panel) prior to degradation. It has been demonstrated with insulin that all cellularly accumulated label, whether receptor bound or not, was subject to degradation (Terris & Steiner, 1975). [^{125}I]hGH was degraded by hepatocytes over a wide concentration range, suggesting that most degradation was not receptor mediated and was related to a low-affinity process. We have chosen incubation conditions designed to minimize such uptake. At low temperatures and hormone concentrations interaction with a high-affinity saturable site was related to accumulation of slowly dissociable [^{125}I]hGH. Slowly dissociable [^{125}I]hGH accumulated on rat liver plasma membranes with a time course similar to that in Figure 4 (top panel) (not shown). While some intracytoplasmic uptake of [^{125}I]hGH may occur under the conditions described, changes in receptor binding properties at the exterior surface of the hepatocyte undoubtedly account for much of the hormone retention. It will be important to distinguish changes in receptor affinity from internalization as the former has been directly related to the onset and offset of biologic response to peptide hormones (Cuatrecasas, 1972; Rodbell, 1973) and the latter to hormone degradation (Terris & Steiner, 1975).

It is noteworthy that slowly dissociable insulin (unpublished results) and glucagon (Martin et al., 1978a,b) accumulate on hepatocytes subsequent to receptor binding. The physical process described here may therefore be related to a binding phenomenon of some generality. Previous studies of hormone binding and regulation of cellular response have emphasized the presence of hormone in rapid equilibrium with the medium. This study suggests that the fraction of bound hormone not in rapid equilibrium with the medium may also be of fundamental importance.

Acknowledgments

We thank Dr. J. McDonald, R. Reiman, and M. Mandel of the Sloan-Kettering Institute Department of Biophysics and Core Computer Facility for assistance in the development of the computer program described.

References

Ansorge, S., Bohley, P., Kirschke, H., Langner, J., & Hanson, H. (1971) *Eur. J. Biochem. 19*, 283.

Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Wiederanders, B., & Hanson, H. (1973) *Eur. J. Biochem. 32*, 27.

Arrenbrecht, S. (1974) *Nature (London) 252*, 255.

Berry, M. N., & Friend, D. S. (1969) *J. Cell Biol. 43*, 506.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Brush, J. S. (1971) *Diabetes 20*, 140.

Crofford, O. B., Rogers, N. L., & Russell, W. G. (1972) *Diabetes 21*, 403.

Cuatrecasas, P. (1972) *Diabetes 21*, Suppl. 2, 396.

Cuatrecasas, P. (1974) *Annu. Rev. Biochem. 43*, 169.

De Rubertis, F. R., Chayoth, R., Zor, U., & Field, J. B. (1975) *Endocrinology 96*, 1579.

Donner, D. B., & Sonenberg, M. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 274.

Donner, D. B., Martin, D. W., & Sonenberg, M. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 672.

Donner, D. B., Nakayama, K., Tani, S., Lutz, U., & Sonenberg, M. (1978b) *J. Biol. Chem. 253*, 6717.

Donner, D. B., Nakayama, K., Lutz, U., & Sonenberg, M. (1978c) *Biochim. Biophys. Acta 507*, 322.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114.

Hammond, J. M., & Jarett, L. (1975) *Diabetes 24*, 1011.
Herington, A. C., Phillips, L. S., & Daughaday, W. H. (1976) *Metabolism 25*, 341.
Kahn, C. R. (1976) *J. Cell Biol. 70*, 261.
Kahn, C. R., & Baird, K. (1978) *J. Biol. Chem. 253*, 4900.
Lesniak, M. A., Roth, J., Gorden, P., & Gavin, J. R., III (1973) *Nature (London), New Biol. 241*, 20.
Lesniak, M. A., Gorden, P., Roth, J., & Gavin, J. R., III (1974) *J. Biol. Chem. 249*, 1661.
Martin, D. W., Sonenberg, M., & Donner, D. B. (1978a) *Diabetes 27*, Suppl. 2, 491.
Martin, D. W., Sonenberg, M., & Donner, D. B. (1978b) *Abstracts, 60th Annu. Meet. Endocr. Soc., Miami*, 161.
Merchant, D. J., Kahn, R. H., & Murphy, W. H. (1964) *Handbook of Cell and Organ Cultures*, 2nd ed., Burgess Publishing Co., Minneapolis, MN.
Ranke, M. B., Stanley, C. R., Rodbard, D., Baker, L., Bongiovanni, A., & Parks, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 847.
Rodbard, D., & Weiss, G. H. (1973) *Anal. Biochem. 52*, 10.
Rodbell, M. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1854.
Rodbell, M., Kraus, M. J., Pohl, S. L., & Birnbaumer, L. J. (1971) *J. Biol. Chem. 246*, 1861.
Saxena, B. B., & Henneman, P. H. (1966) *Biochem. J. 100*, 711.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Schecter, Y., Hernaez, L., & Cuatrecasas, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5788.
Silverstein, S. C., Steinman, R. M., & Cohn, Z. A. (1977) *Annu. Rev. Biochem. 46*, 669.
Suzuki, K., & Kono, T. (1979) *J. Biol. Chem. 254*, 9786.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.
Terris, S., & Steiner, D. F. (1975) *J. Biol. Chem. 250*, 8389.
Tweto, J., & Doyle, D. (1977) in *The Synthesis, Assembly and Turnover of Cell Surface Components* (Poste, G., & Nicolson, G. L., Eds) Elsevier/North-Holland Biomedical Press.
Varandani, P. T., & Nafz, M. A. (1976) *Diabetes 25*, 173.

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

David B. Donner‡

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.

† From the Memorial Sloan-Kettering Cancer Center and the Cornell University Graduate School of Medical Sciences, New York, New York 10021. *Received September 19, 1979.* Supported in part by grants AM 19846, AM 22121, AM 15773, and CA 08778 from the National Institutes of Health.
‡ D.B.D. is the recipient of a Research and Development Award from the American Diabetes Association.

[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.

0006-2960/80/0419-3300$01.00/0