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Optimization of a Radioreceptor Assay for Human Growth Hormone Using Male Rat Liver Membranes[1,2,3] (41359)

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Abstract. Previous investigators have reported that specific somatogenic receptors are present in liver membrane preparations from male rats. However, specific binding was less than 3% of total counts per minute added, and the radioreceptor assay using such receptor preparations was impractical to use. Livers from female rats bound 25% of ^{125}I-labeled human growth hormone added, but binding was not specific in that both prolactin and growth hormone competed. Starting with membrane preparations from male rats, we have systematically studied and improved (a) preparation of the fraction, (b) the method of separation of bound and free, (c) time, temperature, and conditions of incubation, (d) the amount of membrane preparation and treatment of rats before sacrifice, and (e) the quality of the labeled growth hormone. As a result, specific binding increased from less than 3 to 60%. This is offered as a model system for optimization of radioreceptor assay systems. The data indicate that ^{125}I-hGH binding to male rat liver membranes was low, as previously reported, because the preparation and assay system had not been optimized, and not because of an inadequate number of receptors in the male liver.

Binding of protein hormones to specific membrane receptors is the first step in hormone action. Receptor binding is a reversible and saturable process of high affinity, with the amount of binding dependent upon the amount and properties of hormone and receptor preparation, as well as the environment in which association and dissociation occurs. For growth hormone receptor interaction, the effect of species, age, sex, and gestation or gestational hormones has been described for many tissues.

However, a variety of other factors, including the means of and medium for preparation of membrane receptors, the time and temperature of incubation, the amount of receptor protein, the presence or absence of cations (especially Ca^{2+} and Mg^{2+}), the quality of the radioactive labeled hormone, and the means used to separate bound from free hormone, may affect binding and need to be optimized[4] for any assay system.

Friesen *et al.* (1–2) have noted that liver receptor preparations from male rats bound human growth hormone (hGH) specifically, but that maximum binding was only 1–3% per 150 μg membrane protein. Under the same conditions, liver receptor preparations from female rats bound 25.3%, but this binding was not specific; an excess of either hGH *or prolactin* competitively displaced labeled hGH. The investigators concluded that these hGH binding sites in females were predominantly lactogenic sites, not somatogenic. From these studies, it has been concluded that receptor preparations from male rat livers contain predominantly somatogenic binding sites, while those from female or pregnant female rats contain predominantly lactogenic binding sites (1–3).

Since specific binding was so low (less

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[3] These studies were performed during a sabbatical leave of Dr. Odell at the University of Auckland.

[4] "Optimize"—to obtain the "optimum" conditions for utilizing those receptors present in the liver; in a sense, finding a method in which the maximum number of receptors present in the liver *in vivo* could be utilized, and developing the best possible incubation conditions with which to do this.

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than 3%), however, useful receptor assays for hGH could not be prepared from male rat liver.

We report herein that hGH receptor binding was low, not because sufficient binding sites were unavailable, but because the preparative and assay systems had not been optimized. Taking hGH binding to male rat liver as a model system, we have systematically optimized preparation with the result that specific hGH binding increased from <3 to 60%.

Materials and Methods. Immunochemically pure human GH (hGH) was kindly supplied by George Chapman, Ph.D., through the New Zealand National Hormone Laboratory. This hGH had a potency by bioassay of 2.5–3 IU/mg by tibial cartilage bioassay. This material was used both to prepare ^{125}I-hGH and as unlabeled hGH laboratory standard.

Bovine serum albumin (fraction V) was obtained from Sigma, St. Louis, Missouri. All other chemicals were reagent grade.

Method of tissue fractionation. For all studies, livers were removed from male Sprague–Dawley rats weighing 200–300 g immediately after the animals were sacrificed by head blow. For the first studies, they were placed directly in 0.01 *M* phosphate-buffered saline containing 0.15 *M* NaCl at 4° in a ratio of one gram tissue to 4 ml buffer. In later experiments, 0.3 *M* sucrose was evaluated and used in the same tissue–volume ratio. The liver was homogenized at 4° using a Brinkman polytron homogenizer at speed setting 4–5 for 15–30 sec. The homogenate was then centrifuged at 1500*g* for 15 min at 4° to remove cell debris. The resulting supernatant was decanted and recentrifuged at 30,000*g* for 30 min and the final membrane pellet resuspended in 0.01 *M* phosphate buffer to give a ratio of 5 g initial wet weight of liver to 10 ml of membrane preparation. Analysis showed the 30,000*g* supernatant to contain less than one-fifth of the specific binding activity of the pellet. A 25-μl aliquot of receptor preparation was solubilized with 75 μl of distilled H_2O + 0.3 ml of IN NaOH, and was boiled for 30 min. Protein content was estimated by the method of Lowry *et al.* (4). In some later experiments, rats were injected intraperitoneally with reserpine (Serpasil, Ciba-Geigy) at a dose of 10 mg/kg, 12 hr prior to sacrifice.

^{125}I-labeling of hGH. A modification of the chloramine-T method was used (5, 6). Five micrograms of hGH, 1 mCi of ^{125}I, and 10 μg of freshly prepared chloramine-T were used. The radioiodinated hGH was purified on a Sephadex G-100 column (50 × 0.7 cm) with a flow rate of 0.25 ml/min. The immunoreactivity was 80–86%, determined immediately following iodination by incubation with excess guinea pig anti-hGH. When immunoreactivity was below 80%, nonspecific binding in the radioreceptor assay was increased.

Results. *A. Standard binding assay.* Reagents were added to assay tubes in the following order:

(a) 0.01 *M* phosphate–saline buffer, pH 7.4, with 0.1% sodium azide (PBS) to make a final volume of 1 ml;

(b) Human growth hormone in concentrations varying between 0 and 1 μg per assay tube;

(c) Other reagents as investigated, i.e., 10–300 μl of $CaCl_2$ solution of strength 100 m*M*, 100 μl of 0.1 *N* EDTA (disodium salt $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) solution, pH 7.4, or an isosmolar equivalent of sodium chloride solution (586 mOsm) in 5% BSA in PBS buffer;

(d) ^{125}I-labeled growth hormone with specific activities of 100 to 180 μCi/μg. In different experiments, 0–800 μl (each 100 μl = 1.08 μg) of receptor was incubated with constant shaking at 4, 22, or 37° for a duration varying between half an hour and 24 hr. For all studies, experiments were independently performed at least twice for verification of results.

B. Separation of bound from free hormone. A variety of methods of separating bound from free hormone were considered (7). From a practical standpoint, considering rapidity, expense, and precision of separation, two methods were more attractive: (a) nonspecific or chemical separation based on size-solubility; (b) absorption techniques based on size and charge differences. Since absorption techniques are

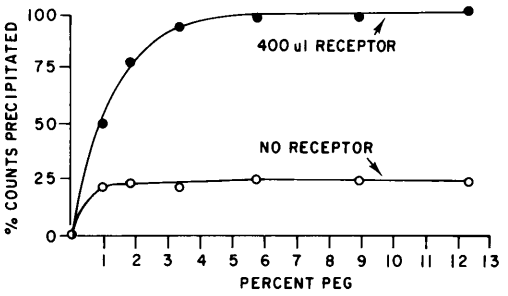

FIG. 1. Determination of suitability of polyethylene glycol (PEG) as a separation method. After incubation of the two series of tubes (see text), varying amounts of polyethylene glycol were added. Note that counts precipitated increased, reaching a plateau on both curves at over 6% PEG. Counts precipitated with 13% PEG maximum (the PEG which it is practical to pipet) have been defined as 100%.

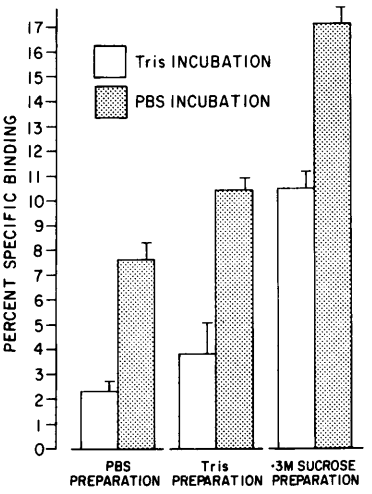

FIG. 2. Evaluation of membrane preparation medium and incubation medium. Rat livers were homogenized and centrifuged in phosphate-buffered saline (PBS), Tris, or 0.3 *M* sucrose. After centrifugation, the pellets were suspended in either PBS or Tris and the radioreceptor assay performed. Note the increase in specific binding with PBS incubation, no matter what preparation media was used. However, 0.3 *M* sucrose proved to be the best preparation medium studied. This was true after correction for protein content of receptor prepared from different media.

greatly affected by protein concentration, while chemical precipitations are less affected, we decided to optimize a chemical separation method—polyethylene glycol (PEG). To determine the optimal PEG concentration, a series of 100% tubes (receptor, ^{125}I-hGH, and buffer) and a series of 0% tubes (no receptor, just ^{125}I-hGH and buffer) were incubated at room temperature for 20 hr. Incubation was then terminated by the addition of varying amounts (0–1600 μl) of cold 20% polyethylene glycol, to give a final PEG concentration of 0 to 12.3%. All tubes were then centrifuged at 2000 rpm for 15 min at 4°, the supernatant decanted, and the tubes left to stand upside down on absorbable tissue paper for 30 sec before determining radioactivity in a gamma spectrometer. The data obtained are shown in Fig. 1. From these data, we selected a final concentration of 8.9% (800 μl) of PEG as giving an optimum precipitation independent of small changes in the amount of PEG added (i.e., precision PEG pipetting was not essential). This separation method was technically easier, and resulted in higher specific binding than the dilution centrifugation method.

C. Determination of "excess" hGH concentration. A 400-μl (4.32 mg) aliquot of membrane preparation was incubated for 6 hr at room temperature with hGH at concentrations of 10^{-5} to 10^{-11} g/ml. Specific binding was 4% in tubes containing no unlabeled hGH; increasing doses of unlabeled hGH produced competitive displacement of ^{125}I-hGH, in doses less than 1 μg. Doses greater than 1 μg produced no greater effect. A concentration of 10^{-6} g/ml (1 μg/tube) was therefore chosen as excess growth hormone.

D. Preparation of membrane fraction. Three extraction media were evaluated: 0.01 *M* phosphate/0.15 *M* NaCl buffer, pH 7.4; 0.3 *M* sucrose; and .025 *M* Tris, pH 7.4. Equal amounts of liver were homogenized and centrifuged in each medium. The final membrane pellets obtained were then resuspended in PBS or Tris and incubated for 2 hr with label and unlabeled hormone. An example of these data is shown in Fig. 2. Sucrose, 0.3 *M*, proved to be the best preparation medium, even after correction for protein content of receptor prepared from the different media.

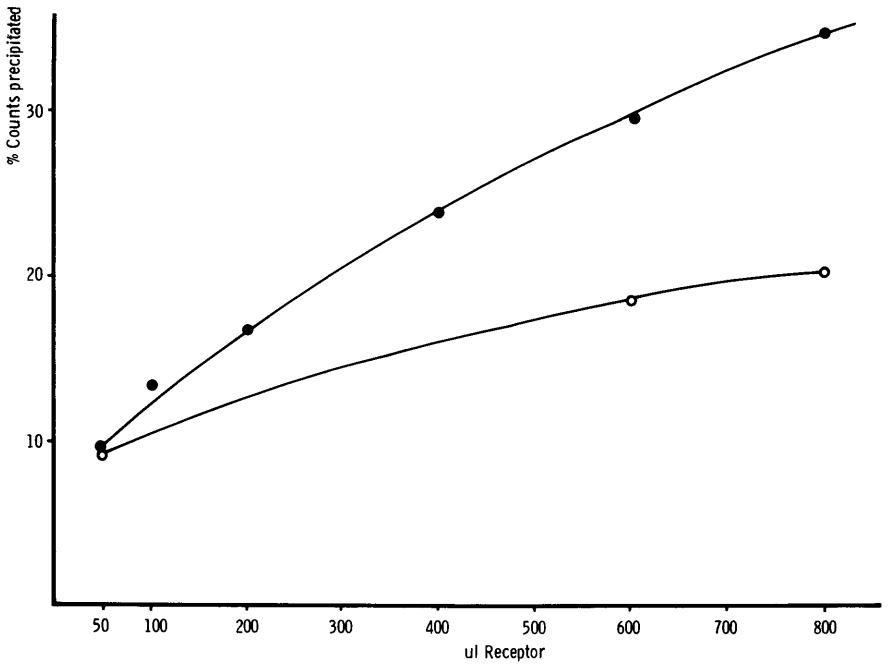

FIG. 3. Effects of varying amounts of receptor preparation. Note that specific binding increased steadily as more receptor preparation was added. No plateau was evident. Each 100 μl of receptor preparation contained an average of 1.07 mg protein.

E. Incubation medium. The effect of varying the incubation medium was also investigated by comparing 0.01 *M* PBS buffer with 0.025 *M* Tris–HCl/10 m*M* Ca^{2+}, as described by Posner *et al.* (1). These data are also shown in Fig. 3. Incubation in PBS gave higher specific binding than did incubation in Tris.

F. Effect of amount of receptor protein. Increasing amounts of receptor protein were incubated for 6 hr at room temperature. Specific binding increased progressively with the amount of receptor protein (Fig. 3). A 600-μl (6.48 ± 0.78 [SD] mg)[5] aliquot of receptor preparation was chosen as the optimum practical amount of receptor, since total volume per assay tube was arbitrarily set at 1.0 ml.

G. Effect of temperature. A standard amount of membrane protein (600 μl) was incubated at 4°, 20°, and 37° for 2, 6, and 12 hr (Fig. 5). For each time of incubation, specific binding was highest at room temperature. At room temperature and 4°, there was no significant change in nonspecific binding with time or temperature. However, nonspecific binding increased with time at 37°. Since total binding remained constant, there was a decrease in specific binding. At 4° specific binding continued to increase, but at each time interval was lower than at room temperature. We then selected room temperature for routine use.

H. Effect of duration of incubation. To ascertain the effect of time on the amount of specific binding, varying time periods up to 24 hr were studied. Binding increased rapidly for 2–3 hr and thereafter rose slowly, reaching maximum at 24 hr. A 6-hr incubation was chosen as a convenient time for later experiments (Fig. 4).

I. Effect of BSA concentration. The effect of the amount of BSA in the incubation medium was investigated in concentrations

[5] SD was calculated from independently prepared membrane preparations. Coefficient of variation from membrane preparation to preparation was 10%.

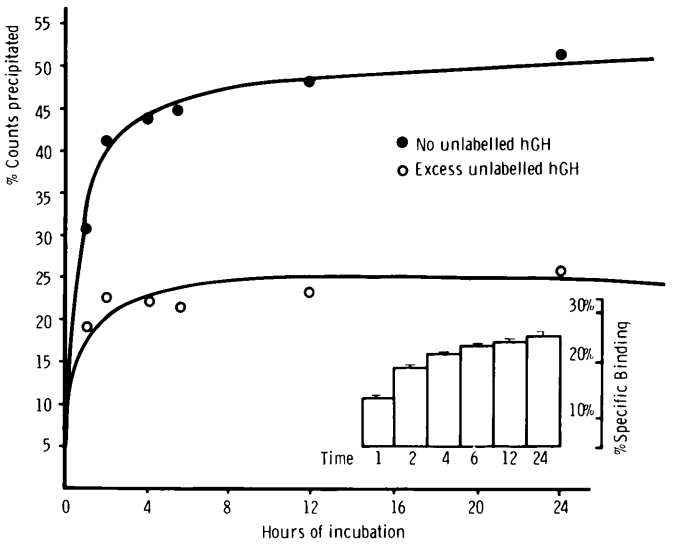

FIG. 4. Effects of time of incubation on specific binding. Counts precipitated are given as percentage of total counts added in the large graph. Specific binding is shown as percentage of total in the inset.

of 0.05 to 2.0%. BSA concentration was found to have no effect either on specific binding or nonspecific binding.

J. Effect of calcium and EDTA. Calcium chloride ($CaCl_2 \cdot 2H_2O$) was added to the assay medium in final concentrations from 0 to 30 m*M*. Even small amounts of calcium decreased specific binding, largely by increasing the amount of nonspecific binding relative to total binding, and this effect increased as calcium concentration increased (Fig. 5). Since Ca^{2+} ions greatly reduced specific binding, 100 μl of 0.1 *N* EDTA (pH 7.4) were added in later experiments. Specific binding at 6 hr was consistently increased by 4% to a mean of 26%, with both a slight (1–2%) drop in nonspecific binding and increase (2%) in total binding (Fig. 5). A control solution of sodium chloride in phosphate buffer isosmolar to the EDTA had no effect on specific binding, showing the effect was not due to ionic changes alone. The addition of other enzyme inhibitors (*N*-ethylmaleimide 2 m*M;* phenylmethylsulfonyl fluoride 1 m*M*) also had no effect on binding, suggesting that the effect of EDTA was not simply one of inhibiting the degradation of receptor by calcium-dependent proteases.

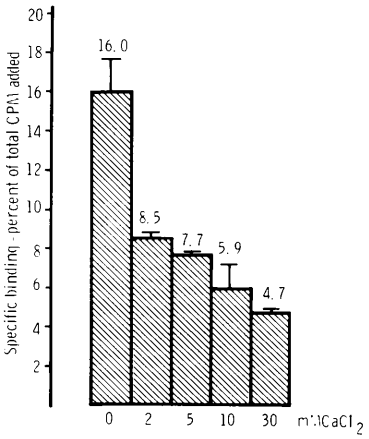

FIG. 5. Effects of calcium on specific binding. As little as 2 m*M* $CaCl_2$ decreased specific binding.

K. Effect of reserpine. Eden (8) has shown that treatment of rats with reserpine at doses of 10 mg/kg will completely abolish normal GH surges. The possibility that pretreatment of rats with reserpine (by lowering endogenous GH concentrations) might increase hGH receptor binding was therefore investigated. At a dose of 10 mg/kg, reserpine (Serpasil; Ciba-Geigy) was given intraperitoneally 15 hr prior to

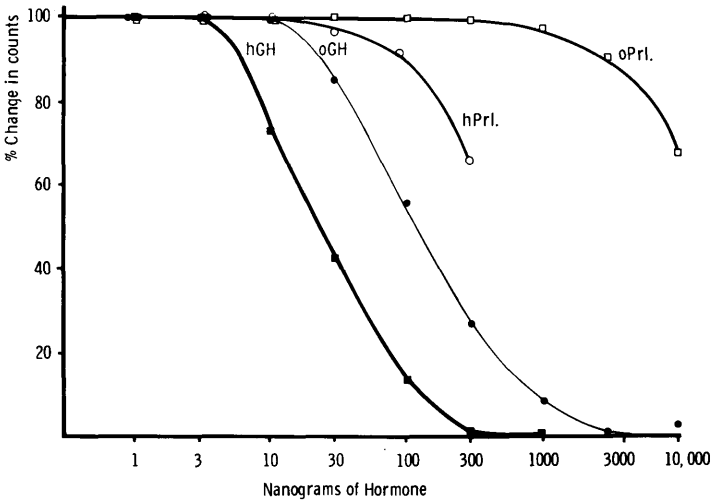

FIG. 6. Dose–response curve for human growth hormone (hGH), ovine growth hormone (oGH), human prolactin (hPrl), and ovine prolactin (oPrl) in the radioreceptor assay. Specific binding in the assay under optimized conditions ranged 55–60%.

sacrifice. Reserpine pretreatment gave a 4% increase in specific binding over control rats of identical age to a mean value of 25% at 6 hr. This effect was additive to that produced by EDTA, such that specific binding was 28% if EDTA was added. In all cases averaging approximately 20–30 ng/ml, there was no significant change in the 50% response figure. Analysis of Scatchard plots confirmed that while reserpine had no effect on affinity, it did result in an increase in available membrane binding sites.

L. Quality of radioiodinated hGH. The radioiodinated hGH used in the previous studies (A–K) was that used for routine hGH radioimmunoassays. For that assay, binding in the presence of excess antibody ranged between 40 and 65%. Special attention was next directed toward improving quality of the ^{125}I-hGH. The general scheme for improving radiolabel quality previously described (6) was used with the result that excess antibody binding increased to 85–90%. When this ^{125}I preparation was used in the radioreceptor assay, specific binding increased from the 26–30% range (illustrated in Fig. 6) to 55–60%.

Discussion. Previous investigators have reported male rat liver to be a poor source of growth hormone receptor binding sites with generally less than 3% specific binding (1–3). The studies reported herein show that systematic attention to (i) the method of separation of bound and free, (ii) method of membrane preparation, (iii) type of incubation medium, (iv) amount of receptor protein, (v) temperature of incubation, (vi) duration of incubation, (vii) presence or absence of calcium during incubation, (viii) handling of animals prior to sacrifice, and (ix) quality of the ^{125}I-hGH resulted in increase of specific binding from 2 to 3% to 55–60%. Previous investigators (1–3) have also shown that membrane preparations of rat liver from females gave much higher specific binding (e.g., 25 vs. 3% for male rat liver preparations) due to the presence of lactogenic receptor sites, which also bound hGH. The increased specific binding we described for male rat liver membrane preparations is not due to the presence of lactogenic receptor sites, as evidenced by the small cross-reaction of ovine prolactin (0.2%).

The effects of incubation time and temperature have been well characterized in a number of systems (9–11). Many investigators have preferred incubation at room temperature owing to the much slower rise in specific binding at 4° and the dispropor-

tionate increase in receptor degradation at 37° relative to the increase in binding rate (12, 13).

Specific binding was directly proportional to the concentration of membrane protein up to 6400 μg protein when plotted on an arithmetic graph. Above this level as receptor concentration was increased relative to the amount of growth hormone, there was a progressive plateauing of binding. However, if binding was plotted against log of receptor concentration, no plateau was seen, suggesting that hormone binding will continue to increase if receptor concentration is high enough.

The addition of calcium ions resulted in a decrease in specific binding due to an increase in nonspecific binding. This effect is contrary to that expected from the data of other investigators who precipitated bound hormone by the addition of 6 vol of ice-cold 0.1% BSA–Tris–10 m*M* Ca^{2+} buffer, followed by slow centrifugation. They found that the presence of divalent cations more than doubled the amount of binding to membrane (1, 13).

In our experiments, the presence of calcium seems to have had some nonspecific effect on protein aggregation, resulting in increased precipitation of nonspecifically bound label by PEG. There seems to be no necessity for the presence of calcium (other divalent cations not tested) for binding of hormone to receptor.

The effect of reserpine is explicable in one of three ways:

(a) Pretreatment with large doses of reserpine could have resulted in a precipitous fall in endogenous growth hormone concentrations in the rat, leading to a rebound increase in receptor number and/or affinity (14).

(b) The fall in rat growth hormone levels may have had no effect on total receptor concentration, but merely left more of the existing receptors unoccupied and available for binding by labeled hGH. Hormone can remain attached to receptor for many hours, especially at the low temperatures used in the preparation of membranes (15). In female rats, Eden *et al.* (16) have shown an increase in tissue responsiveness to exogenous GH when endogenous GH levels are low. Which of the above mechanisms was responsible is uncertain.

(c) The reserpine improves specific binding by virtue of being present as a chemical species during preparation and incubation of membranes, and improving membrane recovery or reducing proteolytic breakdown. Dose–response curves show an increase in the total number of available binding sites, but no significant change in affinity as measured by 50% dose–response level.

The high nonspecific binding in this assay was probably due mainly to the large amount of protein used and was approximately 20% of the counts per minute bound. This is similar to that obtained for ACTH by Wolfsen *et al.* (17) (who described a radioreceptor assay for ACTH using adrenal cortex as a receptor source), and by Carr and Friesen (15) for hGH using human liver as receptor source. However, most investigators record a nonspecific binding level of below 5–10% with protein concentrations of 200–400 μg/tube. In the assay system described herein, nonspecific binding was 18–20% because the low concentration of hGH receptors present necessitated using a large amount of membrane protein. This was, however, a relatively constant percentage of total counts added and therefore still permitted good precision.

In summary, the final assay system employed PEG final concentration 8.9% as a separation method. Membranes were prepared in 0.3 *M* sucrose, incubated in PBS containing EDTA (0.01 *N*, pH 7.4) for 6 hr at 22°, and reserpine (10 mg/kg) was administered to the animals 15 hr prior to sacrifice. The labeled GH preparation was purified to give 85–90% immunoreactivity. The result of these methodological changes increased specific binding from 3 to 60%, resulting in a usable, specific radioreceptor assay for GH.

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