

Identification and Characterization of Specific Binding Proteins for Growth Hormone in Normal Human Sera

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Abstract

The well-recognized "big" forms (45,000–100,000 mol wt) of immunoreactive human growth hormone (hGH) in human serum have been reported to be random aggregates or formal polymers. However, we have now investigated the possibility that they are protein-bound forms. After incubation of monomeric ^{125}I -hGH with normal serum, gel chromatography indicated a peak of bound ^{125}I -hGH (at $\sim 120,000$ mol wt), which was completely displaced by excess unlabeled hGH. When serum alone was chromatographed two peaks of specific binding were subsequently detected, the major peak, eluting between 74,000 and 85,000 mol wt corresponded to the ^{125}I -hGH-binding protein complex observed at $\sim 120,000$ mol wt. Using a mini-gel filtration system for separating bound from free hormone, binding of ^{125}I -hGH by normal human serum was dependent on time (equilibrium was reached in 2 h at 21°C), temperature ($21^\circ\text{C} > 37^\circ\text{C}$), Ca^{2+} and serum concentrations. Binding was reversible and highly specific for hGH, not being displaced by GH or prolactins from several species. Scatchard analysis revealed linear plots with an affinity (K_A) of $0.32 \pm 0.06 \times 10^9 \text{ M}^{-1}$ ($n = 7$). Human serum with low endogenous hGH levels, when added to rabbit liver membranes, decreased the binding of ^{125}I -hGH in this tissue in a dose-dependent manner. These data indicate that human sera contain a specific, high affinity binding protein for hGH and that this may account, at least in part, for the known size heterogeneity of GH in serum. Its effect on GH binding to target tissues may indicate a role for the binding protein in the regulation of GH action.

Introduction

It has been recognized for more than a decade that there is considerable size and charge heterogeneity of growth hormone (GH)¹ in human serum (for reviews see 1–3). Marked discrepancies have also been reported in both rat and human sera between radioimmunoassayable GH activity and growth-promoting bioactivity (4), although no discrepancy was observed for metabolic (5) or lactogenic bioactivities (6). These observations raise the possibility that forms of GH with altered activity (e.g., reduced immunoactivity or enhanced bioactivity) may be involved in, and important for, the overall growth promoting activity of serum. These aspects of the chemical nature and activity of GH, or GH-like molecules, are still poorly understood.

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1. Abbreviations used in this paper: BSA, bovine serum albumin; GH, growth hormone; hGH, human GH; K_A , affinity binding coefficient; oPRL, ovine prolactin; PL, placental lactogen; PRL, prolactin.

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Several groups (7–14) have shown that gel chromatography of human serum, or human pituitary extracts, yields three major immunoreactive species of hGH—little or monomeric GH (22,000 mol wt), big GH ($\sim 45,000$ mol wt) and big-big GH (variable but $\geq 60,000$ mol wt). Little GH has been shown more recently to exist in several forms—the 22,000-mol wt single chain major form, the 20,000-mol wt variant, and probably three proteolytically cleaved two-chain forms (1). The 20,000-mol wt form is considered to have normal growth promoting activity but diminished insulinlike metabolic activity (3). Some (15, 16), but not all reports (17), suggest that the two-chain forms may have enhanced biological activity. In contrast, several of the studies (11–14) of the big and big-big forms reported a diminished activity in radioreceptor assays relative to immunoassays, thus leading to the suggestion that the larger forms may be less biologically active.

Overall, the chemical nature of these larger forms of GH has been controversial. The possibilities considered were that they represented hGH precursors, hGH aggregates (either random or formal polymers, including disulfide-linked forms), hGH bound to one or more serum proteins or immunoassay artifacts. The most widely held view has been that they are hGH aggregates and this was recently supported by a detailed investigation by Stolar et al. (18) who deduced that big GH was an hGH dimer and big-big GH was a mixture of tri-, tetra-, and pentamers. Although it appeared that unlabeled hGH, when incubated with plasma (1 h, 22°C) did not appreciably change its molecular size (as determined by radioimmunoassay of fractions from Sephadex G-100 gel filtration), close inspection of their data indicates that one cannot rule out the possibility that GH binding to one or more plasma proteins, as opposed to simple aggregation, would be a sufficient and satisfactory explanation for the size heterogeneity.

This possibility is supported by the recent demonstration in this laboratory of an abundant, high affinity, and specific GH-binding protein in the sera of normal and pregnant rabbits (19). This binding protein had binding and structural characteristics similar to the GH receptor of rabbit liver membranes. It was not detectable, however, by traditional polyethylene glycol or charcoal separation techniques, thereby providing a possible explanation for the previous failure of others to detect this binding protein. As a result of this observation we have examined whether human serum contains a similar GH-binding protein that might account, at least in part, for the size heterogeneity of serum hGH. We now report the identification and characterization of such a binding protein.

Methods

Reagents. hGH (NIAMDD-hGH-I-1) used for iodination, and ovine prolactin (oPRL) (NIAMDD-oPRL-15), bovine GH (NIH-GH-B-18), and ovine GH (NIH-GH-S-11) used for unlabeled preparations were gifts of the National Hormone and Pituitary Program (NIADDK, Na-

tional Institutes of Health, Bethesda, MD). Bovine GH used for iodination was a generous gift from Dr. M. Sonenberg, Memorial Sloan-Kettering Cancer Center, New York; ^{125}I -human PRL and unlabeled hPRL were obtained through the World Health Organization (WHO) Matched Reagent Program by courtesy of Professor H. G. Burger; hGH used for unlabeled standards and bovine serum albumin (BSA, 68,000 mol wt) were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Human serum was obtained from normal adult males and females. In some initial experiments, prior to use for binding studies, human serum was centrifuged at 200,000 *g* and the supernatant filtered through a 0.22- μm Millipore filter (Millipore Corp., Bedford, MA). This procedure ensured the complete solubility and absence of blood cells from the serum preparation. All other reagents, including molecular weight markers for gel chromatography, were obtained as described previously (19).

Iodination and binding studies. hGH was iodinated as previously described (20), using the Iodogen method (21). The ^{125}I -labeled protein peak was separated on a Sephadex G-50 column (0.8 $\times$ 19 cm) and subsequently purified on Ultrogel AcA54 (1 $\times$ 55 cm) (LKB Produktur, Bromma, Sweden) to yield a peak of monomeric ^{125}I -hGH (22,000 mol wt). Specific activities of 30–50 $\mu\text{Ci}/\mu\text{g}$ were achieved as estimated by trichloroacetic acid precipitation of the iodination mixture or by a tracer dilution method (22).

Binding studies were performed usually at 21–23°C using 25 mM Tris/HCl buffer pH 7.5 containing 10 mM CaCl_2 , 0.02% (wt:vol) sodium azide and 0.1% (wt:vol) BSA, with ^{125}I -hGH (normally 20,000 cpm; 12–20 fmol), human serum (50 or 100 μl), and unlabeled GH as required, in a final volume of 250 μl . Bound and free hormone were separated by gel filtration on AcA44 mini-columns (0.6 $\times$ 22 cm) at 21–23°C as described previously (19). No differences in radioactive profile or calculated percent binding were noted if the mini-columns were run at the less convenient temperature of 4°C. Specific binding was calculated as the difference in binding in the presence (nonspecific binding) and absence (total binding) of an excess (0.1 μM) of unlabeled GH. All protein estimations were performed using the Coomassie Blue method (23).

Gel filtration of serum alone or serum preincubated with ^{125}I -hGH. Initial gel filtration studies were performed at 4°C on an Ultrogel AcA34 column (1 $\times$ 90 cm) equilibrated and eluted with 50 mM Tris/HCl buffer pH 7.5 containing 0.02% azide, exactly as described previously for rabbit liver cytosol or serum binding proteins (19, 20). When ^{125}I -hGH-serum complexes (250 μl ; 20,000 cpm) were gel filtered the elution profile of serum GH binding proteins was determined by simply counting the radioactivity of each fraction. When serum alone (0.6 ml) was chromatographed the GH binding profile was determined by taking 100- μl aliquots from each 1-ml column fraction, incubating with ^{125}I -hGH for 2 h and then measuring specific binding by separation of bound and free GH on AcA44 mini-columns. The AcA34 column was calibrated with blue dextran (V_0), riboflavin (V_i), and molecular weight markers as listed previously (19).

Results

Incubation of normal human serum (2 h, 21°C) with ^{125}I -hGH followed by gel chromatography on Ultrogel AcA34 gave rise to three peaks of radioactivity (Fig. 1 *a*). Peak 1, eluting at a position corresponding to $\sim 120,000$ mol wt was not present when excess unlabeled hGH was included in the incubation and therefore represents a specifically bound peak of ^{125}I -hGH. This peak was not symmetrical, having a shoulder (which was also displaceable by unlabeled hGH), tailing into peak 2, the largest peak that represents unbound ^{125}I -hGH. Peak 3 appeared at the total volume of the column and represents free iodine or low molecular weight degradation products.

In contrast, two peaks of specific binding were observed after incubation of ^{125}I -hGH with individual column fractions ob-

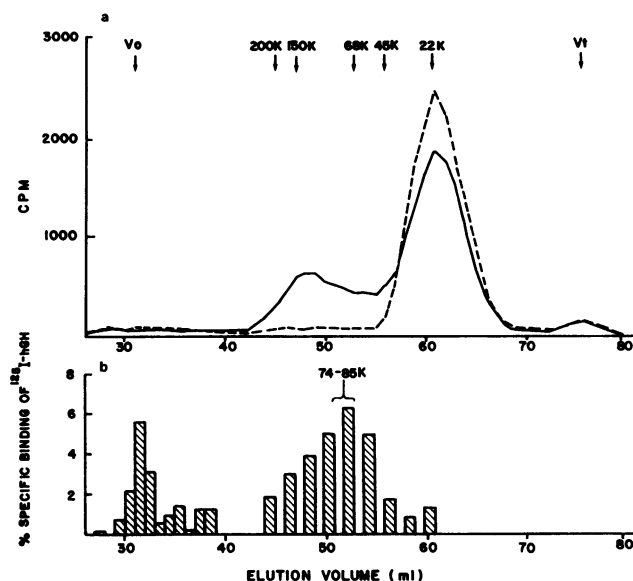


Figure 1. A representative gel filtration profile on Ultrogel AcA34 (1 $\times$ 90 cm) of (a) human serum- ^{125}I -hGH incubation mixture in the absence (—, total binding) and presence (---, nonspecific binding) of excess (0.1 μM) unlabeled hGH and (b) human serum alone, with specific binding of ^{125}I -hGH (■) being determined in 100- μl aliquots from individual column fractions. The elution position of molecular weight markers as listed in ref. 19 are shown by the arrows. Similar data were obtained in many (a) and three (b) such experiments.

tained from gel filtration of serum alone (Fig. 1 *b*). The major peak, eluting at 74,000–85,000 mol wt, was broad but corresponded in principle with the position one would expect for a binding protein that formed an $\sim 120,000$ -mol wt complex with hGH (Fig. 1 *a*). Rather surprisingly, a second peak of binding activity was detected near the void volume of the column ($>400,000$ mol wt); this peak was never seen after gel filtration of ^{125}I -hGH-serum incubation mixtures (as in Fig. 1 *a*). We currently have no adequate explanation for this anomaly unless formation of this high molecular weight species is prevented in whole serum by the presence of the more abundant lower molecular weight binding species. Attempts to determine the affinity of this high molecular weight binding protein by Scatchard analysis have been relatively unsuccessful due to poor reproducibility and an apparent lability of this binding species (not shown). Since this form is not detected by incubations of ^{125}I -hGH with whole serum (Fig. 1 *a*) it has been assumed in the subsequent experiments reported, that binding characteristics of the lower molecular weight ($\sim 120,000$) ^{125}I -hGH-binding protein complex only were being analyzed.

In a similar manner to that described previously for rabbit tissue cytosols (20, 24) and serum (19), the GH binding activity of human serum was not detectable using a 12.5% (wt/wt) final concentration of polyethylene glycol 6000 or a dextran-charcoal separation system (data not shown). For this reason the AcA44 mini-column system (19) was used to further characterize the GH binding protein of normal human serum.

In addition to the demonstration of serum binding of ^{125}I -labeled hGH, incubation of serum with unlabeled, monomeric (22,000 mol wt) hGH led to a prominent shift in the radioimmunoassayable GH profile into a higher molecular weight gel filtration fraction (Table I). hGH alone, when chromatographed

Table I. The Gel Chromatographic Distribution of Monomeric Unlabeled hGH Following Incubation with and without Human Serum Binding Protein

	hGH content (% total recovered)				
	Fraction no.				
	1	2	3	4	5
GH alone	0.3	0.5	1.8	52.9	44.5
GH + human serum	4.3	5.0	3.3	36.4	50.1
GH + affinity-purified binding protein	0.8	7.7	4.5	40.2	46.9

Unlabeled hGH (20 ng) was incubated for 2 h at 21°C–23°C in 25 mM Tris/HCl buffer pH 7.5 containing 10 mM CaCl₂, 0.02% sodium azide and 0.1% BSA in the presence or absence of normal human serum (150 μ l) or partially purified serum binding protein (50 μ l, 2 μ g protein) in a total volume of 250 μ l. The partially purified binding protein was obtained by hGH-Affigel affinity chromatography.² After incubation, 200 μ l of the incubation mixture was applied to an Ultrogel AcA44 mini-column and five fractions collected as previously described (19). GH binding protein is known to elute primarily in fraction 2; monomeric hGH is known to elute in fractions 4 and 5. Each column fraction was dialyzed, lyophilized, and measured in duplicate for hGH content by a standard hGH radioimmunoassay. The hGH content of each fraction is given as a percentage of total hGH recovered; very similar data were obtained in a second experiment.

on Ultrogel AcA44 mini-columns eluted in fractions 4 and 5, whereas after incubation with serum (or with hGH-Affigel affinity-purified binding protein²) a small but unambiguous proportion eluted in fraction 1–3. The proportion of unlabeled hGH measured in fraction 2 is slightly overestimated since the binding protein itself, which is known to elute in this same fraction on mini-columns (see ref. 19), can be partially detected as an apparent GH peak by GH radioimmunoassay due to sequestration of ¹²⁵I-hGH tracer. However, the data of Table I indicate that the GH binding phenomenon can occur with unlabeled, as well as ¹²⁵I-labeled, hGH.

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Fig. 2 *a* illustrates the time and temperature dependence of ¹²⁵I-hGH binding. Equilibrium was reached generally within 2 h at room temperature (21–23°C) and was maintained for up to 24 h in other experiments (e.g., Fig. 2 *b*). The mean level of binding at equilibrium for 50 μ l normal human serum was 8.0 $\pm$ 0.5% (mean $\pm$ SEM, *n* = 7). Binding at physiological temperature was somewhat lower but tended to reach equilibrium more rapidly. The binding of ¹²⁵I-hGH was rapidly reversible (as shown in Fig. 2 *b*) with a half-time for dissociation of 27–44 min. There was no evidence for any tracer degradation at either temperature, as determined by trichloroacetic acid precipitation of the incubation medium. Fig. 3 *a* shows the dependence of specific binding on the amount of serum used with binding being linear up to 100 μ l (in 250 μ l total volume) and being detectable with as little as 6 μ l. Fig. 3 *b* indicates that, unlike tissue GH binding proteins in other species (25–27), there was only a minor effect of Ca²⁺ on hGH binding, which increased in a linear manner (by up to 44%) as the Ca²⁺ concentration rose from a physiological level (2.5 mM) up to 40 mM.

The hormonal specificity of binding of ¹²⁵I-hGH by human serum is shown in Fig. 4. Binding was inhibited only by unlabeled hGH and not by other human hormones (hPRL, hPL) or by GHs or PRLs of other species (bovine GH, ovine GH, ovine PRL). Furthermore, no specific binding of ¹²⁵I-bGH or ¹²⁵I-hPRL was detectable in several experiments (data not shown). Nor was any binding of ¹²⁵I-hGH observed following incubation with human albumin, one of the major proteins of serum (data not shown). This indicates a further degree of specificity of the serum binding of hGH.

Scatchard analysis of data obtained from a classical displacement curve (Fig. 5 *a*) yielded linear plots (Fig. 5 *b*). The mean ($\pm$ SEM) binding parameters calculated for seven determinations on four different normal human sera (including one pool of >50 different normal samples) were: affinity (*K_A*) 0.32 $\pm$ 0.06 $\times$ 10⁹ M⁻¹, capacity 26.5 $\pm$ 4.5 fmol/mg serum protein or 1841 $\pm$ 320 fmol/ml serum. Specific binding of hGH has been detected in all 39 human sera so far examined (Fig. 6). These sera were obtained from 29 different subjects with varying levels of radioimmunoassayable hGH (GH-deficient, normal, acromegalic). A weak negative linear correlation (*r* = -0.526) was observed between GH binding and GH content of these sera (Fig. 6), suggesting that the binding protein levels themselves may vary with circulating GH levels or, more likely, that there are differing

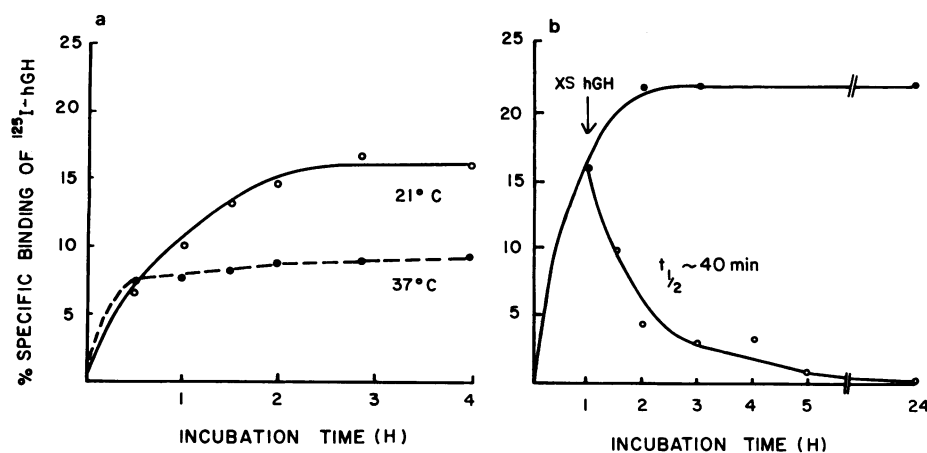


Figure 2. The time course of association (*a*) and dissociation (*b*) for the specific binding of ¹²⁵I-hGH to normal human serum. For the dissociation experiments, binding was performed at 21°C and dissociation was initiated in one set of tubes by the addition at 1 h of an excess (2 μ g) of unlabeled hGH in a 10- μ l vol to avoid dilution effects. The data points for both panels represent the mean of duplicates that differed by <8% in all cases; very similar data were obtained in a second experiment.

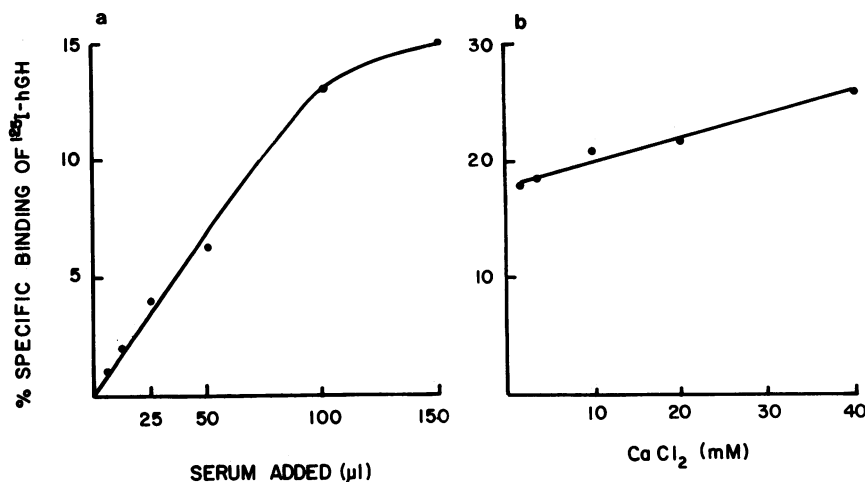


Figure 3. The effect of increasing serum concentration (a) and calcium ion concentration (b) on the specific binding of ^{125}I -hGH to normal human serum. The data are expressed as for Fig. 2. Calcium concentration was increased by addition of CaCl_2 to the 50 mM Tris/HCl incubation buffer (pH 7.5). The experiment was not carried out in the complete absence of Ca^{2+} due to the Ca^{2+} content of the normal serum used. Similar data were obtained in three experiments.

degrees of occupancy with different circulating GH concentrations. Although this relationship was significant at the level of $P < 0.001$, the relatively large scatter observed would suggest that any implications of particular physiological significance should be made with caution at this time.

In an attempt to assess the possibility that the GH binding protein might modulate the interaction and/or action of GH at its target cells, the effect of normal human serum on the binding of ^{125}I -hGH to rabbit liver membranes was determined. As shown in Fig. 7, there was a dose-dependent partial inhibition of binding of ^{125}I -hGH to the membrane receptor that could not be accounted for by the known hGH content of the serum (<2 ng/ml). This effect was significant ($P < 0.05$ to $P < 0.005$) in three experiments at serum doses of 50–150 μl . No such inhibition occurred for ^{125}I -bGH binding to the same membranes, demonstrating that this was a specific effect of the hGH binding protein.

Discussion

These data indicate that normal human sera contain a highly specific hGH-binding protein with a relatively high affinity for hGH ($0.3 \times 10^9 \text{ M}^{-1}$), although it is only one-third to one-fifth of that for the GH receptor of human fibroblasts (28), liver (29), or cultured IM-9 lymphocytes (30). Binding of both ^{125}I -labeled hGH and unlabeled hGH has been detected. While serum binding of GH has been suggested previously (31–39) this is the first report to detail the nature of the binding characteristics of the binding protein in human serum. We have shown that GH binding is dependent on time, temperature, and serum concentration, and is completely reversible, even at 21°C . The binding protein, which has recently been partially purified using hGH-affinity chromatography³ was of 74,000–85,000 mol wt and was distinct from the major serum protein, human albumin. The binding protein was not detectable by charcoal or polyethylene

glycol precipitation. This provides a convincing reason why the binding protein has not been recognized or adequately characterized in the past; many workers have used these methods alone for detection of possible soluble binding proteins. In other instances, where gel filtration has been used to attempt separation of bound forms of GH, the experimental conditions (e.g., time, temperature, ligand and serum concentrations) for incubation of labeled or unlabeled hGH with serum have been inadequate to permit a binding reaction to occur or else have not been reported (7, 8, 18). In our studies, a mini-gel filtration system (Ultrogel AcA44, 0.6×22 cm) has been used to achieve an appropriately rapid, simple, quantitative, and discriminatory separation. This system was devised originally for quantitative measurement of soluble GH binding proteins in rabbit serum (19) and tissue cytosols (24).

The levels of binding protein detected in normal human serum are quite significant, particularly considering that presumably only unoccupied binding protein would be measured under the conditions of incubation. That some binding protein is occupied by endogenous hormone is suggested by the inverse correlation between binding protein and circulating GH levels. Indeed, the calculated binding capacity of normal sera ($\sim 1,800$ fmol/ml serum), which would be underestimated given the likelihood of endogenous occupancy, would be sufficient to bind all of the GH present in normal serum (~ 500 – $1,000$ fmol/ml). However, if one also accounts for the binding affinity (K_A $0.3 \times 10^9 \text{ M}^{-1}$), then at 500 fmol GH/ml the binding protein would be $\sim 10\%$ occupied. On the basis of a 1:1 binding stoichiometry between GH and its binding protein, one can then estimate that in normal sera ~ 30 – 40% of GH may be in a bound form. This compares with values for the percent big-big (i.e., $\geq 60,000$ mol wt and presumed aggregated) GH forms reported by others: 13–36% (13), $>50\%$ (37), 18–25% (40), $<20\%$ (41). If one makes the same calculation in acromegalics (assuming a GH content of 5 pmol/ml, and the same total binding capacity and affinity as in normal serum) then 20–26% of GH would be in the bound form. This compares with the values of 5–27% (13), 17% (37), 5–25% (40) and 0–37% (42) previously recorded for big-big GH forms in acromegaly. These data are consistent, therefore, with the view that the big forms of GH previously reported in serum,

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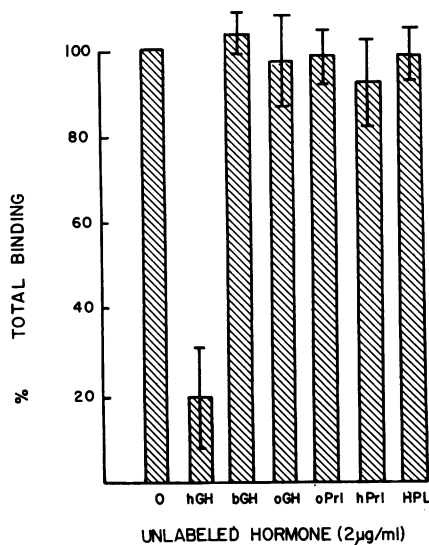


Figure 4. The hormonal specificity of binding of ^{125}I -hGH to normal human serum. All unlabeled hormones were added at a final concentration of 2 $\mu\text{g}/\text{ml}$. Binding is expressed as a percentage of total binding in the absence of unlabeled hormone (100%). Each histogram is the mean of two experiments (values shown by the vertical bar) that were each performed in duplicate.

with 60,000–100,000 mol wt, include protein-bound forms rather than just aggregated/polymeric forms.

The concept of binding proteins in serum is not new for hormones such as steroids and thyroid hormones, nor for the polypeptide growth factors, insulinlike growth factors (43), platelet (44), epidermal, and nerve growth factors (45). Although not yet a recognized phenomenon for larger polypeptide hormones such as GH, in fact there are, apart from our earlier study in rabbit serum (19), several references in the older literature (31–38) to indicate that gel filtration profiles and electrophoretic patterns of ^{131}I -, ^{125}I - or ^{14}C -labeled hGH could be altered by the addition of, or preincubation with, human, rabbit, rat, and

guinea pig sera. Indeed, Beitins et al. (35) reported for human sera, gel filtration data similar to that of the present study. However, none of these earlier studies reported any details of the nature, characterization, or specificity of the changes in hGH profiles. More recently, Peeters and Friesen (39) identified and partially characterized, using a charcoal separation procedure, a GH binding protein ($\sim 60,000$ mol wt) in serum from pregnant, but not nonpregnant mice. Very large amounts of unlabeled hGH (25 μg) were necessary, however, to effect displacement of the bound tracer, suggesting that the binding protein was of high capacity and very low affinity. All of these reports have been somewhat controversial and certainly not all *in vitro* studies have reported size changes in GH preincubated with serum (e.g., 7, 8, 18). However, it is clear from the data in this paper that a highly specific binding protein for hGH is present in human serum.

The physiological role of such a binding protein is not yet clear, however, given that more than half of the hGH remains in the free or monomeric state. Several possibilities are worth considering. First, the binding protein may have a protective effect against very high levels of hGH; it is well-recognized that the big forms of hGH reported previously are less biologically active (11–14). Dwarfism due to an increase in the presence of big and big-big hGH forms has been reported (46). Such a role for big GH, however, would not appear to explain the dichotomy between hGH levels and clinical symptoms in acromegaly since the dichotomy remains if one measures only the monomeric form (42). Second, the binding protein may be designed to maintain a steady baseline of GH in the face of the pulsatile secretion from the pituitary: it has been shown recently (47) that the larger forms of hGH are cleared more slowly from the circulation. Third, it may be that bound forms of hGH are protected from degradation or alternatively are preferred for the *in vivo* generation of various biologically active GH fragments (3). A further consequence of the presence of unoccupied GH binding protein in serum may be a significant effect on the receptor- and immunoassayable levels of GH; the binding protein, via competition for the labeled ligand, leading to an overestimation of hGH. This would be analogous to the situation with the mea-

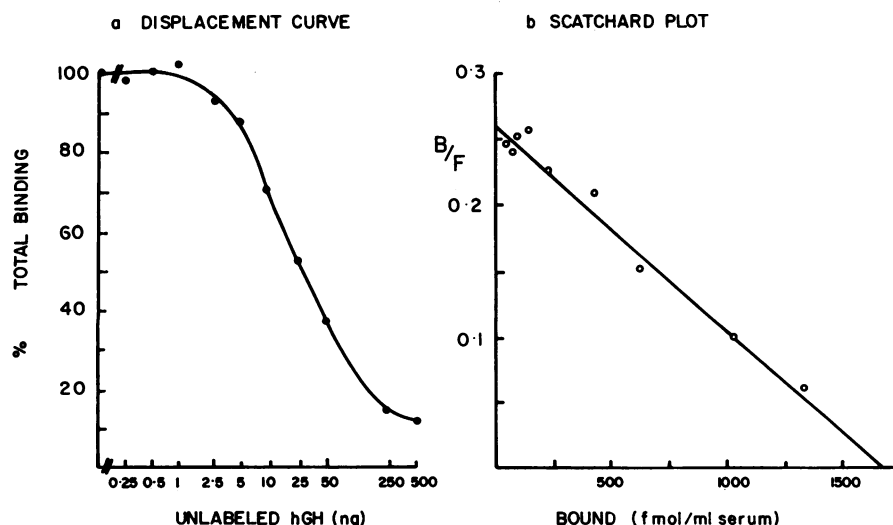


Figure 5. A representative displacement curve (a) and the subsequently derived Scatchard plot (b) for binding of ^{125}I -hGH to normal human serum. The Scatchard plot abscissa is expressed as femtomoles hGH bound per milliliter of serum. Mean binding parameters for affinity and capacity for seven such analyses are given in the text. Each data point is the mean of duplicate determinations that differed by <8%.

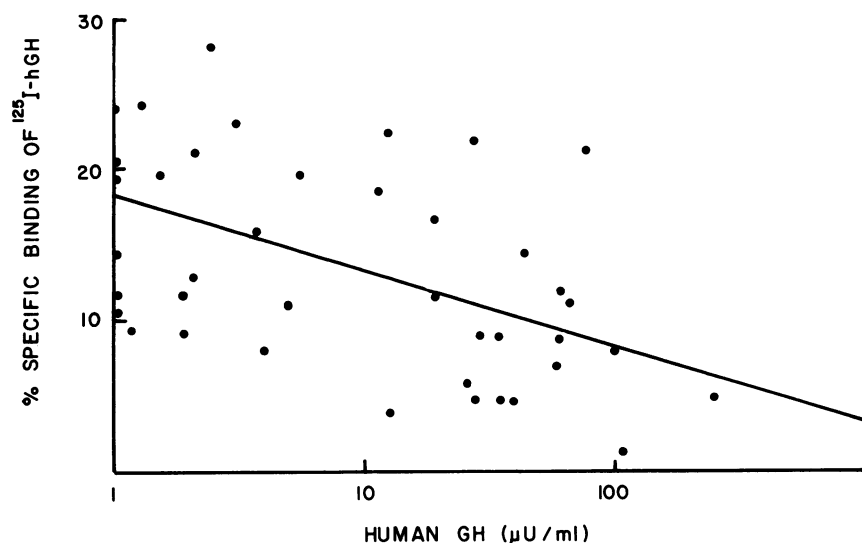


Figure 6. Specific binding of ¹²⁵I-hGH to 39 human sera as a function of the radioimmunoassayable GH content of the sera (expressed as microunits per milliliter). The correlation coefficient ($r = -0.526$) and regression line were calculated by desk-top computer. The abscissa is expressed on a log scale; 100 μl of serum was used for binding in each case.

surement of the insulin-like growth factors in the presence of their binding protein(s) (48).

Finally, we know nothing as yet regarding the origin of the binding protein. One possibility is that like GH itself it is secreted from the pituitary gland since it is known that higher molecular weight forms of GH are present in pituitary extracts (7, 11). A possibility considered more likely, however, is that it is secreted by or shed from the liver and other GH target tissues. This suggestion is based on the very close antigenic and structural relationship between the rabbit liver membrane GH receptor, the rabbit serum GH binding protein (19), and a GH binding protein found in target tissue high-speed cytosol preparations (24).

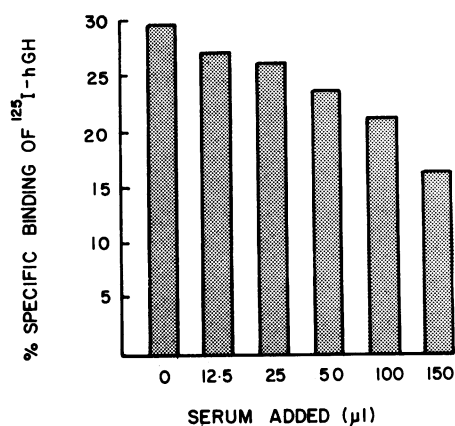


Figure 7. A representative experiment showing the dose-related effect of normal human serum on the specific binding of ¹²⁵I-hGH to pregnant female rabbit liver membranes. Binding was carried out at room temperature for 2 h with a final membrane protein concentration of 400 μg/ml as described previously (14), but in the presence or absence of human serum. Each histogram is the mean of duplicate determinations that differed by <6% in all cases. Data from three such experiments that have been carried out indicated that the effect of serum was significant (Student's *t* test) at serum levels of 50 μl ($P < 0.05$), 100 μl ($P < 0.01$), and 150 μl ($P < 0.005$).

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