RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE

VOL. I

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CORRIGENDUM

RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE, VOL. I.

(STI/PUB/350)

Paper IAEA-SM-177/87 by Badawi et al.

Page 417, line 7

For MRX 71/222 read MRC 71/222

Page 417, lines 8 - 11

The sentence should be as follows:

It was also found that 1.0 milli-ampoule of the serum standard MRC 71/167 was equivalent to 0.09 ng and 0.9 μU of pituitary standard MRC 71/222 with 95% fiducial limits at 0.06 - 0.14 and 0.6 - 1.4, respectively.

RADIOIMMUNOASSAY AND RELATED PROCEDURES IN MEDICINE
IAEA, VIENNA, 1974
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GLYCOPROTEIN HORMONES (Session III)

PEPTIDE HORMONES (Sessions IV and V)

RADIOIMMUNOLOGICAL BEHAVIOUR OF ENDOGENOUS AND EXOGENOUS HUMAN GROWTH HORMONE*

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Abstract

RADIOIMMUNOLOGICAL BEHAVIOUR OF ENDOGENOUS AND EXOGENOUS HUMAN GROWTH HORMONE.

The immunoreactivity of endogenous human growth hormone (HGH) and of exogenous HGH after infusion into patients has been investigated. Four different HGH preparations gave identical standard curves documenting immunoidentity. Complete immunoidentity could not be demonstrated for "big" HGH which was separated from regular HGH by dextran gel filtration. The HGH immunoreactivity, which was eluted from the column ahead of "big" HGH, showed an even more pronounced change in immunoreactivity. Exogenous and endogenous human placental lactogen (HPL) showed the expected deviation from the HGH standard curve, whereas bovine prolactin (BPr) and high endogenous immunoassayable HPr (3200 ng/ml) did not cross-react with the HGH antibody. Immunoidentity of HGH with the standards was documented in 10 patients with acromegaly. This was true for samples from the jugular vein as well as from the periphery. Eight insulin tolerance tests (ITT) were performed in patients with normal HGH secretion. Samples at 30, 45, 60 and 90 min were diluted and the resulting slopes compared with the standard curve. Whereas the slopes of the 30-, 45- and 60-min samples were parallel, the slopes of the 90-min samples deviated from the standard curve. Exogenous HGH was infused (2 mg for 30 min for each person) into five normal subjects (I) and five patients with HGH deficiency (II). HGH levels rose to 171.6 ± 19.8 ng/ml (I) and 192.4 ± 34.8 ng/ml (II). HGH half-time of disappearance was 14.9 ± 3.9 min (I) and 19.1 ± 6 min (II). There was no change of HGH immunoreactivity over a period of 2 hours.

In summary: (1) Cross-reactivity of HPL but not of HPr with HGH antibodies was demonstrated. (2) HGH in acromegaly and exogenous HGH after infusion have identical immunoreactivity. (3) The change in endogenous HGH immunoreactivity 90 min after stimulation is apparently due to the secretion of a different HGH immunoreactivity that is most likely residing in a larger molecular weight fraction.

Radioimmunoassay has become the method of choice for measuring peptide hormones and other substances in biological fluids since the development of the first radioimmunological determination for insulin by Yalow and Berson [1]. One of the criteria for the validity of radioimmunoassays is the identity of the immunoreactivity of the standard preparation and the unknown substance which is measured in the body fluid [2, 3]. In the case of immunoidentity, the standard curve and the dilution curve of the unknown will run parallel. A deviation of the dilution curve from the standard curve is either due to a different antigen with similar, but not identical, immunoreactivity or to catabolic processes in the body fluid changing the immunodeterminant site of the original antigen.

Human growth hormone is a single-chain peptide with a molecular weight of 21 500 [4]. It has been shown by several investigators that HGH-immunoreactivity is also found in a larger molecular weight fraction if pituitary- or serum-HGH is subjected to dextran gel filtration [5, 6, 7]. The existence of "big" hormones, as have been demonstrated for insulin [8], parathyroid hormone [9], gastrin [10], ACTH [11] and HGH [5, 6, 7], is

* Supported by DFG/SFB 51.

not fully explained. They can represent prohormones as has been shown for insulin [8], hormones bound covalently to proteins [11] or aggregated hormone complexes. Since prohormones should have a different tertiary structure because they contain additional amino acids a difference in immunoreactivity compared with the final hormone could be encountered. The amino acid sequence and the immunoreactivity of HGH is different from the other pituitary hormones except that human prolactin (HPr) seems to have a similar structure [12]. Even more related in biological activity, amino acid sequence and immunoreactivity is human placental lactogen (HPL) [13]. Thus, cross-reactivity of HPL with HGH antibodies has been found [14, 15].

The following study was designed to investigate the immunoreactivity of various pituitary HGH preparations before and after separation into "big" and regular HGH. The immunoreactivity was investigated by comparing the slope of the respective dilution curves with the standard curve of a reference preparation. This method was also applied to study the immunoreactivity of endogenous serum HGH. HGH of patients with acromegaly as well as endogenous HGH after maximal stimulation in patients with normal pituitary function were investigated. In addition to the endogenous HGH exogenous HGH after infusion into human subjects was investigated. To determine the degree of cross-reactivity of HPL and HPr with our HGH antibody, dilution curves of endogenous and exogenous HPL and endogenous HPr were compared with the HGH standard curve.

MATERIALS AND METHODS

HGH was measured by a semiautomated double antibody radioimmunoassay [16]. Three different HGH preparations were used: Roos HGH [17] containing 2 IU/mg protein (Kabi, Sweden, Lot No. 19156), Wilhelmi HGH (Societa Ricerche Impianti Nucleari, Saluggia, Italy) and Raben HGH containing 2 IU/mg protein. As reference preparation, highly purified Wilhelmi HGH from the National Institute of Health (batch NIH-GH-HS/394) was used. HGH standards as well as the serum samples were diluted with 0.13M borate buffer, pH 8.4, containing 0.5% bovine albumin. It could be demonstrated that the recovery of exogenous HGH added to HGH-free serum was not significantly influenced by a dilution up to 1:200 with the assay buffer. The graphs of the dilution curves were made in the following way: the B/B_0 ratio of the highest serum dilution was used to determine the HGH concentration. The HGH concentration of this sample was read from the steepest and most accurate part of the standard curve between 0.5 and 1.5 ng and was regarded as the reference concentration. From this reference concentration the concentrations of the other dilutions were calculated and the actual B/B_0 ratio was marked in the co-ordinate system. The resulting dilution curves as well as the standard curves were logit transformed according to Rodbard [18] which made the standard curve linear from 1 to 10 ng/ml.

Insulin hypoglycaemia tests were performed according to Greenwood et al. [19]. The diagnosis of active acromegaly was confirmed by glucose non-suppressable elevated HGH levels [20]. Dextran gel filtration was performed on a 3 cm × 110 cm column of Sephadex G-75. The buffer used for the gel filtration and in the radioimmunoassay of the eluate was 0.05M

sodium phosphate buffer with 0.1% sodium azide, pH 7.5. For radioimmunoassay 0.5% bovine albumin was added to this buffer. The column was saturated with 5 ml of 5% albumin before chromatography. ^{125}I-HGH was labelled according to Hunter and Greenwood [21] and prepurified on a Sephadex G-50 column. The pooled protein peak was then eluted on the G-75-column and fractions of 10 ml were collected. The radioactive material eluting at K_D 0.8 (peak III) was used as tracer for HGH radioimmunoassay. Forty μg of clinical-grade pituitary HGH (Roos) and 2 ml of serum from an acromegalic patient were used for Sephadex chromatography. For the radioimmunoassay of the column eluate two different HGH antibodies – goat anti-HGH and guinea-pig anti-HGH serum – were used. The recovery of the labelled HGH ranged from 90 to 100%, that of the non-labelled HGH from 80 to 90%.

The HGH used for infusion was the same Roos preparation as that used for chromatography (German Kabi, Ltd., Munich). 2 mg of this preparation in 250 ml of saline were infused for 30 min into five subjects with normal HGH secretion and five patients with HGH deficiency. Blood samples for radioimmunoassay were taken until 3 hours after the start of the infusion. The biological activity of the HGH was verified by measuring the increase in concentration of free fatty acids in serum and by its effect upon glucose-stimulated insulin secretion [22].

RESULTS

The dilution curves (= standard curves) of the three HGH preparations and the NIH reference HGH were shown to be identical which demonstrates immunoidentity of the standard preparations. The dilution curve of the TSH preparation (NIH, biological activity 3.5 IU/mg protein) shows an inhibition parallel to the HGH standard curve. This is due to contamination of the TSH with HGH (Fig. 1). From the TSH inhibition curve it can be deducted that 100 μU of the TSH contain 1 ng of HGH. There is partial

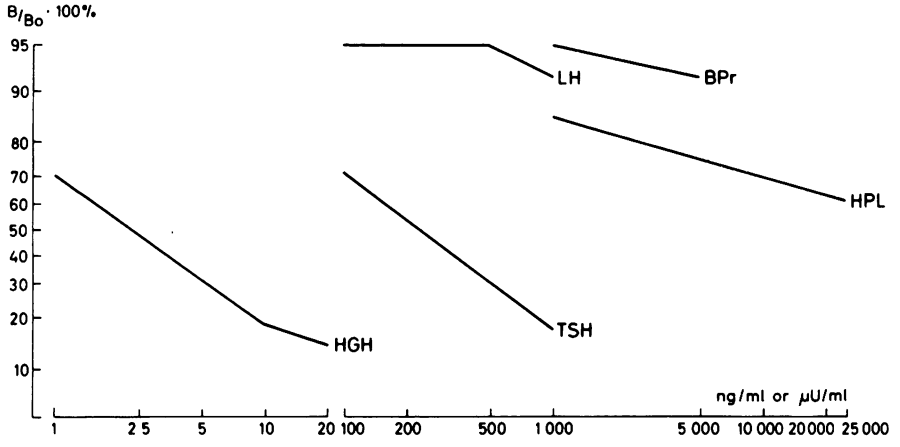

FIG. 1. HGH standard curve and inhibition curves of TSH, LH BPr and HPL with the HGH antibody.

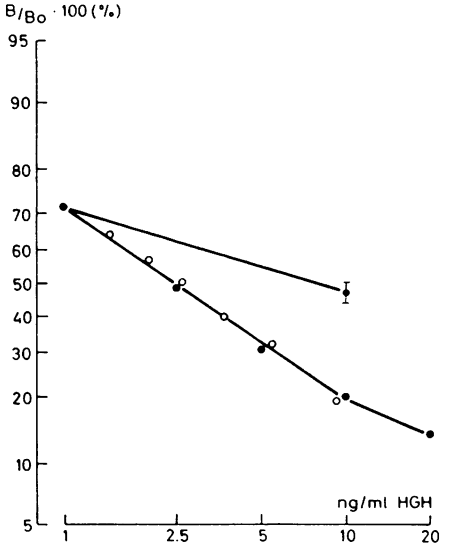

FIG. 2. Dilution curve of a serum containing 5.6 ng HGH/ml and 3200 ng HPr/ml (○) showing parallelism to the HGH standard curve (●). Deviating dilution curves of eight serum samples from pregnant women (—●— ± SE).

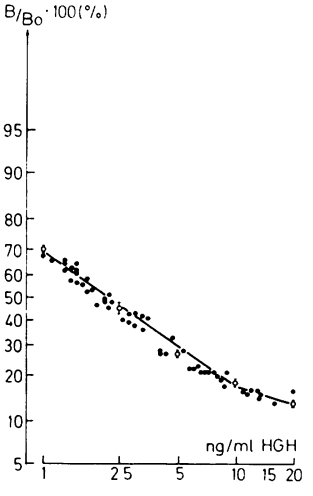

FIG. 3. HGH standard curve (○————○). The points (●) result from serum dilutions of ten acromegalic patients (± SE).

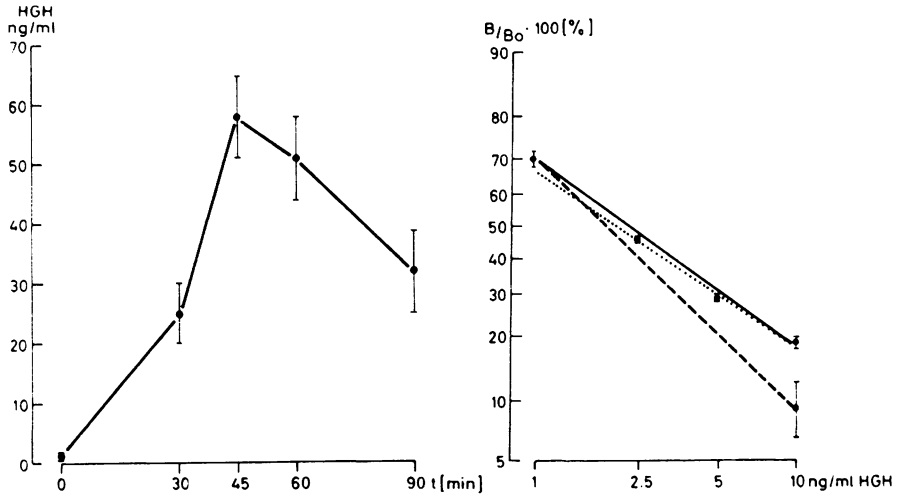

FIG. 4. Left: HGH response to insulin hypoglycaemia in eight subjects (●——●). Right: dilution curve of all 30-, 45- and 60-min samples (●·····●) and the 90-min samples (●— — —●) compared with the standard curve (——) (± SE).

cross-reactivity with HPL and no cross-reactivity with LH (3.7 NIH-LH-S_1/mg protein) and bovine prolactin (BPr). Cross-reactivity of endogenous HPL with the HGH antibody was demonstrated by the deviation of the dilution curves of eight serum samples from pregnant women with a mean HPL concentration of 12.3 ± 2.3 μg/ml ± SD (Fig. 2). The serum of a patient with a prolactin-producing adenoma containing 5.6 ng HGH/ml and high endogenous immunoassayable human prolactin (3200 ng/ml) showed no deviation from the standard curve (fig. 2). The dilution curves of 10 serum samples from patients with active acromegaly also did not deviate from the slope of the standard dilutions (Fig. 3). The HGH concentration ranged from 14 ng/ml to 200 ng/ml. In three patients samples were also taken from the jugular vein where the HGH concentration was 1.1 to 2.4 times higher than in the cubital vein. These findings demonstrate immunoidentity between HGH from acromegalics' serum and HGH standards.

The HGH response to insulin-induced hypoglycaemia of eight non-obese subjects with normal pituitary function is depicted in the left part of Fig. 4. These insulin hypoglycaemia tests were used for our dilution studies since they showed a rather high rise of HGH compared with that of 54 subjects who had a mean maximal HGH rise to 39.9 ± 3.0 ng/ml ± SE ranging from 16.5 to 92.0 ng/ml. The serum samples of the eight subjects were diluted and the resulting dilution curves of the 30- 45- and 60-min samples were shown to be parallel to the standard curve. Only the dilution curves of the 90-min sample deviated significantly from the standard curve and the other dilution curves (Fig. 4).

Dextran gel filtration of labelled growth hormone revealed three radioactive peaks (Fig. 5). The first peak bound only poorly to the antibody. A second peak eluted at K_D 0.45 and the third and largest radioactive peak eluted at K_D 0.8, representing the labelled regular HGH. Dextran gel

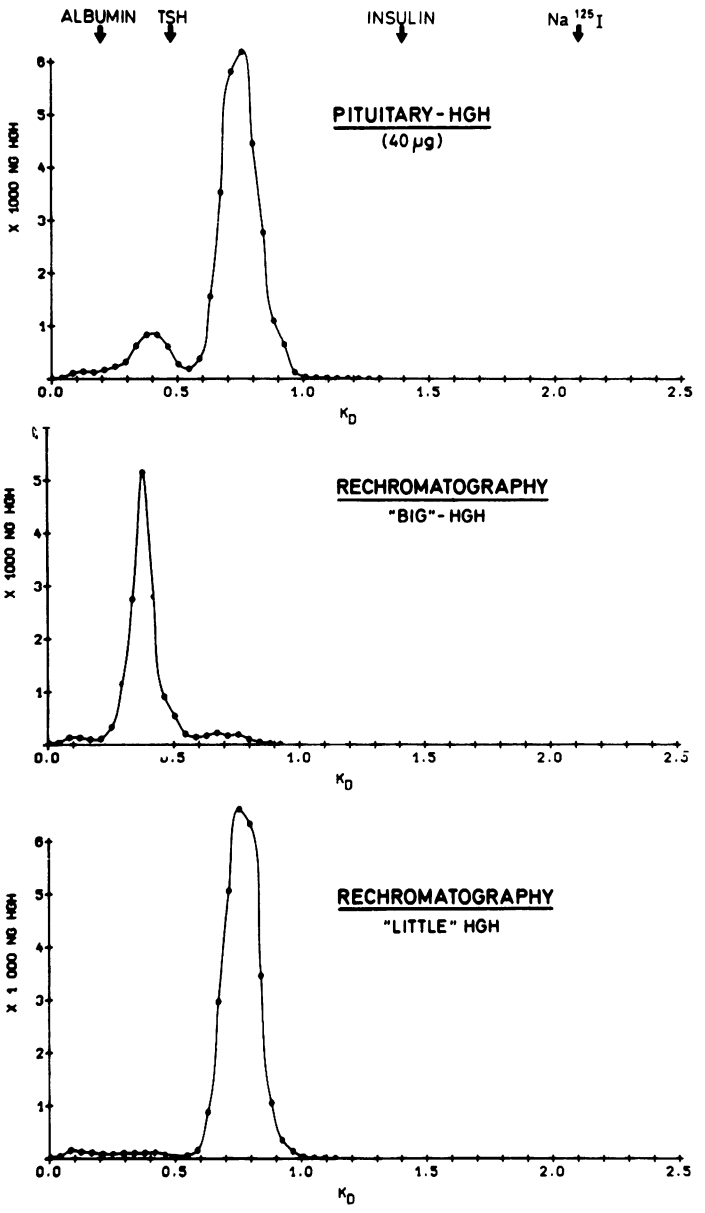

FIG. 5. Chromatography of ^{125}I-HGH on Sephadex G-75 (top). Three distinct peaks can be seen, the first eluting at K_D 0.18 (peak I), the second eluting at K_D 0.45 (peak II) and the third eluting at K_D 0.8 (peak III) representing regular HGH, used as tracer for radioimmunoassay. Pituitary HGH (middle) and acromegalic serum HGH (bottom) elutes in a similar pattern. The immunoreactivity in the area under peak I is small in relation to the radioactivity, but was found consistently.

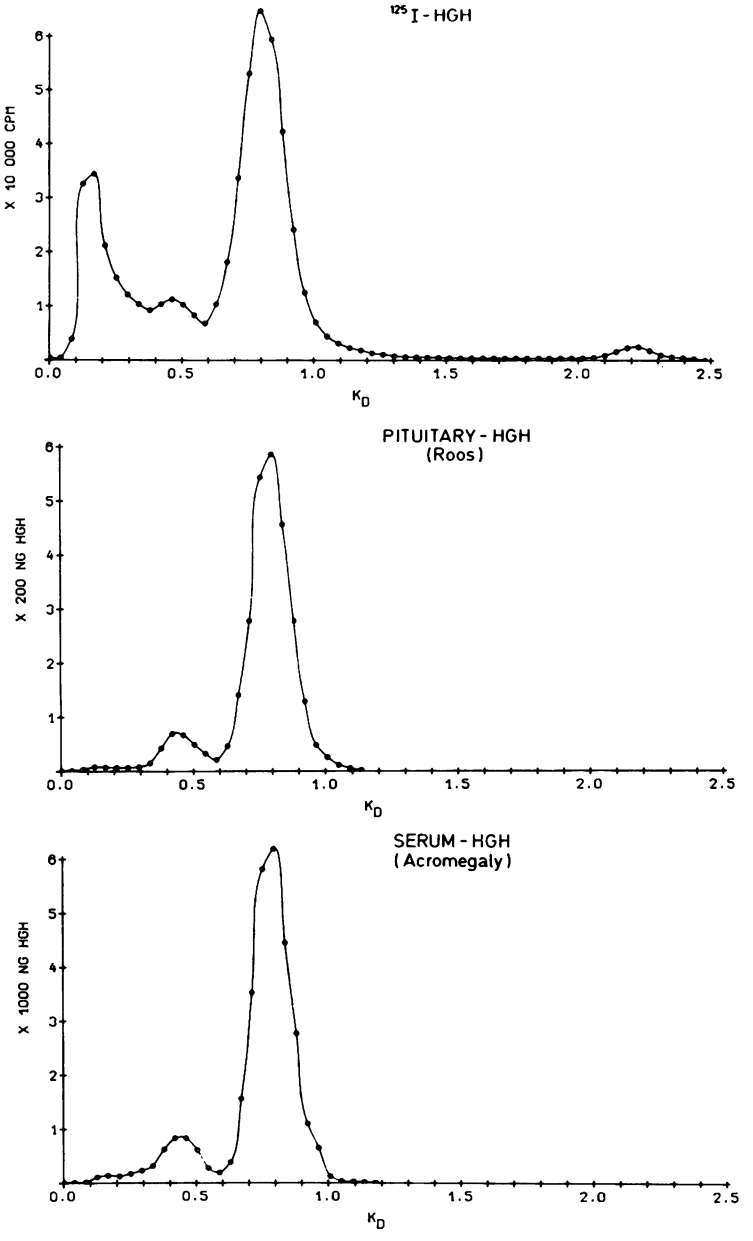

FIG. 6. Dextran gel filtration of 40 μg of pituitary human growth hormone. The arrows depict the eluted volume (Ve) of the various markers used. After rechromatography "big" HGH (peak II) and "little" HGH (peak III) elute as one single peak from the column.

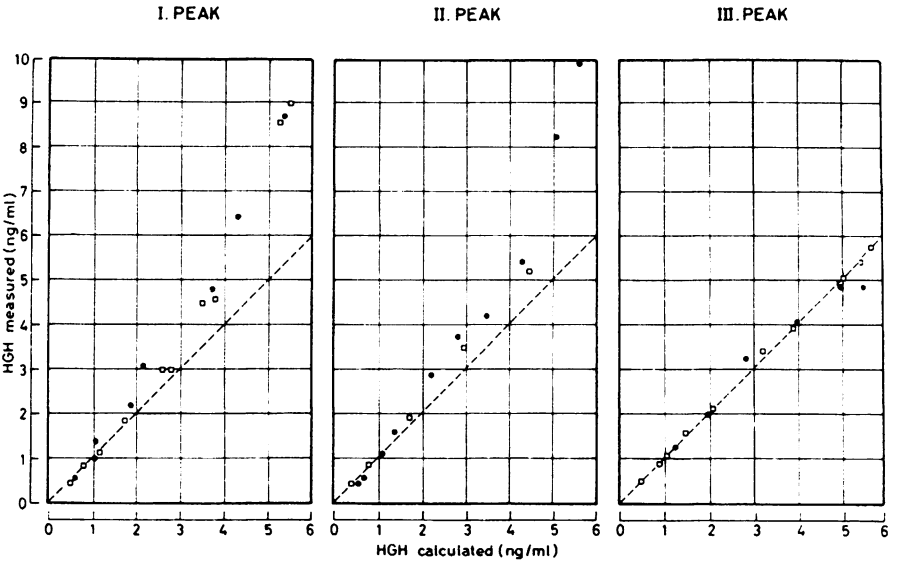

FIG. 7. Discrepancy of measured and calculated concentration of HGH immunoreactivity of peak I and peak II. Peak III shows good agreement between measured and calculated results. Two different antibodies were used: goat anti-HGH (●) and guinea-pig anti-HGH (□).

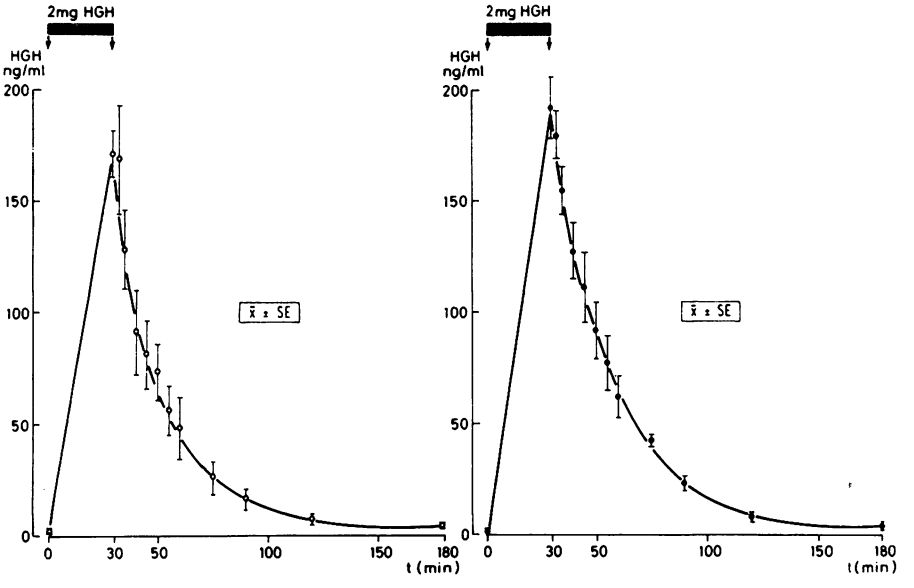

FIG. 8. HGH levels in subjects with normal pituitary function (○——○) and patients with HGH deficiency (●——●) after infusion of 2 mg of pituitary HGH.

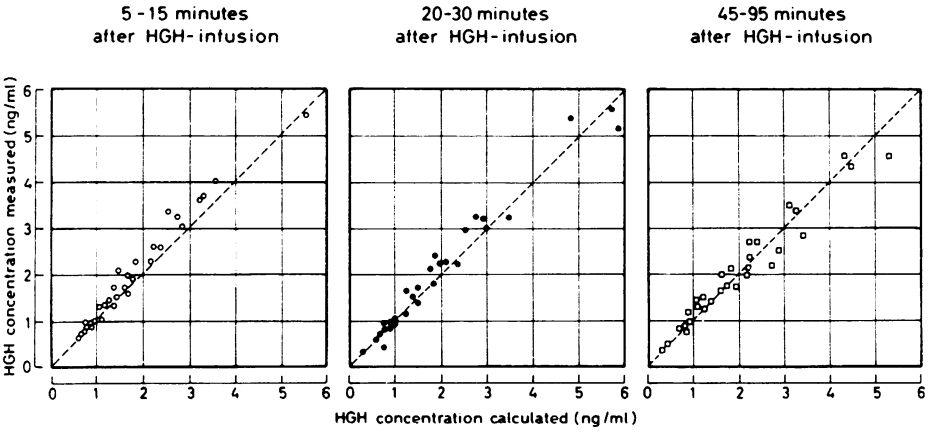

FIG. 9. Good agreement between measured and calculated HGH concentrations in the different dilutions of serum samples taken after infusion with 2 mg of pituitary HGH in subjects with and without endogenous HGH.

filtration of clinical-grade pituitary growth hormone and endogenous HGH from a patient with acromegaly and extremely elevated HGH levels (3100 ng/ml) also showed three peaks (Fig. 5). The homogeneity of immunoreactive fractions obtained under peak II and peak III was confirmed by rechromatography (Fig. 6). If the dilutions of the fractions containing peak III were analysed for their HGH concentration the measured HGH concentration was found to be identical with the calculated HGH content (Fig. 7). The dilution curve of the immunoreactivity of peak II showed a deviation from the standard dilution curve resulting in discrepancy between measured and calculated HGH concentrations in the different dilutions. This was even more pronounced for the immunoreactivity of peak I. The immunoreactivity of the larger molecular weight fractions was demonstrated to be different from that of the HGH standard with two different antibodies (Fig. 7).

The infusion of 2 mg of HGH into patients with regular HGH secretion resulted in a HGH rise of 171.6 ± 19.8 ng/ml. The HGH level in patients with endogenous HGH deficiency rose after infusion to 192.4 ± 34.8 ng/ml (Fig. 8). The difference in half-time of the disappearance from the serum of the HGH immunoreactivity between the two groups was not significant: 14.9 ± 3.9 min in subjects with normal HGH secretion and 19.1 ± 6 min in patients without endogenous HGH secretion. The dilution curves of the serum samples of both groups taken until 2 hours after the start of the infusion were parallel to the dilution of the infused HGH standard. There was therefore no difference between measured and calculated HGH concentration in the serum dilutions (Fig. 9).

DISCUSSION

Specificity is essential for radioimmunoassay of hormones. It relies on an antigenic determinant part – in the case of peptide hormones a certain

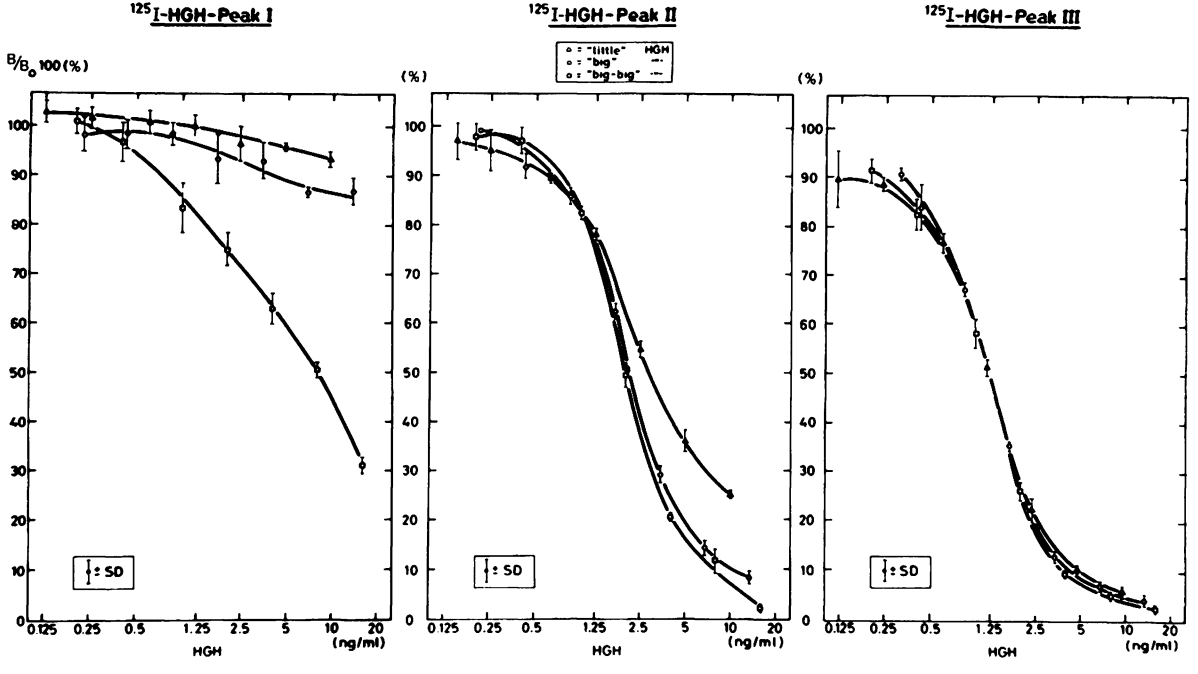

FIG. 10. Standard curves of "big-big" (□———□), "big" (○———○) and "little" (△———△) HGH with different ^{125}I-HGH tracers. Only "big-big" HGH inhibits ^{125}I-HGH-peak I binding to the antibody. The inhibition curves of the different HGH fractions are superimposable only when ^{125}I-HGH-peak III, i.e. the purified labelled "little" HGH, is used.

amino acid sequence – which is unique for the hormone to be measured. The structure of HGH has been revised recently [4]. According to Li et al. it is a single-chain peptide with 190 amino acids, two disulphide bridges and a molecular weight of 21 500. Bovine and human prolactin (BPr, HPr) seem to be very similar to HGH [12]. It is therefore surprising that HGH antibodies do not cross-react with BPr and HPr. The fact that there is no cross-reactivity is proven by the failure of BPr to inhibit ^{125}I-HGH binding to the antibody and by identity of the slope of the serum dilution curve prepared with HGH in the presence of a high concentration of HPr with the slope of the HGH standard curve. If the HPr were to react with the HGH antibody the serum dilution would deviate from the standard slope and the measurement of HGH would not be possible since one of the basic criteria for the validity of radioimmunoassays – identity of the dilution curves of standard and unknown – is not fulfilled.

HPL is a peptide hormone with 190 amino acids, two disulphide bridges and a molecular weight of 21 000 [13]. Since 160 of the 190 amino-acid residues of HPL occupy positions that are identical to those in HGH it is not surprising that both hormones have similar antigenic determinants. Because of the resulting cross-reactivity HGH measurements during pregnancy will remain difficult. This has been demonstrated before and is again pointed out by our results which show inhibition of ^{125}I-HGH binding to the HGH antibody by HPL standards (Fig.1) and non-parallelism of the HGH standard curves and the serum dilution curves for pregnant women. Since the other pituitary hormones have no structural similarity to HGH, cross-reactivity of these hormones with the HGH antibody should not occur. The displacement of ^{125}I-HGH by the TSH standard must be due to contamination of this preparation, as is demonstrated by the parallel TSH-inhibition curve (Fig.1) and the absence of interference from elevated endogenous TSH in the HGH immunoassay (unpublished data). According to these findings the only material in non-pregnant women that reacts with the HGH antibody should be HGH. Unless the HGH is altered, dilution curves of serum containing HGH and standards should be parallel. The immunoidentity of four different HGH preparations was documented in this manner. In contrast to Greenwood et al. [14], who described one acromegalic patient with circulating HGH immunologically different from the standard preparation, we found identical HGH immunoreactivity in all our patients (Fig.3). When subjects with normal HGH secretion were investigated we found that individuals with very pronounced HGH responses to insulin hypoglycaemia showed a change in immunoreactivity 90 min after insulin was given (Fig.4). Landon et al. [23] also found a change in ACTH immunoreactivity after maximal stimulation of its secretion. These findings could be explained by two different mechanisms:

1. Catabolism of the circulating antigenic peptide gives rise to fragments of the antigen which only have partial immunoreactivity.
2. After maximal stimulation the pituitary secretes precursor hormones which differ immunologically from the original hormone.

Heterogeneity in molecular size of peptide hormones has been described [7-11]. HGH immunoreactivity has been shown to be present in various molecular fractions [5, 6, 7, 24]. Our results demonstrating HGH immunoreactivity eluting in a less retarded peak from the Sephadex column are in

good agreement with these of Gorden et al. [6] and Goodman et al. [7], who found the less retarded "big" HGH (peak II, Fig.5) in pituitary HGH, in serum of acromegalics and in serum of subjects with normal HGH secretion. They also found immunoreactivity being eluted before "big" HGH in some of their samples. In our studies significant amounts of immunoreactive material were always eluted just ahead of albumin (peak I, Fig.5), which has also been demonstrated by Berson and Yalow [24]. According to Goodman et al. [7] "big" and regular HGH have identical immunoreactivity [7]. By using the receptor assay Gordon et al. [6] showed in preliminary studies a lower affinity to the receptor of "big" compared with regular HGH. Our studies demonstrate with two different antisera to HGH that "big" (peak II) and more pronounced "big-big" (peak I) react differently with the HGH-antibodies, compared to regular HGH (peak III). Since the difference in immunoreactivity between "big" and regular HGH is not so obvious and will differ depending on the antibody used, this may explain the discrepancy between our results and those of Goodman et al. The immunoreactivity of "big-big" HGH has not been investigated by these authors. Of interest is the finding that serum of acromegalics contain less "big" HGH than serum of patients after insulin hypoglycaemia [6]. One could assume, therefore, that the change in HGH immunoreactivity during insulin hypoglycaemia is due to the increase of "big" and, maybe, "big-big" HGH in the serum. "Big" forms of HGH are able to cause better inhibition of ^{125}I-HGH peak II than "little" HGH which causes a change in the slope of the inhibition curve (Fig.10). The conversion of infused HGH into larger immunoreactive molecules in the circulation is improbable since no immunological changes in circulating HGH can be observed over a period of 2 hours after infusion (Fig.9).

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DISCUSSION

Rosalyn S. YALOW: Your larger growth hormone (GH) components seem to be much more stable than the "big" GH of other workers. Are there any particular methods which help in maintaining the stability of the "big" form?

Frohman and Friesen carried out independent biosynthetic studies to establish the precursor nature of the two larger hormonal forms, and also noted a usually rapid appearance of labelled amino acids in the "little" form, again suggesting lack of stability of the "bigger" forms, as compared to the "bigger" forms of other hormones, such as insulin or gastrin.

The question of stability is important in considering whether the "bigger" forms result from covalent bonding.

K. von WERDER: We have, in fact, seen very little conversion of "big" into "little" HGH, even after storage of the "big" HGH preparation for four weeks. These results are, however, in contrast to the findings of Goodman et al.[1] and Gorden et al.[2]. Our methodology differs from theirs in that we use 0.05 M phosphate buffer for elution, and perform our gel filtration at 24°C.

Since Goodman et al. recently reported at Chicago that there is a labile "big" HGH, which is convertible into "little" HGH by 6 M urea, as well as by freezing and thawing the "big" HGH preparation, and that there is in addition a stable form of the "big" hormone which is not so converted, it might be that we are dealing mainly with the latter. Our serum samples were frozen and thawed at least once and so most of the convertible "big" HGH may have been converted. The "big" HGH remaining was therefore in the stable form. We believe, as suggested by Goodman, that we are dealing with two forms of "big" HGH, one of which is not covalently bound to another protein, while the other is. The latter is the stable form and, in our opinion, a true precursor hormone.

[1] See Ref. [7] of the paper.
[2] See Ref. [6] of the paper.