A Radio-Immunoelectrophoretic Assay for Human Growth Hormone

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A number of attempts have been made to measure the amount of growth hormone in human plasma or in extracts thereof.

By using the tibia-line biological assay Gemzell (1959) and Forsman & Gemzell (1959) detected growth hormone in extracts from subjects with acromegaly or diabetes but not in extracts of normal plasma. Owing to the lack of sensitivity and specificity of biological assays for growth hormone no further attempts with these methods have been reported. The sulphation factor in serum has been used as an indication of human growth hormone (Daughaday, Salmon & Alexander, 1959; Almquist & Falkheden, 1961), but despite its usefulness the values obtained are not direct measurements of the hormone.

An immunological approach to the problem was provided by the work of Hayashida & Li (1958) and of Read & Stone (1958), who produced antiserum to purified human growth hormone in the rabbit. The reaction between growth hormone and its antibody has formed the basis of a number of assays for the hormone. Read & Stone (1958) and Read & Bryan (1960) have used the haemagglutination-inhibition technique for this purpose. Subsequent experience (cf. Read, Eash & Najjar, 1962; Grumbach & Kaplan, 1962) showed that it was subject to gross interference by plasma. However, Ehrlich & Randle (1962) obtained reproducible results with the method and were able to use it for comparative purposes in studying diabetes mellitus. The removal of non-specific inhibition by an extraction procedure has been reported by Dominguez & Pearson (1962), and the concentrations of growth hormone in normal adults were found to be less than 20 $\mu\text{mg./ml.}$ of serum.

Methods based on the quantitative precipitin reaction (Li, Moudgal & Papkoff, 1960), complement fixation (Trenkle, Moudgal, Sadri & Li, 1961) and the precipitin reaction with ^{125}I -labelled antibody (Greenspan, Cofell, Lew & Peng, 1962) do not appear to be subject to interference by plasma. However, the sensitivity of these methods is not adequate for the measurement of growth hormone in plasma. Greater sensitivity was achieved by Utiger, Parker & Daughaday (1962) by using im-

muno-precipitation and ^{131}I -labelled growth hormone. With plasma extracted for growth hormone by the method of Gemzell (1959) interference was decreased to the limit of sensitivity of the method, i.e. 7.5 $\mu\text{mg.}$ of growth hormone/ml. of plasma. Growth hormone was found in 12 out of 30 normal adults and where detected ranged from 7.5 to 50 $\mu\text{mg./ml.}$ of plasma.

The radio-immunoelectrophoretic technique developed by Yalow & Berson (1960) for the assay of human insulin has been applied to human growth hormone (Hunter & Greenwood, 1962*a*). Greater sensitivity has been achieved (1 $\mu\text{mg./ml.}$) and lower concentrations of growth hormone in extracts of plasma from normal adults obtained (2.4–5.6 $\mu\text{mg./ml.}$ of plasma). A method for the assay of growth hormone based on these principles has also been developed by Glick, Roth, Yalow & Berson (1963) and a lower limit of sensitivity of 0.1 $\mu\text{mg./ml.}$ reported. The present paper describes the radio-immunoelectrophoretic assay for growth hormone and subsequent developments that enable growth hormone to be measured in extracts of plasma from normal adults and in unextracted diluted plasma from children and acromegalic subjects. The quantitative lower limit of sensitivity is defined as that amount of growth hormone which is distinguishable from zero and measured with a maximum percentage error of $\pm 50\%$ ($P 0.05$). The sensitivity calculated from a large series of assays for growth hormone is 0.22 $\mu\text{mg./ml.}$ in plasma as extracts and 0.07 $\mu\text{mg./ml.}$ in aqueous solutions for duplicate estimations.

MATERIALS

Unless otherwise stated, sodium salts were used in the preparation of buffers.

Human growth hormone. Initially a gift of growth hormone from the Clinical Endocrinology Committee of the Medical Research Council was used. Growth hormone was subsequently prepared from acetone-dried human pituitaries by the method of Raben (1959) and is referred to as purified or standard growth hormone. Homogeneity studies on such preparations have been carried out (Hunter, 1963). All batches studied contained a component (peak I) separable from growth hormone by gel-filtration on Sephadex G-200 with 0.05 M-borate-0.05 M-potassium chloride buffer, pH 8.6. This component had a similar sedimentation behaviour to growth hormone (peak II)

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although it was unretarded on Sephadex G-200. It did not react with antiserum raised against standard growth hormone. Growth-hormone preparations (peak II) obtained from such Sephadex columns have not been used as standards in the present assay but have been used in the preparation of ^{131}I -labelled growth hormone. In an experiment carried out in conjunction with Dr H. E. C. Cargill Thompson (Clinical Endocrinology Research Unit, Edinburgh), peak I and peak II materials were assayed in terms of the starting material by the radio-immunoassay and by the tibia-line bioassay. Peak I was immunologically 18.4% and biologically 19.7% (fiducial limits 13–30%) of the starting material. Peak II was immunologically 120% and biologically 142% (fiducial limits 111–228%) of the starting material.

^{131}I -labelled growth hormone. This was prepared weekly by the method of Hunter & Greenwood (1962b) except that the buffering capacity of the reaction mixture was increased by the addition of 0.025 ml. of 0.5 M-phosphate buffer, pH 7.5. The amount of [^{131}I]iodide allowed to react with 5 μg . of growth hormone was kept at 2 mc of IBS-3 (The Radiochemical Centre, Amersham) for the reasons described by Greenwood, Hunter & Glover (1963). Specific activities ranged from 200 to 280 $\mu\text{C}/\mu\text{g}$.

Rabbit antiserum to growth hormone. Antiserum to growth hormone was obtained in adult rabbits essentially by the method described by Read & Bryan (1960). In most cases Freund's complete adjuvant (Difco) was used as a vehicle for the three subcutaneous injections. Antiserum III used in the assay was obtained from an adult male rabbit bled 6 days after the second intravenous injection of growth hormone (Medical Research Council, batch 1), the latter injections being separated by an interval of 4 days. Antisera were heated in a water bath at 56° for 30 min. to inactivate complement, centrifuged at 8720 g_{av} in a Spinco model L centrifuge (no. 30 rotor) for 20 min. Samples (1:1, 1:20 or 1:1000 in diluent) were stored at –20° without preservatives.

Human and monkey pituitary extract. An aqueous alkaline extract was obtained from a single human pituitary and from two fresh monkey pituitaries kindly supplied by the Division of Immunological Products Control, Medical Research Council. The pituitaries were each homogenized in 2 ml. of water, 0.2 ml. of 0.1 N-NaOH was added and the homogenate was diluted to 10 ml. with 0.07 M-barbitone buffer, pH 8.6. The homogenate was then centrifuged at 2000 g in a refrigerated centrifuge and the supernatant retained.

Animal plasmas. Horse, sheep, rabbit and calf sera were obtained from Burroughs Wellcome, Beckenham, Kent. Turkey, chicken, guinea-pig and rat plasma were kindly supplied by colleagues. Monkey plasma was kindly supplied by the Department of Biological Standards, Medical Research Council.

Diluent. This was a solution of crystallized bovine albumin (Armour and Co., Eastbourne, Sussex) (0.5 mg./ml.) in 0.07 M-barbitone buffer, pH 8.6.

METHODS

Radio-immuno-electrophoretic assay for growth hormone. The assay is carried out with three different amounts of added ^{131}I -labelled growth hormone (2.0, 0.2 and 0.05 μmg .) in an incubation mixture of final volume 0.5 ml. These

three concentrations give ranges for amounts of standard growth hormone respectively of 2.0–32, 0.2–3.2 and 0.05–0.8 $\mu\text{mg.}/0.4$ ml. added to the incubation mixture. These ranges have been found to be convenient for the following respective groups of test samples: pituitary extracts; children, adolescents and acromegalic subjects; normal adults, hypopituitary and hypophysectomized subjects. In each assay test samples are estimated at a minimum of two concentrations together with an appropriate series of standards with Raben growth hormone. All additions to the incubation tubes (8 cm. $\times$ 1 cm.) are made in diluent.

Assay with 0.2 μmg . of ^{131}I -labelled growth hormone. The incubation mixture consists of solutions of standard growth hormone, ^{131}I -labelled growth hormone and antiserum. Standard growth hormone (0.0, 0.2, 0.4, 0.8, 1.6 and 3.2 μmg . in 0.4 ml. of diluent) or plasma (0.2 and 0.4 ml., or 0.1, 0.2 and 0.4 ml. of a 1:4 dilution) are first added to the incubation tubes. For acromegalic plasma doubling volumes from 0.05 to 0.4 ml. of a 1:16 dilution of plasma are taken. All volumes are made up to 0.4 ml. by the addition of diluent. The ^{131}I -labelled growth hormone (0.05 ml. of a solution containing 4 $\mu\text{mg.}/\text{ml}$. of diluent) is then added with an Agla micrometer syringe. The contents of the tubes are gently mixed after this addition. Rabbit antiserum to growth hormone (0.05 ml. of a 1:20 000 dilution of antiserum, giving a final dilution in the incubation mixture of 1:200 000) is then added with an Agla micrometer syringe. The antiserum is previously titrated against 0.2 μmg . of ^{131}I -labelled growth hormone as described below. The contents of the tubes are mixed gently, capped with Parafilm (Gallenkamp-Towers, London) and incubated for 5–7 days at 4°.

The determination of the percentage of the radioactivity of the ^{131}I -labelled growth hormone bound to the immune γ -globulin after incubation is determined by electrophoresis and radioactivity determinations. Horse serum (0.1 ml.) is added to each tube after incubation to facilitate the electrophoretic separation. A sample (0.025 ml.) from each tube is applied to the centre of cellulose acetate strips (Oxoid) (20 cm. $\times$ 2.5 cm.) previously soaked in buffer (0.023 M-barbitone, pH 8.6) and the excess removed by lightly blotting the strips on filter paper dampened with the buffer. Electrophoresis is carried out at room temperature in Shandon Universal electrophoresis tanks with a bridge gap of 17.5 cm. A number of tanks are used, usually six in parallel each with seven strips with two unused outer-edge strips (20 cm. $\times$ 1.25 cm.). Electrophoretic separation of unbound ^{131}I -labelled growth hormone from that bound to antibody requires 3½ hr. at a constant output voltage of 400 v with 0.023 M-barbitone buffer, pH 8.6. The strips are dried in air at room temperature and cut into two at a line 1.5 cm. on the cathode side of the centre sample line. The smaller section contains the ^{131}I -labelled growth hormone bound to the γ -globulin, and the larger section contains unbound or free ^{131}I -labelled growth hormone. The sections are rolled into scrolls, tied with cotton and placed into counting vials (20 ml.) containing liquid phosphor (Panax TTP/4) (15 ml.). Samples are counted for 10 min. in a Packard Tri-Carb automatic liquid-scintillation system, which for 0.4 μmc of IBS-3 (The Radiochemical Centre, Amersham) gives 2000 counts/min. and a background count of 30 counts/min.

The percentage of the radioactivity of the ^{131}I -labelled growth hormone bound to immune γ -globulin in the presence

of standard growth hormone is calculated and plotted against the amounts of standard added by using a logarithmic scale for the latter (Fig. 1). The amounts of growth hormone in the test samples are calculated from this curve.

Assay with 0.05 $\mu\text{mg.}$ of ^{131}I -labelled growth hormone. The procedure as described above is used with the following modifications. The amounts of standard growth hormone are 0.0, 0.05, 0.1, 0.2, 0.4 and 0.8 $\mu\text{mg.}/0.4$ ml. of diluent. Test samples are extracts from plasmas obtained by the procedure described below and estimated at 0.2 and 0.4 ml. volumes of the final extract. The amount of ^{131}I -labelled growth hormone is 0.05 $\mu\text{mg.}/\text{incubation volume of } 0.5$ ml. (0.05 ml. of a solution of ^{131}I -labelled growth hormone containing 1 $\mu\text{mg.}/\text{ml.}$ of diluent). Rabbit antiserum (0.05 ml.) is added as a 1:70 000 dilution to give a final dilution of 1:700 000. The incubation time is extended to 12–14 days. No antibacterial agents were present in any incubation mixture. After the addition of horse serum (0.1 ml.) samples of the mixture (0.05 ml.) are applied to cellulose acetate strips (20 cm. $\times$ 5 cm.). Three such strips are used for each tank with two unused outer-edge strips (20 cm. $\times$ 2.5 cm.). The strips are cut as before and each section is counted for 30 min. The standard curve for this assay is shown in Fig. 1.

Assay with 2.0 $\mu\text{mg.}$ of ^{131}I -labelled growth hormone. The amounts of standard growth hormone, labelled hormone and antiserum are as follows: standards, 0, 2, 3, 4, 6, 8, 12, 16 and 32 $\mu\text{mg.}$; ^{131}I -labelled growth hormone, 0.05 ml. of a solution containing 40 $\mu\text{mg.}/\text{ml.}$ in diluent; antiserum, 0.05 ml. of a 1:2000 dilution of antiserum, the final dilution in the incubation mixture being 1:20 000. The incubation time is 3–5 days. Electrophoresis of 0.02 ml. samples, after the addition of horse serum, is carried out on cellulose acetate strips (20 cm. $\times$ 1.7 cm.). Each tank contains ten such strips with two unused outer-edge strips (20 cm. $\times$ 1.7 cm.). The counting time for the sectioned strips is 5 min. The standard curve for this assay is shown in Fig. 1.

Extraction of plasma for growth hormone. Extraction is based on the procedure for the isolation of pituitary growth hormone (Raben, 1959). Volumes of plasma down to 3 ml. can be handled. All solutions and steps are at 4°. To the plasma (10 ml.) is added rapidly with stirring 30 ml. of acetic acid-acetone (1:1, v/v) and the mixture left for 10 min. to flocculate. The precipitate formed is recovered by centrifugation (1800g for 10 min.) and re-extracted twice with successive volumes of acid acetone (20 ml. and 10 ml.), with centrifuging after each extraction. The supernatants (60 ml.) are combined in a centrifuge bottle and the total protein is precipitated with ether (300 ml.), added rapidly with stirring. After standing for 1 hr. the precipitate is centrifuged (1200g for 10 min.), the supernatant discarded and the precipitate washed with acetone (1 $\times$ 100 ml.) and ether (1 $\times$ 100 ml.) at the centrifuge (1200g for 5 min.). To the precipitate is added 3 ml. of 0.07M-barbitone buffer, pH 8.6, and the residual ether is removed under reduced pressure in a vacuum desiccator until frothing ceases. The suspension is dissolved by adjusting the pH to 8.6 $\pm$ 0.05 with N-NaOH and mixing with a pipette. The measurement of the pH of small volumes of solution in large centrifuge bottles is facilitated by using extended microelectrodes (Electronic Instruments Ltd., Richmond, Surrey). The contents of the centrifuge bottle are transferred to a graduated, conical centrifuge tube (10 ml.) with two washes of buffer (2 ml.) and centrifuged at

2000g for 30 min. The final volume is recorded and samples (0.2 and 0.4 ml.) are added to the incubation mixture.

Preliminary titration of antiserum. This is carried out for each new antiserum and when a new batch of growth hormone is used for ^{131}I -labelling. The ^{131}I -labelled growth hormone (0.1 ml. of a solution containing 2 $\mu\text{mg.}/\text{ml.}$ of diluent) is incubated with 0.4 ml. volumes of antiserum at final dilutions, in an incubation mixture of total volume 0.5 ml., from 1:20 to 1:5 120 000. Incubation is carried out for 5–7 days at 4° and the percentage of the radioactivity of the ^{131}I -labelled hormone bound to the immune γ -globulin is determined by electrophoresis as described above.

Fig. 2 shows a plot of the results obtained by using two rabbit antisera titrated against 0.2 $\mu\text{mg.}$ of ^{131}I -labelled

Fig. 1. Standard assay curves for growth hormone: comparison of the amounts of growth hormone required to decrease the amount of ^{131}I -labelled growth hormone bound to immune γ -globulin. Δ , ^{131}I -labelled growth hormone (2 $\mu\text{mg.}$) and antiserum (1:20 000 dilution); $\square$, ^{131}I -labelled growth hormone (0.2 $\mu\text{mg.}$) and antiserum (1:200 000 dilution); $\circ$, ^{131}I -labelled growth hormone (0.05 $\mu\text{mg.}$) and antiserum (1:700 000 dilution).

Fig. 2. Titration of rabbit antisera to growth hormone with ^{131}I -labelled growth hormone: the relationship between the percentage of the radioactivity of ^{131}I -labelled growth hormone (0.2 $\mu\text{mg.}$) bound to the immune γ -globulin and the antiserum dilution. $\square$, Rabbit antiserum VII; $\circ$, rabbit antiserum III.

growth hormone. The strongest antiserum (III) of the 13 tested was used for the routine assay and a final antiserum dilution selected for this (1:200 000) such that 60–70 % of 0.2 μ mg. of ^{125}I -labelled growth hormone was bound to immune γ -globulin in the absence of unlabelled growth hormone. The final antiserum dilution for the 2 μ mg. assay is then 1:20 000 and for the 0.05 μ g. assay is theoretically 1:800 000. In practice a slightly higher antibody concentration (1:700 000 dilution of antiserum) is required to bind 60–70 % of the added ^{125}I -labelled growth hormone.

RESULTS

Separation of unbound ^{125}I -labelled growth hormone from that bound to γ -globulin

Paper electrophoresis will readily separate γ -globulin from large amounts of growth hormone (50 μ g.) but not from the micromilligram amounts of ^{125}I -labelled growth hormone present in incubation mixtures. The adsorption of insulin to paper was utilized by Yalow & Berson (1960) in a chromatoelectrophoretic system for separating free ^{125}I -labelled insulin from that bound to antibody. With this technique with paper or cellulose acetate it was found that migration of all of the bound ^{125}I -labelled growth hormone was not possible without releasing some unbound ^{125}I -labelled growth hormone adsorbed at the start line. Electrophoresis on cellulose acetate (Kohn, 1958) was then attempted with 0.07 M-barbitone buffer, pH 8.6. Samples of incubation mixtures were taken after the addition of 0.1 ml. of horse serum to compensate for differences in the protein concentration of the incubation mixtures. Samples were applied to cellulose acetate strips (20 cm. $\times$ 2.5 cm.) at a point 2 cm. on the cathode side of the centre. Under these conditions antibody-bound ^{125}I -labelled growth hormone moved 1 cm. towards the cathode, by electroendosmosis, and the free ^{125}I -labelled growth hormone migrated as a broad peak to the anode with a marked trail to the line of application. The electrophoretic migration of free ^{125}I -labelled growth hormone to the anode was therefore decreased and the electroendosmotic migration of bound ^{125}I -labelled growth hormone to the cathode increased by decreasing the molarity of the buffer (to 0.023 M-barbitone) and applying the sample to the centre of the strip. After electrophoresis the free ^{125}I -labelled growth hormone was found as an asymmetric peak 1 cm. on the anode side of the start line. This peak was spread to a point 1.5 cm. on the cathode side. With antibody in excess (1:200 dilution of antiserum) γ -globulin together with bound ^{125}I -labelled growth hormone migrated 4–5 cm. towards the cathode and a sharp symmetrical peak of radioactivity was found (Fig. 3). Under the conditions of the assay in which a limited amount of antibody is present, the anti-

gen-antibody complex migrates between the β - and γ -positions. Over a period of 5 months such strips together with controls of free ^{125}I -labelled growth hormone alone and bound ^{125}I -labelled growth hormone alone were stained and scanned for radioactivity by using the 4 π Nuclear-Chicago Actigraph (1032 and 8408). It was found that cutting the strip 1.5 cm. on the cathode side of the centre starting line coincided with a well-marked trough between the free and bound ^{125}I -labelled growth hormone areas. The anodic section contained approx. 98 % of the free ^{125}I -labelled growth hormone. The separation is less than ideal and the conditions are empirical, but once determined they yield reproducible results. Only a fraction of the radioactivity of the incubation mixture is used. Attempts were made to separate all of the free and bound ^{125}I -labelled growth hormone on columns of Sephadex G-200 (2.5 g.). However, under the buffer conditions used, 0.05 M-borate-0.05 M-potassium chloride, an immunologically inert component

Fig. 3. Separation of free ^{125}I -labelled growth hormone from ^{125}I -labelled growth hormone bound to immune γ -globulin. Samples from incubation mixtures of ^{125}I -labelled growth hormone and antiserum were applied to cellulose acetate strips at the points marked by arrows. After electrophoresis the strips were scanned for radioactivity from the anode (+) to the cathode (-). The broken line represents the cut taken as a routine to separate bound and free ^{125}I -labelled growth hormone. The final dilutions of antiserum in the incubation mixtures were 1:20, 1:20 000 and 1: ∞ in (a), (b) and (c) respectively.

was found in free ^{131}I -labelled growth hormone which was unretarded on the column and formed part of the γ -globulin area. With the removal of this component, by prior separation on Sephadex G-200, separation of bound and free ^{131}I -labelled growth hormone on Sephadex G-200 with 0.07 M-barbitone buffer, pH 8.6, was superior to that obtained by electrophoresis.

Extraction of plasma with acid acetone

Acid-ethanol extracts of human plasma yield essentially the albumin fraction together with growth hormone (Gemzell, 1959). Although such extracts are inefficient in terms of concentration of the hormone ($\times 2$) their stability and solubility are useful. To avoid the dialysis and freeze-drying steps involved in Gemzell's (1959) method the present version was based on the method of isolating human pituitary growth hormone (Raben, 1959). The proportion of acetic acid in acetone and the volume of ether used to precipitate this extract were obtained separately from recoveries of added ^{131}I -labelled growth hormone. A 1:1(v/v) mixture of acetic acid and acetone gave the highest recovery (90%) while removing most of the plasma proteins. In the precipitation step increasing recoveries of ^{131}I -labelled growth hormone were obtained as the volume of ether was increased. A precipitation of 91% of the added ^{131}I -labelled growth hormone, obtained with 5 vol. of ether, was accepted, and enabled the precipitate to be harvested in a single centrifugation. The overall recovery of ^{131}I -labelled growth hormone measured as ^{131}I was shown to be 82%. The results of re-

covery experiments of growth hormone added to plasma are shown in Table 1. The quantitative recovery of the endogenous growth hormone of acromegalic subjects suggests that all of it is available in plasma for reaction with antibody and that it has solubility properties in common with the pituitary hormone.

Incubation time

In the quantitative precipitin reaction between antibody to growth hormone and growth hormone a finite time, usually 4 days, is required for maximal precipitation as measured by a micro-Kjeldahl method (cf. Grumbach & Kaplan, 1962). In the radio-immunoassay at the concentration of reactants used (micromilligram amounts) no insoluble precipitates are obtained but maximum reaction, as judged by the percentage of the antigen bound to antiserum, requires a finite incubation time. Longer periods of incubation were required as the sensitivity of the assay was increased by decreasing the concentrations of the reactants. The period of incubation has to be determined for the particular concentrations since the reaction did not follow simple kinetics. A time-course of the reaction between ^{131}I -labelled growth hormone (2.5 $\mu\text{mg.}$) and antiserum (1:12000) at room temperature showed that 42% of the ^{131}I -labelled growth hormone was bound to immune γ -globulin within the first hour, that the reaction was essentially linear up to 8 hr. (63% bound) and that a further 40 hr. was required for maximum binding (77%). However, an assay can be based on a short but precise period (18 hr.) of incubation at room tem-

Table 1. *Recovery of added and endogenous growth hormone from plasma and plasma extracts*

Growth hormone in the amounts indicated was added to whole plasma and estimated together with control plasma without added growth hormone. Estimations were carried out in duplicate either on plasma (1:4 to 1:8 dilutions) or on extracts prepared by the method described in the text.

Plasma source	Amount of growth hormone added ($\mu\text{mg./ml.}$)	Material assayed	Growth hormone ($\mu\text{mg./ml.}$)		Recovery (%)
			Endogenous	Total recovered	
Normal adult	0.5	Extract	0.22	0.62	80
	2.0	Extract	0.22	1.78	78
Normal child	2.0	Plasma	2.6	4.7	105
	4.0	Plasma		6.6	100
	8.0	Plasma		10.4	98
	16.0	Plasma		16.4	84
Lactating woman	4.0	Plasma	4.7	8.3	90
	8.0	Plasma		13.1	105
	16.0	Plasma		20.8	100
	32.0	Plasma		32.4	87
Acromegalic subject	—	Plasma	31.3	—	96
	—	Extract	29.9	—	
Acromegalic subject	—	Plasma	64.0	—	105
	—	Extract	67.0	—	
Rhesus monkey	—	Plasma	30.4	—	65
	—	Extract	19.8	—	

perature. This requires a greater amount of antiserum to achieve the same percentage binding of the added ^{131}I -labelled growth hormone than an assay incubated for longer periods at 4° . Such a procedure provided more rapid results but the increased amounts of growth hormone, either the purified standard or that present in plasma, required to inhibit with the higher amount of antiserum entailed a marked decrease in sensitivity.

The time required for the system ^{131}I -labelled growth hormone-standard growth hormone-antiserum to reach equilibrium is determined by analysing reaction mixtures at daily intervals until the values for the percentage bound show no further change. At equilibrium the slope of the standard curve reaches a maximum.

The limited reversibility of the reaction was demonstrated by first allowing ^{131}I -labelled growth hormone ($0.5\text{ }\mu\text{mg.}$) to react with antiserum (1:35 000 dilution). After 4 days of incubation at 4° some 65% of the ^{131}I -labelled growth hormone was bound to antiserum. The addition of $264\text{ }\mu\text{mg.}$ of standard growth hormone followed by a further 4 days of incubation resulted in a fall in the percentage of the ^{131}I -labelled growth hormone bound from 65 to 50%. In a control incubation the same decrease in the percentage bound was obtained by adding $0.64\text{ }\mu\text{mg.}$ of growth hormone to ^{131}I -labelled growth hormone ($0.5\text{ }\mu\text{mg.}$) before the addition of antiserum (1:35 000 dilution).

Sensitivity of the assay

The sensitivity of the system ^{131}I -labelled growth hormone-antiserum to inhibition by unlabelled growth hormone can be increased by decreasing the amount of ^{131}I -labelled growth hormone. The antiserum dilution was simply related to the amount of ^{131}I -labelled growth hormone (Fig. 4). The increased sensitivity of the system to the inhibition permitted smaller amounts of unlabelled growth hormone to be used for the standard curve. However, the time required for maximum reaction sets a practical limit to increasing the sensitivity. This equilibration time is a function of the avidity of the particular antiserum used. For the antiserum shown in Fig. 4, equilibration was incomplete at 7 and 12 days for $0.02\text{ }\mu\text{mg.}$ of ^{131}I -labelled growth hormone. The addition of standard growth hormone increases the total growth hormone concentration and it was found empirically that equilibration was attained in 12 days with $0.05\text{ }\mu\text{mg.}$ of ^{131}I -labelled growth hormone, $0.05\text{ }\mu\text{mg.}$ of standard growth hormone and antiserum (1:700 000 dilution). The range of the standard curve was then $0.05\text{--}0.8\text{ }\mu\text{mg.}$ and no change in the position or slope of the standard curve was detected between 12 and 14 days. Incubation times longer than 14 days were impracticable with ^{131}I (half-life

8.04 days). The antiserum dilution taken for the assay was such that 60–75% of the amount of added ^{131}I -labelled growth hormone was bound in the absence of standard growth hormone. Increasing the dilution at this point decreased the effective range of the standard curve; decreasing the dilution decreased the sensitivity since the first standard was insufficient to inhibit the antibody.

Slope and estimate of precision of the standard curve for purified growth hormone. By using linear scales the relation between the amounts of growth hormone and the percentages of the added ^{131}I -labelled growth hormone bound to γ -globulin had the form shown for the insulin-anti-insulin system (Yalow & Berson, 1960). When the amounts of growth hormone were plotted on a logarithmic scale the curve approximated closely to a straight line (Fig. 1). This facilitated tests for parallelism between unknowns and standards and allowed the slope to be expressed as a fall in the percentage bound for doubling amounts of growth hormone. The mean slope is a convenient method for expressing the avidity of different antisera for growth hormone. In routine assays the best straight line was fitted over the range $0.05\text{--}0.4\text{ }\mu\text{mg.}$ of growth hormone since the range $0.4\text{--}0.8\text{ }\mu\text{mg.}$ was non-linear on a logarithmic scale (Table 2). The mean slope over the range $0.05\text{--}0.4\text{ }\mu\text{mg.}$ was 11.2 ± 1.6 (s.d.)% in 28 experiments, but statistically the logarithmic plot was non-linear since the mean slopes between doubling amounts of growth hormone, although differing by 1 or 2%, were significantly different (Table 2). This is also shown for logarithmic plots of single standard curves (Fig. 1). The reproducibility of the slope is shown by the results in Table 2, and its position relative

Fig. 4. Titration of rabbit antiserum to growth hormone with various amounts of ^{131}I -labelled growth hormone. Rabbit antiserum was titrated against the following amounts of ^{131}I -labelled growth hormones: $\circ$, $20\text{ }\mu\text{mg.}$; $\square$, $2\text{ }\mu\text{mg.}$; $\triangle$, $0.2\text{ }\mu\text{mg.}$; $\bullet$, $0.02\text{ }\mu\text{mg.}$ The incubation period was 7 days.

Table 2. *Slope and estimate of precision of the standard curve for purified growth hormone*

The results were obtained from weekly experiments in duplicate with ¹³¹I-labelled growth hormone (0.05 µmg.), antiserum (1:700 000 dilution) and the amounts of growth hormone shown. The mean slope is expressed as the fall ± s.d. in the percentage bound over the range given. Each slope was obtained by fitting the best line over the range given; the results are given as means ± s.d. The precision of the measurement of the percentage bound at each concentration of growth hormone is expressed as an estimate, *s*, of the s.d. of results from their means (from duplicate measurements). The percentage bound at each point is the mean ± s.d. The numbers of single experiments in each set of results are shown in parentheses. The differences between the results obtained in the different groups were analysed statistically by Student's *t* test: for A and B, not significant; for B and C, 0.1 > *P* > 0.05; for C and D, 0.01 > *P* > 0.001; for D and E, 0.01 > *P* > 0.001.

Amounts of growth hormone (µmg.)	Group	Slope (%)	Amount of growth hormone added (µmg.)	Precision (<i>s</i>)	Percentage bound
0.05-0.4		11.2 ± 1.6 (28)	0.0	± 3.2 (26)	68.2 ± 8.2 (27)
0.0 -0.05	A	11.8 ± 3.2 (26)	0.05	± 2.4 (26)	56.8 ± 6.9 (26)
0.05-0.1	B	11.1 ± 3.6 (25)	0.1	± 2.9 (26)	45.5 ± 6.8 (25)
0.1 -0.2	C	12.7 ± 2.7 (24)	0.2	± 1.8 (24)	33.3 ± 4.9 (25)
0.2 -0.4	D	10.2 ± 2.4 (24)	0.4	± 1.3 (26)	23.2 ± 3.4 (27)
0.4 -0.8	E	8.1 ± 2.2 (25)	0.8	± 1.2 (24)	15.2 ± 2.2 (25)

to the abscissa is shown by the values for the mean percentage bound at each point on the standard curve.

The sensitivity of the system to purified growth hormone is given by the amount of growth hormone which is significantly different from the '0' standard and is a function of the slope (the avidity of the antiserum) and the precision of the measurement of the percentage bound. The difference, *d*, between a number of duplicate measurements, *n*, of the percentage bound over the range of the standard curve provide an estimate of the s.d., *s*, of results from their means and hence the precision (Table 2). This is calculated from

$$s = (\Sigma d^2/2n)^{\frac{1}{2}}$$

(Brown, Bulbrook & Greenwood, 1957). A significant difference (*P* 0.05) between single estimations of the '0' standard and a first standard is given by $t(s_1^2 + s_2^2)^{\frac{1}{2}}$, where *t* is 1.96, *s*₁ is 3.2 % and *s*₂ is 2.4 %, and is equal to a fall in the percentage bound of not less than 8.9 % or not less than 0.035 µmg. of growth hormone in 0.4 ml. of the 0.5 ml. of incubation mixture (0.088 µmg./ml.). The maximum percentage error (*P* 0.05) at this level is ± 56 %. The quantitative sensitivity of the system to purified growth hormone is such that 0.069 µmg. of growth hormone/ml. can be measured with a maximum percentage error (*P* 0.05) of ± 50 % with a duplicate estimation and 0.098 µmg./ml. for a single estimation.

Precision and sensitivity of the assay when applied to plasma. For the assay of growth hormone in plasma the volume of standard growth hormone in the incubation mixture was replaced by plasma or plasma extract at two amounts (0.2 and 0.4 ml.) and the volumes made up to 0.4 ml. In the most sensitive system 0.4 ml. of an extract, equivalent to

Table 3. *Precision of the assay expressed as estimates of the standard deviations of results from their means obtained by analyses at two concentrations*

The numbers of assays at two concentrations of plasma are given in parentheses. Values of *s* are calculated in terms of µmg. of growth hormone/ml. of plasma.

Concn. range of growth hormone (µmg./ml.)	Estimate of s.d. (<i>s</i>)
0.0-1.0	± 0.08 (47)
1-5	± 0.51 (57)
5-10	± 0.95 (40)
10-40	± 2.22 (82)
40-1000	± 9.34 (37)

approx. 0.6 ml. of original plasma, was used. The lower limit of quantitative sensitivity theoretically given by the sensitivity of the standard curve is then 0.05 µmg./ml. of original plasma measured as an extract in duplicate.

Estimations at two different concentrations provide a continuous check on the parallelism of the unknown and standard and can also be regarded as duplicate estimations. From a series of 263 assays at two concentrations of plasma or plasma extracts the precision of the method obtained in practice was calculated as described above (Table 3). The quantitative sensitivity of the method is then calculated to be 0.22 µmg. of growth hormone/ml. of plasma as an extract which is measured with a maximum percentage error (*P* 0.05) of ± 50 % when estimates at two concentrations are performed. This is calculated from the relationship:

$$M = \pm \frac{100ts}{xn}$$

where *M* is the mean amount measured, *t* is 1.96, *s* is 0.08 µmg./ml. (Table 2), *x* is the percentage error and *n* is the number of determinations.

A replication experiment was carried out on a pool of plasma from normal children which was stored as samples at -20° . Five assays were carried out over a period of 3 months, each at three concentrations, namely with 0.1, 0.2 and 0.4 ml. of a 1:4 dilution of plasma, and the mean value for each assay was calculated. Over the period the mean of the five assays was 10.2 ± 1.0 (s.d.) $\mu\text{mg./ml.}$ and no effect of storage on the amount of growth hormone was detectable. Since each assay was read at three different points on the standard curve the mean values given at these points over the five assays could be calculated. The mean values $\pm$ s.d. in $\mu\text{mg./ml.}$ of plasma for 0.1, 0.2 and 0.4 ml. of plasma were 10.2 ± 1.7 , 10.5 ± 0.8 and 9.9 ± 2.1 respectively. The means were not significantly different but the variance ratios for the values with 0.2 and 0.4 ml. of plasma were significantly different ($P < 0.05$). This illustrates the degree of imprecision inherent in an asymptotic standard curve either in its linear or logarithmic form. A similar replication experiment with samples from a frozen pool of plasma extracts gave a mean value of 0.81 ± 0.04 (s.d.) $\mu\text{mg./ml.}$ for five assays at two concentrations over 3 months.

The lower limit of quantitative sensitivity calculated from the precision of the method during routine use has been taken as $0.22 \mu\text{mg./ml.}$ rather than that calculated from the sensitivity of the standard curve, i.e. $0.05 \mu\text{mg./ml.}$ The difference between these values suggests that measurement of growth hormone in plasma is subject to a greater degree of imprecision than its measurement in pure solution, or that there is some degree of non-specificity detectable at the lower limit of the method, or both. Evidence bearing on the specificity is given in the next section.

Specificity of the radio-immunoassay

The application of the radio-immunoassay to plasma assumes that, if human plasma or extract inhibits the reaction between ^{125}I -labelled growth hormone and rabbit anti-(pituitary growth hormone) serum with a slope parallel to that given by pituitary growth hormone, then there is a protein present in the plasma immunologically indistinguishable from pituitary growth hormone isolated by Raben's (1959) method. There is no evidence that such a protein in plasma is or is not equipotent biologically with pituitary growth hormone as isolated. Such a test with the tibia-line assay would require 1–100 l. of plasma.

Tests for parallelism. These tests were carried out on representative plasma samples from the groups under investigation: children, normal adults, breast-cancer subjects and acromegalic subjects. An inhibition curve for each sample was obtained over the range of inhibition of the standard curve as

shown in Fig. 5 for adolescent plasma extracts and acromegalic plasma.

All representative samples showed the same slope as that illustrated and statistical tests for parallelism were not carried out. As a routine the assay of individual plasmas at a minimum of two concentrations provides a check on parallelism. The lack of parallelism of certain groups of plasmas reported below was detected by this routine check.

The decreased slope of the inhibition curve with pregnancy plasma (Fig. 5) is interpreted as being due to a protein present only in the maternal circulation which originates from the placenta and from which it can be isolated. The protein cross-reacted with some of the antibody to growth hormone, or with all of this antibody but with a lower affinity, and was thus closely related immunologically to pituitary growth hormone. The material was not related to human chorionic gonadotrophin (Fig. 6). These results confirm similar findings by Josimovich & MacLaren (1962), who also report that the placental material was active in biological assays for prolactin but not for growth hormone.

The inhibition curves for extracts of monkey and human pituitaries were parallel to the standard curve, but in the absence of a purified preparation of monkey growth hormone a quantitative relationship could not be established (Fig. 6). Plasma

Fig. 5. Inhibition of the ^{125}I -labelled growth hormone-antiserum system. Antiserum to growth hormone (1:200 000 dilution) was incubated with ^{125}I -labelled growth hormone ($0.2 \mu\text{mg.}$) and growth hormone (○), plasma extract from adolescent subjects (△), plasma from acromegalic subjects (●) or plasma extract from pregnant subjects (□) in the amounts or dilutions shown. Parallel inhibition is shown between growth hormone and plasma from adolescent subjects or plasma from acromegalic subjects. The inhibition by plasma from pregnant subjects is not parallel to that given by growth hormone.

from a non-pregnant monkey assayed in terms of human growth hormone showed a parallel slope and gave a value of $50.4 \mu\text{mg./ml.}$ of plasma. A sample from a pregnant monkey had a similar slope to that obtained from human pregnancy plasma and extracts of human and monkey placentas. These preliminary results suggest that the method could be applied to the measurement of growth hormone in plasma from a non-pregnant monkey.

Homogeneity of the growth hormone. The specificity of the radio-immunoassay is dependent on the homogeneity of the growth hormone used for ^{125}I -labelling and the standard curve but not on the homogeneity of the antiserum. The assay is not susceptible to trace contaminants in the growth hormone preparations (cf. Grumbach & Kaplan, 1962) since only the reaction between ^{125}I -labelled growth hormone and antiserum is estimated. Antisera to growth hormone contain antibodies to human albumin and γ -globulin, although these contaminants are not detected by physicochemical methods in growth hormone. Antibodies to albumin in antiserum II were detected and titrated against ^{125}I -labelled human plasma albumin, but in a growth hormone assay the latter will be present in only trace amounts in the ^{125}I -labelled growth hormone and will travel with the excess of albumin

present in plasma samples for assay and not interfere with it.

The homogeneity of the growth hormone-anti-(growth hormone) system has been extensively studied (cf. Wolstenholme & Cameron, 1962). The implications of the removal of a biologically and immunologically inactive component from growth hormone, prepared by Raben's (1959) procedure, with Sephadex G-200 is not clear. The increase in biological and immunological potency obtained (approx. 130 %) suggests a true but marginal purification. An immunologically inert contaminant is not, of course, detectable by immunological methods and affects only the accuracy of the measurement for growth hormone. The absence of antibody to a component constituting 20 % by weight of the antigen and of similar molecular weight is surprising in view of the antibodies found to a trace contaminant, albumin, in the antigen. For the present the peak II material from the Sephadex G-200 column is used for ^{125}I -labelling but not for the standard curve. Values obtained for growth hormone in plasma should theoretically be corrected by a factor of $\frac{2}{3}$ but this would seem premature until further investigations are completed.

Effect of animal sera in the assay. The reaction between growth hormone, adsorbed on to the tanned red cell, and antibody is specifically inhibited by added growth hormone. Non-specific inhibition of the reaction was shown by human and animal sera when measured as apparent growth hormone in hypophysectomized subjects ($15\text{--}600 \mu\text{mg./ml.}$) and in animal sera (e.g., in guinea pig, $240 \mu\text{mg./ml.}$). These values were generally decreased to the limit of sensitivity when extracts were used (Read *et al.* 1962).

The radio-immunoassay was similarly tested for non-specific interference by using the most sensitive procedure, extracts of plasma or serum in a ' $0.05 \mu\text{mg.}$ ' assay (Table 4). With one exception the values obtained were not distinguishable from zero, being below the calculated quantitative sensitivity of the method, i.e. $0.22 \mu\text{mg./ml.}$

The effect of undiluted animal serum (0.4 ml.) in an incubation mixture of total volume 0.5 ml. on the percentage of ^{125}I -labelled growth hormone bound to antiserum at the dilution used in an assay is to decrease this value by 20. The same volume of animal serum diluted 1:2 (v/v) decreased the percentage bound by 12.9, and the decrease was negligible (2.2) at 1:4 (v/v) dilution and above. The significant effect with undiluted plasma and at 1:2 (v/v) dilution was independent of the amount of ^{125}I -labelled growth hormone and the antiserum used for the ' 0 ' standard. The apparent values for growth hormone calculated from the falls in the percentage bound caused by animal sera were

Fig. 6. Inhibition of the ^{125}I -labelled growth hormone-antiserum system. Antiserum to growth hormone (1:20 000 dilution) was incubated with ^{125}I -labelled growth hormone ($2 \mu\text{mg.}$) and growth hormone (O), human pituitary extract (Δ), monkey pituitary extract ($\bullet$) or human chorionic gonadotrophin ($\square$) in the amounts or dilutions indicated. Parallel inhibition between growth hormone and human and monkey pituitary extracts and the absence of inhibition between growth hormone and human chorionic gonadotrophin is shown.

therefore decreased as the sensitivity of the method was increased. The effect of human plasma, undiluted or at 1:2 (v/v) dilution, by inference from the results of animal sera, would be to give a fall in the percentage bound in the absence of any growth hormone in the plasma and that this effect would not be significant at 1:4 (v/v) dilution and above. This has been shown for 1:4 (v/v) dilution and above since some samples of human plasma gave non-significant falls in the percentage bound at this

Table 4. *Values for growth hormone in plasma from different animals and after hypophysectomy in women*

Extracts of plasma or serum were made by the method described in the text. Samples (0.4 ml.) were assayed, equivalent to 0.6 ml. of original plasma, by using ^{131}I -labelled growth hormone (0.05 $\mu\text{mg.}$) and antiserum (1:700 000 dilution). Values are expressed as $\mu\text{mg.}$ of growth hormone/ml. of plasma; those below 0.22 $\mu\text{mg./ml.}$ are not significantly different (P 0.05) from zero.

Source of sample	Concn. of growth hormone ($\mu\text{mg./ml.}$)
Sheep	0.09
Calf	0.06
Turkey	0.15
Chicken	0.08
Rat	0.14
Guinea pig	0.11
Horse	0.11
Rhesus monkey	50.5
Rhesus monkey (pregnant)	—*
Women after	0.19
hypophysectomy	0.14
	0.00
	0.21
	0.10
	0.10

* Slope not parallel to that obtained with growth hormone but similar to that given by plasma from pregnant women and human and monkey placental extract.

dilution. Routine estimations on unextracted plasma were therefore carried out at dilutions of 1:4 (v/v) and above.

Attempts were made to elucidate the nature of the effect of undiluted animal sera by incubating serum and ^{131}I -labelled growth hormone before adding antiserum. The results of a typical experiment with horse serum are shown in Table 5. When ^{131}I -labelled growth hormone was incubated for 3 days in horse serum (tube 2) the scan of the electrophoresis of the incubate showed a broadening of the free ^{131}I -labelled growth hormone compared with that incubated in diluent. The broader peak showed a spread into the γ -globulin area, as shown by the increase in percentage bound from 2.2 to 11.0% although no antibody was present. When antibody in excess is added at the start of such incubations (tubes 3 and 4) the percentage of the ^{131}I -labelled growth hormone transferred to the immune γ -globulin is the same whether animal sera or diluent is used. It is possible that some of the bound ^{131}I -labelled growth hormone was the result of the spreading into the γ -globulin area observed in the absence of antibody or that the addition of excess of antibody 'protected' the ^{131}I -labelled growth hormone. That there was an effect on ^{131}I -labelled growth hormone in the absence of antibody which makes some part of it unavailable for subsequent binding to antibody was shown by tube 5. Antiserum added after 3 days, the equilibration time for the concentrations used, followed by a further 3 days of incubation, gave a decrease in the percentage bound and an increase in the percentage free of 17.2% compared with tubes in which antiserum was added at the start (tubes 3 and 4). The electrophoresis scan showed an additional peak of radioactivity on the starting line between the 'free' and the 'bound' that would be calculated if present in an assay mainly as 'free' and give an apparent growth-hormone concentra-

Table 5. *Effect of horse serum on ^{131}I -labelled growth hormone*

The ^{131}I -labelled growth hormone (2.5 $\mu\text{mg.}$) was added at the start to all incubation mixtures. The presence (+) or absence (−) of horse serum, diluent and antiserum in the incubation mixture is indicated. The volume of horse serum or diluent was 0.4 ml. The final dilution of antiserum, where used, was 1:200. The distribution of radioactivity after electrophoresis of samples from the incubation mixture was determined by scanning and expressed as percentage of radioactivity bound to γ -globulin.

Tube no.	Horse serum	Diluent	Antiserum	Incubation time (days)	Percentage bound
1	−	+	−	3	2.2
2	+	−	−	3	11.0
3	−	+	+	3-6	93.5
4	+	−	+	3-6	93.8
5	+	−	+	6	76.6
6	−	+	+	6†	94.9
7	+	−	+	6†	88.7
8	Extract	−	+	6	96.5

* Antiserum added after 3 days of incubation.

† Incubation mixture centrifuged before samples taken for electrophoresis.

tion in the animal serum used. The additional component produced under these conditions could be partially removed by centrifugation before electrophoresis (cf. tubes 7 and 8). Centrifugation did not affect the percentage bound obtained in diluent (cf. tubes 6 and 3). Results comparable with these without centrifugation were obtained with extracts of the horse serum (tube 9).

We have no evidence that the appearance of a third component produced by preincubation with animal sera is progressive with time but it does appear to be dependent on the protein concentration and solubility of the incubated material. In soluble extracts or in diluted animal sera no effects on ^{131}I -labelled growth hormone were seen. It is suggested that undiluted plasma undergoes changes on standing to produce partially soluble aggregates to which ^{131}I -labelled growth hormone is adsorbed. Two observations are in accordance with this speculation. Minor alterations in the starch-gel pattern of normal serum on standing at 4° have been noted by Richter, Gold, Rankowicz & Rose (1962). Gibson, Ekstedt & Stollerman (1962) report that human serum kept in an ice bath for 18–20 hr. at pH 7.8–8.0 after the addition of minute amounts of formalin is partially denatured and exhibits the properties of an antiserum.

The percentage of the ^{131}I -labelled growth hormone affected is independent of the amount present over the range 0.05–20.0 $\mu\text{mg.}$ and it seems possible that this is due to a selective adsorption by plasma protein aggregates in undiluted plasma of the ^{131}I -labelled growth hormone component (approx. 20%), designated peak I material, on Sephadex G-200. This component of growth hormone is more strongly adsorbed to surfaces than is the main component (peak II). The present conclusion would be that the ^{131}I -labelled growth hormone used in the radio-immunoassay is susceptible to damage by undiluted or 1:2 (v/v) dilutions of plasma which could give over-estimates of growth hormone concentrations. The effect may

be related to the heterogeneity of the standard growth hormone used for ^{131}I -labelling, and the effect is not significant when soluble plasma extracts or diluted plasma samples are used.

Results obtained on normal and pathological plasma. A summary of the results obtained with the radio-immunoassay with normal and pathological plasma samples is given in Tables 6 and 7. There was a general agreement between the difference in the concentrations found in particular physiological or pathological situations and what might be expected by deduction from the known biological effects of human growth hormone. For example, the concentrations in children are highest at birth and during the period of rapid growth in adolescence; acromegalic subjects have markedly elevated concentrations; a diurnal variation in growth hormone concentrations was observed; and higher-than-normal values were observed during lactation and in subjects with galactorrhea (Table 6). The marginal elevation of concentrations in cancer subjects is in accordance with the resistance to insulin and diminished tolerance to oral glucose in such subjects (Pastorelle, 1962). The half-life of injected growth hormone is in accordance with previous findings (cf. Parker, Utiger & Daughaday, 1962), and the effect of tolbutamide (Table 7) provides additional evidence for the response of growth hormone to hypoglycaemia found by Roth, Glick, Yalow & Berson (1963). A fivefold increase in the growth hormone concentration during starvation for 60 hr., accompanied by a fall in the starvation blood-sugar concentration of approx. 12 mg./100 ml., has been recorded by Roth *et al.* (1963). However, an increase in the concentration of growth hormone was obtained after starvation for 12 hr. and a period of exercise without hypoglycaemia (Table 7).

Such agreement between expected and observed concentrations does not, in itself, prove that the method is specific for growth hormone but shows only that the method can provide measurements

Table 6. *Values for the concentration of growth hormone in plasma obtained by the assay*

The concentrations of growth hormone ($\mu\text{mg./ml.}$ of plasma) are given as means $\pm$ S.D. The numbers of subjects studied are given in parentheses.

Source of plasma	Concn. of growth hormone ($\mu\text{mg./ml.}$)	Comment
Cord blood	49.7 $\pm$ 57.8 (10)	—
Normal children	18.6 $\pm$ 12.5 (10)	Age < 1 year old
Normal children	8.5 $\pm$ 7.8 (26)	Age 1–8 years
Normal children	2.8 $\pm$ 2.8 (14)	Age 9–10 years
Normal children	14.0 $\pm$ 13.4 (34)	Age 11–17 years
Adults in hospital	0.55 $\pm$ 0.68 (18)	Age 24–68 years
Women with breast growth	1.5 $\pm$ 2.5 (35)	Benign and malignant
Men with lung cancer	1.3 $\pm$ 1.4 (7)	Age 42–66 years
Women in lactation	4.5 $\pm$ 2.1 (11)	1–6 weeks <i>post partum</i>
Women with galactorrhea	7.0 $\pm$ 3.4 (7)	—
Acromegalic subjects	72.3 $\pm$ 69.3 (23)	Active and inactive

Table 7. *Serial estimations of growth hormone in metabolic experiments*

Growth-hormone concentrations were assayed in plasma by using the routine methods and are expressed as $\mu\text{mg./ml.}$ of plasma. Blood-sugar concentrations (mg./100 ml.) are given in parentheses in (c) and (d).

	Concn. of growth hormone ($\mu\text{mg./ml.}$)
(a) Half-life of injected growth hormone	
Control plasma	7.8
Growth hormone (5 mg.) given intravenously at time t_0	10.0
At $t_0 + 2$ min.	10.1
At $t_0 + 6$ min.	147.0
At $t_0 + 10.5$ min.	130.0
At $t_0 + 31$ min.	74.0
At $t_0 + 64$ min.	34.0
At $t_0 + 126$ min.	18.0
At $t_0 + 259$ min.	8.8
At $t_0 + 730$ min.	8.0
(b) Diurnal variation in two adolescents	
Subject 1	
After 14 hr. without food	45.0
3 $\frac{3}{4}$ hr. after first meal	1.5
1 $\frac{1}{2}$ hr. after second meal	1.5
1 hr. after third meal	26.0
Subject 2	
After 14 hr. without food	21
2 $\frac{1}{2}$ hr. after first meal	6.5
1 hr. after second meal	< 1.0
1 $\frac{1}{2}$ hr. after second meal	3.0
(c) Effect of tolbutamide on normal adult	
After 12 hr. fast	1.7 (66)
Tolbutamide (1 g.) given intravenously at time t_0	
At $t_0 + 30$ min.	3.4 (32)
At $t_0 + 60$ min.	31.0 (50)
At $t_0 + 90$ min.	7.5 (53)
(d) Effect of exercise on normal adult	
After 12 hr. without food	1.2 (70)
After 2 $\frac{1}{4}$ hr. of exercise	4.6 (68)
After 100 g. of glucose given orally	0.9 (96)

which are in general agreement with physiological and clinical evidence. However, the number of such agreements increases thereby the confidence that can be attached to the precise measurements of growth hormone obtained by the radio-immunoassay.

DISCUSSION

The radio-immuno-electrophoretic assay for growth hormone was developed after the failure of the haemagglutination technique in our hands. It appeared that the Yalow & Berson (1960) method for the assay of insulin could be a general technique for the assay of protein hormones. Glick *et al.* (1963) have also applied the technique to growth hormone and reported details of their method and its application (Roth *et al.* 1963). The methods described by Glick *et al.* (1963) and our-

selves have levels of sensitivity not previously attained (Utiger *et al.* 1962), are not subject to interference by plasma and report much lower values for the concentration of growth hormone in plasma from normal adults. The two methods use the same basic principles and the same method for the preparation of ^{131}I -labelled growth hormone. They permit the routine assay of large numbers of samples by using two concentrations of plasma (1:4 dilution) or extracts as described for the present method or by using single or serial estimations on plasma (1:2 dilution) as described by Glick *et al.* (1963). There are differences in the purity of the standard growth hormone, the avidity of the antisera used, the purification of the ^{131}I -labelled growth hormone, the period of incubation, the volume incubated and the electrophoretic technique. Values obtained for growth hormone in many physiological and clinical states studied by both methods are in good agreement. Values for the calculated sensitivity of the method presented above are not available for the method of Glick *et al.* (1963), but they report that growth hormone can be detected in amounts down to $0.1 \mu\text{mg./ml.}$

The differentiation between normal adults and hypophysectomized subjects on single plasma samples is difficult with either method. Glick *et al.* (1963) report that of 64 samples from normal adults one-third had detectable growth hormone, by using their assay at four levels of sensitivity, namely down to 0.4, 1, 2 and $4 \mu\text{mg./ml.}$, and, in 21 samples from hypophysectomized subjects, six had less than $4 \mu\text{mg./ml.}$, eight had less than $2 \mu\text{mg./ml.}$, four had less than $1 \mu\text{mg./ml.}$ and three had less than $0.1 \mu\text{mg./ml.}$ The authors report that, in subsequent random samples from normal subjects, with their most sensitive system, two-thirds had detectable growth hormone. In the smaller series reported in the present paper growth hormone was present in nine of 15 plasmas from hospitalized subjects and not in six samples from hypophysectomized subjects. It would seem that, although either of the methods can be used as a routine on a range of physiological and clinical problems in children and in adults, a further increase in sensitivity is desirable.

One approach would be the development of fractionation methods for growth hormone in plasma giving greater purification and with smaller volumes of plasma than the method described. The marginal purification of the standard growth hormone by the removal of some 20% of an immunologically unreactive component increases the accuracy but not the sensitivity of the method. Higher specific activities of ^{131}I -labelled growth hormone likewise would not increase the sensitivity by permitting a lower amount of tracer to be used

since the length of incubation is limited by the avidity of the antiserum. The avidity and heterogeneity of the antiserum can theoretically be increased by repeated immunization. It has been previously noted that the radio-immunoassay can tolerate a heterogeneous antiserum.

Further studies on increasing the sensitivity are based on the assumption that growth hormone is always present in adult plasma and that the concentration in a single plasma sample measures the growth hormone concentration and pituitary function of the subject. However, Roth *et al.* (1963) have demonstrated acute changes in the growth hormone concentrations of individuals as a function of their nutritional status, after the injection of insulin and after the oral administration of glucose. We have shown a marked diurnal variation in the growth hormone concentrations of some normal adolescents such that single samples are valueless in evaluating the growth hormone status. From these observations it is possible to estimate the half-life of endogenous growth hormone on the assumption of a cessation of growth hormone secretion after the oral administration of glucose or a meal. The value approximates to that shown for exogenous growth hormone (26 min.; see Table 7). A single concentration of growth hormone given by a random sample could be a point in a dynamic change in concentrations during an acute response of the pituitary or could be a 'resting' concentration. On this view the differentiation between normal and hypophysectomized or hypopituitary subjects by single samples is not possible but requires serial estimations throughout treatment with intravenous insulin or starvation (Roth *et al.* 1963) or with intravenous tolbutamide (the present paper) to test pituitary function.

As purified protein hormones become available and radio-immunoprecipitin or immunoelectrophoretic methods developed for their measurement in plasma, detailed studies of the role and interaction between them will become possible. At the present simultaneous estimations on plasma are possible for insulin (Yalow & Berson, 1960; Morgan & Lazarow, 1962; Hales & Randle, 1963), glucagon (Unger *et al.* 1959; Grodsky, Hayashida, Peng & Geschwind, 1961), adrenocorticotrophin (Felber, 1963) and growth hormone (Glick *et al.* 1963; and the present paper).

SUMMARY

1. A method for the measurement of growth hormone in human plasma is described based on the inhibition of the reaction between ^{125}I -labelled growth hormone and rabbit antiserum to purified human growth hormone. The extent of the reaction is determined by electrophoresis and radioactivity

measurements and can also be determined by gel-filtration on Sephadex G-200.

2. The method permits the assay of multiple samples of plasma by using small volumes of plasma or plasma extract at two concentrations.

3. The lower limit of sensitivity for purified growth hormone is $0.07 \mu\text{mg./ml.}$ and this concentration is measured with maximum percentage error (P 0.05) of $\pm 50\%$.

4. The precision of the measurement of growth hormone in plasma has been calculated and the quantitative sensitivity of the method derived from results given by its routine use. At a concentration of $0.22 \mu\text{mg./ml.}$ of plasma, growth hormone is significantly different from zero (P 0.05) and measured with a maximum percentage error (P 0.05) of $\pm 50\%$.

5. Evidence for the specificity of the method has been obtained by comparing the inhibition of the reaction by purified growth hormone and by a variety of plasma samples, from the effect of animal sera in the assay, from the recovery of endogenous growth hormone in a simple extraction procedure, and from the results of assays in normal and pathological plasma.

6. A protein cross-reacting with antibodies to growth hormone which is present in plasma from women and monkeys during pregnancy and in placentas at term was noted.

7. The slope of the inhibition curve obtained with monkey pituitary extract and plasma from a non-pregnant monkey is identical with that obtained from human pituitary extracts and suggests that the method could be applied to the measurement of growth hormone in plasma from non-pregnant monkey.

8. The removal of an immunologically and biologically inactive component present in all growth hormone preparations studied by gel-filtration is reported.

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Inhibition of Glucose Phosphorylation by Mannoheptulose

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D-Mannoheptulose prevents glucose from stimulating the secretion of insulin by rabbit pancreas *in vitro* (Coore, Randle, Simon, Kraicer & Shelesnyak, 1963). This action of mannoheptulose may be largely responsible for the temporary diabetic state induced by injection of the sugar (Simon & Kraicer, 1957; Simon, Scow & Chernick, 1961). Identification of the enzyme in glucose metabolism inhibited by mannoheptulose might thus indicate which pathway of glucose metabolism within the β -cell provides the stimulus for insulin secretion. Because no convenient preparation is available for a study of glucose metabolism in mammalian β -cells we have investigated the effect of mannoheptulose on glucose metabolism in rat-liver slices. Inhibitory effects of mannoheptulose on glucose and mannose uptake by rat-liver slices and on crude rat-liver glucokinase and hexokinase have been demonstrated. These observations suggested that mannoheptulose may also inhibit phosphorylation of glucose in the pancreatic β -cell and therefore that

the sugar cannot be used to determine whether a particular pathway of glucose metabolism beyond glucose 6-phosphate provides the signal for insulin release.

EXPERIMENTAL

Materials. D-Mannoheptulose was given by Professor E. Simon, Rehovoth, Israel. The sources of other chemicals and enzymes were as given by Battaglia & Randle (1960) and by Newsholme & Randle (1961).

Methods. Glucose was determined with glucose oxidase (Huggett & Nixon, 1957), mannoheptulose by the method of Dische (1953) and total reducing sugar by Somogyi's (1945) modification of Nelson's method. Mannose was calculated by difference (total reducing sugar minus glucose and minus mannoheptulose when present). Solutions for sugar analysis were deproteinized with $\text{Zn}(\text{OH})_2$ (Somogyi, 1945). Glycogen was assayed by the method of Walaas & Walaas (1950) except that glucose was assayed after hydrolysis with glucose oxidase. Glucokinase and hexokinase activities were assayed at 20° or 30° by the rate of