THE EXISTENCE OF A NOVEL GROWTH HORMONE BINDING FACTOR IN THE SERUM OF PREGNANT MICE

"THE EXISTENCE OF A NOVEL GROWTH HORMONE BINDING FACTOR IN THE SERUM OF PREGNANT MICE"

by

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MASTER OF SCIENCE

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ABSTRACT

A radioreceptor assay using pregnant rabbit liver membranes as receptor detected what appeared to be a growth hormone-like activity in the serum of pregnant Swiss Webster mice. This activity appeared at mid-pregnancy, and peak concentrations of 4-6 ug/ml were detected one to two days before parturtition. Preliminary attempts at the characterization of this activity by Sephadex G 100 gel filtration led to the observation that, in the presence of pregnant mouse serum, ^{125}I-hGH eluted from the column with a relative elution volume (V_e/V_o) = 1.2, rather than with V_e/V_o = 2.0 observed when ^{125}I-hGH alone was fractionated on the same gel column. More than 75% of the ^{125}I-hGH was recovered in the larger molecular weight fractions. More than 65% of the ^{125}I-hGH in these fractions was precipitable by excess specific antiserum to hGH. When serum was obtained from pregnant mice after injection of 10 ug of unlabelled hGH and was similarly fractionated, immunoreactive hGH, as detected by specific radioimmunoassay, was recovered in eluates corresponding to a V_e/V_o = 1.2. Similar fractionation and assay of serum obtained after injection of hGH into non-pregnant mice led to the recovery of immunoreactive hGH in fractions eluting with V_e/V_o = 2.0. When unlabelled oPRL was used as the injection material and the serum obtained was also

fractionated by Sephadex G 100 gel filtration, the oPRL consistently eluted in fractions corresponding to those in which ^{125}I-oPRL was recovered when the latter was fractionated in buffer alone. Further *in vitro* studies indicated that the serum GH-binding factor competitively inhibited the binding of ^{125}I-hGH to rabbit liver receptor in the standard radioreceptor assay. This competition leads to false estimates of growth hormone-like activity in pregnant mouse serum. *In vivo* experiments showed that the half time disappearance of ^{125}I-hGH was significantly longer in the pregnant than in the non-pregnant mouse. When fresh term pregnant mouse serum was fractionated by Sephadex G 200 gel filtration and the resulting eluates were analyzed by radioimmunoassay for mouse growth hormone, the endogenous mouse growth hormone was found to elute in tubes which also contained the serum binding factor as determined by radioreceptor assay. Serum concentrations of immunoreactive mouse growth hormone increase during pregnancy. Possible consequences of this growth hormone binding factor on metabolism during pregnancy are discussed.

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List of Abbreviations

Hormones

GH	growth hormone
PRL	prolactin
Ins	insulin
FSH	follicle stimulating hormone

Prefix denoting species of origin

b	bovine
h	human
m	mouse
o	ovine
p	porcine
r	rat

Assays and activities

RIA	radioimmunoassay
IR-hormone	immunoreactive hormone
RRA	radioreceptor assay
GH-LA	growth hormone-like activity as detected by RRA
PRL-LA	prolactin-like (lactogenic) activity as detected by RRA
specific binding	cpm bound (e.g. to receptor) that can be displaced by excess hormone.
% specific binding	$\frac{\text{cpm specifically bound}}{\text{total cpm in assay}} \times 100$
cpm	counts per minute

Gel filtration

V_e/V_o	relative elution volume

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$, where V_e = elution volume, V_o = void volume, V_t = bed volume

Units of measure

g	gram
mg	milligram
ug	microgram
ng	nanogram
ml	millilitre
ul	microlitre
V/W	volume per unit weight (ml/g)
cm	centimeter
mm	millimeter
nm	nanometer

Miscellaneous

BSA	bovine serum albumin
day 10, day 18, etc.	defines the day of pregnancy on which sample was obtained
T1/2	time in which circulating amount of hormone is reduced by one half
IEP	immunoelectrophoresis
hypox	hypophysectomized
S.D.	standard deviation

STATEMENT OF PURPOSE

The recent advent of radioreceptor assays for lactogens and for growth hormones has prompted a search for these hormones in the sera of various species. Mouse serum, obtained in the latter half of pregnancy, contained both activities. Sephadex G 100 gel filtration of pregnant mouse serum and radioreceptor assay of the eluates showed the GH-LA to be associated with proteins that eluted close to the void volume of the column. On the other hand, lactogenic activity eluted with molecules having a molecular weight of about 20,000 daltons. In this instance, the GH-LA and PRL-LA appeared to be independent of one another. This observation suggested the existence of a separate "growth hormone of pregnancy". The existence of such a molecule had long been postulated to account for anabolic features of maternal metabolism, particularly during the second half of pregnancy.

This study was initially undertaken to characterize the GH-LA detected in mouse serum during pregnancy. However, it soon became evident that this serum also contained a factor capable of binding growth hormone. It became necessary to examine the possibility that this GH-binding factor could interfere in the radioreceptor assay, thereby producing "false positive" results which could incorrectly

be interpreted to indicate the presence of a GH-LA. The investigation and characterization of this GH-binding activity is the subject of this presentation.

INTRODUCTION

Changes in maternal metabolism during pregnancy have been noted for many years. The major changes are anabolic being characterized by a retention of nitrogen (1,2). The nitrogen storage is associated with utilization of stored fat and increase in lean body mass (3). In the liver of pregnant mice there is a fivefold increase in protein synthesis over that seen in the non-pregnant animal (4). These effects are similar to the metabolic effects seen when GH is present in a number of test systems. Growth hormone was first shown to stimulate the transport of amino acids into cells of several organs by Noall (5). Pituitary somatotropin has been shown to affect the aggregation of polysomes, the synthesis of mRNA and the production of protein both *in vitro* and *in vivo* (6,7,8,9,10). In addition, there is evidence that GH plays a physiological role in the regulation of carbohydrate and lipid metabolism. Prolonged treatment with GH leads to reduced glucose utilization at the expense of stored lipids. Because these effects are the result of chronic treatment with GH, they are thought to represent the actions of GH in the intact animal where GH is, in all phases of development, present in the circulation (11). Because the metabolic changes observed during pregnancy are in many ways similar to those induced by growth hormone in the non-pregnant animal

the existence of a "growth hormone of pregnancy" has long been suspected (12,13).

The search for this postulated growth hormone-like factor prompted experiments designed to detect somatotropic activity in sera obtained during pregnancy and in extracts of placentas. Gemzell, in 1955, demonstrated that human retroplacental plasma did possess some growth promoting activity when tested in the rat tibial assay (14). Shortly afterwards, Contopoulos and Simpson reported that pregnancy increased the growth promoting action of rat plasma threefold (15). According to estimates based on the tibial assay and tail length measurements, these authors estimated that plasma obtained from pregnant rats contained the equivalent of 2.5 - 3.5 ug bovine GH per ml. Somatotropic activity of human placental extracts was first reported by Fukushima and was characterized by Josimovich and MacLaren by its ability to cross react with antiserum to hGH (16,17). However, the somatotropic activity of the purified hormone was disappointing. The pigeon crop sac assay showed the material to be a powerful lactogen but the rat tibia test showed it to possess low growth promoting potency (17). For this reason the material was named placental lactogen by Josimovich et al. (17). Grumbach et al., however, emphasizing the high concentrations of this material in the circulation and its metabolic actions preferred the term "chorionic growth hormone-prolactin" (13). The report of Josimovich and MacLaren suggested that, as fractionation

of the placental extract proceeded, the lactogenic and growth promoting activities could be separated (17).

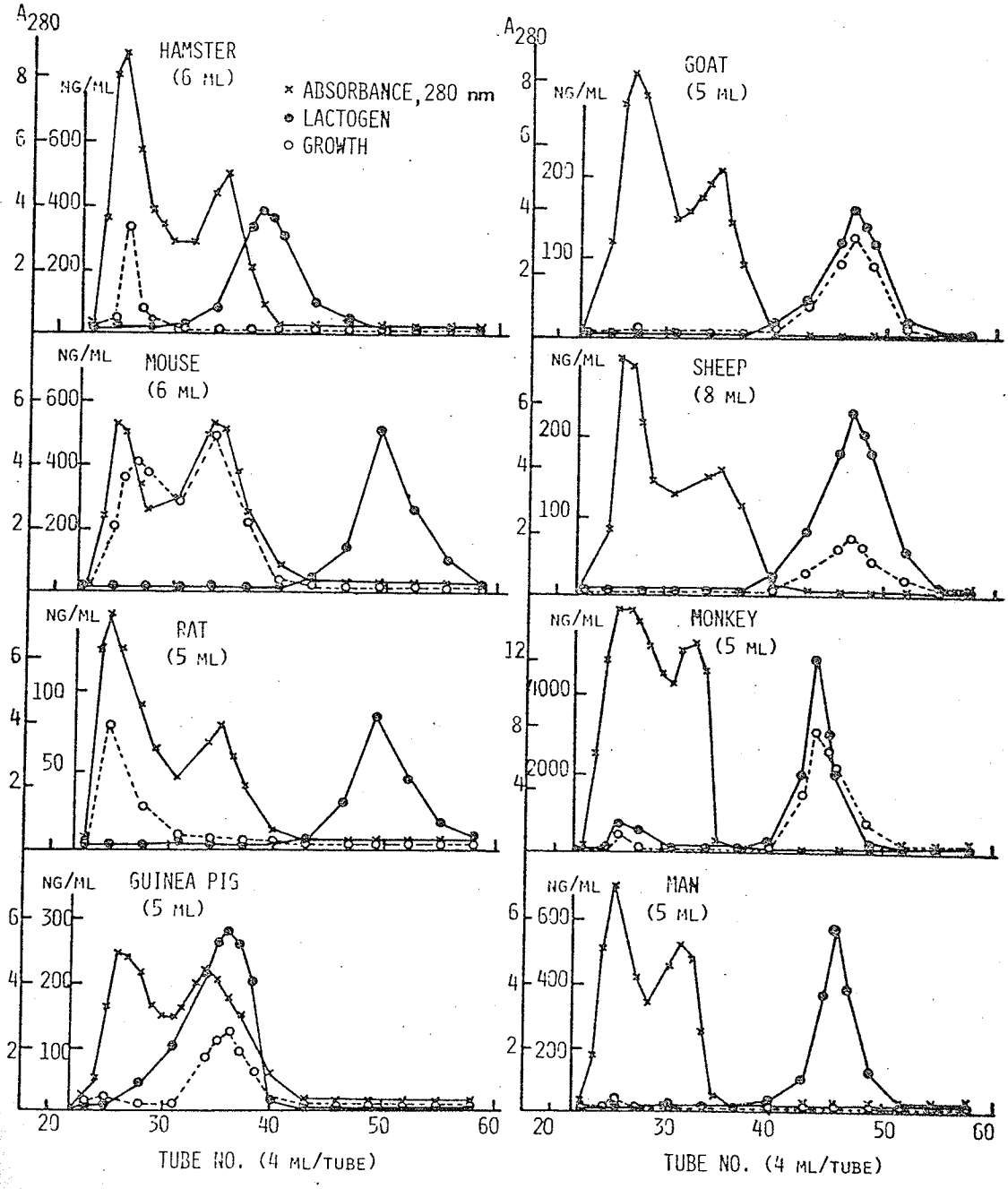
With the advent of immunoassays it became possible to determine more accurately the circulating levels of GH in a number of species. Schalch and Reichlin showed that in the pregnant rat immunoreactive GH accounted for only 1% of the concentrations postulated by Contopoulos and Simpson (18). Moreover, pregnancy did not seem to affect serum concentrations of GH.

During the last 3 years, radioreceptor assays have been developed which specifically detect lactogenic and growth hormones (19,20,21). The activity detected by the assays appears to reflect the biological potency of the test material (22). These assays have been particularly useful in the search for lactogenic and growth hormone-like activities thought to be present in the sera and placentas of many species (23). Tsushima et al. were first able to identify, in the circulation of pregnant rhesus monkeys, a hormone which possessed almost equipotent lactogenic and growth hormone-like activity as determined by RRA (24). This material was found in placental extracts in concentrations up to 80 ug/g wet weight of tissue. When sera or placental extracts were analyzed by Sephadex G 100 fractionation and by polacrylamide gel elctrophoresis, PRL-LA and GH-LA were always recovered in the same fractions. Chan et al. have purified a similar hormone possessing both

PRL-LA and GH-LA from the placental cotyledons of sheep (25). This purified ovine placental hormone is potent both as a lactogen, as determined by its ability to stimulate casein synthesis in organ culture of rabbit mammary gland, and as a growth hormone, as determined by whole body weight gain of hypophysectomized rats. Kelly et al. examined serum samples obtained during pregnancy from several species by fractionating the material on Sephadex G 100 gel columns and assaying aliquots of the eluates by the two radioreceptor assays(23). (Figure 1)

When serum samples from pregnant guinea pigs, goats, sheep and monkeys were fractionated, both PRL-LA and GH-LA eluted in the same fractions. When serum from pregnant women was similarly fractionated and assayed, only lactogenic activity was detected. In eluates obtained after fractionation of pregnant hamster, mouse, and rat sera, both activities were again detected but, this time, in different fractions. The GH-LA eluted as a large molecular weight molecule close to the void volume of the column. Lactogenic activity in mouse and rat sera was recovered in fractions corresponding to a molecular weight of 20,000. The PRL-LA in hamster serum was slightly larger than this but was still separate from the GH-LA. The serum concentrations of GH-LA were highest in the mouse, ranging from 3-6 ug/ml.

Figure 1. Sera from 8 species were applied to Sephadex G 100 columns (2.6 x 64 cm) and were eluted with 0.05 M ammonium bicarbonate. The ordinates indicate the absorbance at 280 nm and the hormone concentration detected by radioreceptor assays in ng/ml. Growth hormone-like activity is determined from a standard curve obtained using pituitary hGH as a reference hormone in the GH-RRA and lactogen is determined from a standard curve obtained using oPRL as a reference hormone in the PRL-RRA. In all other figures this same convention is used to express activity detected by a RRA. The symbols used are noted in the top left panel.


A280
HAMSTER
(6 ML)
NG/ML
× ABSORBANCE, 280 nm
● LACTOGEN
○ GROWTH
GOAT
(5 ML)
MOUSE
(6 ML)
SHEEP
(8 ML)
RAT
(5 ML)
MONKEY
(5 ML)
GUINEA PIG
(5 ML)
MAN
(5 ML)
TUBE NO. (4 ML/TUBE)
TUBE NO. (4 ML/TUBE)

In the rat and hamster GH-LA concentrations were only 10-20% of those found in the mouse. The opportunity to study a separate and distinctive "growth hormone of pregnancy", clearly independent of lactogenic activity, presented itself. This study was undertaken to examine and characterize this GH-LA in the hope of substantiating the initial observations of Contopoulos and Simpson.

MATERIALS AND METHODS

Animals

Swiss Webster mice were obtained from Canadian Breeding Farm and Laboratories Limited (Montreal, Canada). Females were mated at 8 weeks of age with mature males by placing pairs of animals in individual cages for 3 days. The end of the second day was designated day 1 ± 1 of pregnancy. Animals 8-14 weeks of age were used as controls.

Serum samples

All blood sampling was done under light ether anaesthesia. Multiple samples were taken from the orbital sinus using Pasteur pipets or microhematocrit tubes. Animals were exsanguinated from the inferior vena cava using a 21 guage needle and a 1 ml disposable syringe. Pooled blood was obtained from fetal and neonatal mice by decapitation.

Except where specified, all blood was allowed to clot at 22 C for 2 hours before centrifuging to separate the serum. Pools of serum were divided into aliquots and frozen at -20 C. Generally, samples were thawed only once and used immediately. Serum referred to as fresh was kept on ice and used within one hour of sampling.

Sera from species other than the mouse and rat had been obtained by various members of the laboratory personnel for other purposes and had been kept at -20 C for not more than

three months prior to this study.

Tissue extracts

Placenta, liver, kidney, adrenal, spleen, uterus, ovary, heart, submaxillary gland, pituitary and brain were removed after sacrifice and frozen. When thawed, the tissues were blotted, weighed and homogenized for 1 minute in 5 volumes (v/w) of cold 0.025M tris-HCl buffer pH 7.6 using a Brinkman Polytron set at maximum speed. Extraction was carried out overnight with constant mixing at 10 C. The homogenate was centrifuged at 50,000 rpm in a 50 Ti rotor using a Beckman L5-65 ultracentrifuge. The supernatant is designated the extract.

Reference hormones

All the following hormone preparations were obtained from the NIAMDD division of the National Institutes of Health: human growth hormone, 1648E and 1934D; bovine growth hormone, 1003-A and B17; rat growth hormone, RP-1 and I-2; mouse growth hormone AFP-689-B; ovine prolactin, P-S-10; rat follicle stimulating hormone RPI-3. Porcine insulin was purchased from Connaught Laboratories, Toronto, Canada.

Iodinations

Sodium ^{125}Iodide, carrier free, was purchased from New England Nuclear (Dorval, Quebec) and Amersham Searle (Oakville,

Ontario). All hormones were iodinated by the method of Thorell and Johansson (26). Iodinated hormone was separated from the reaction mixture by Sephadex G 100 gel filtration and the iodinated hormone was tested for its ability to bind specifically in the radioreceptor assay, as previously described (21,27). Tested tracer was aliquoted and frozen at -20 C. Iodinated hormones were thawed only once before use. Prior to injection, sufficient sodium chloride was added to the iodinated hormone to make a solution of 0.9%.

Radioreceptor assays

Assays for lactogenic and growth hormone-like activities were carried out using particulate receptor preparations (21,19). Protein content of the preparations of receptor was determined by the method of Lowry et al. (28). A solubilized liver receptor assay was also employed as previously described (29). After the standard incubation time, the entire reaction mixture was fractionated on a Sephadex G 100 column (1 x 45 cm) using 0.025 M tris-HCl pH 7.6 containing 0.1% Triton X-100.

Radioimmunoassays

Radioimminoassays for human growth hormone, and ovine prolactin were carried out, with minor modifications, as previously described for other double antibody radioimmunoassays (30). Mouse growth hormone was assayed using antiserum to rat

growth hormone, a gift from Doctor T. Hayashida. Purified mouse pituitary growth hormone was used as the iodinated hormone and as standard in this assay. The standard curves obtained using purified mouse GH and rat GH were superimposable. (Figure 2). The range of this assay was 1 to 10 ng/ml, of the hGH-RIA was 1 to 30 ng/ml, and of the oPRL-RIA was 5-100 ng/ml.

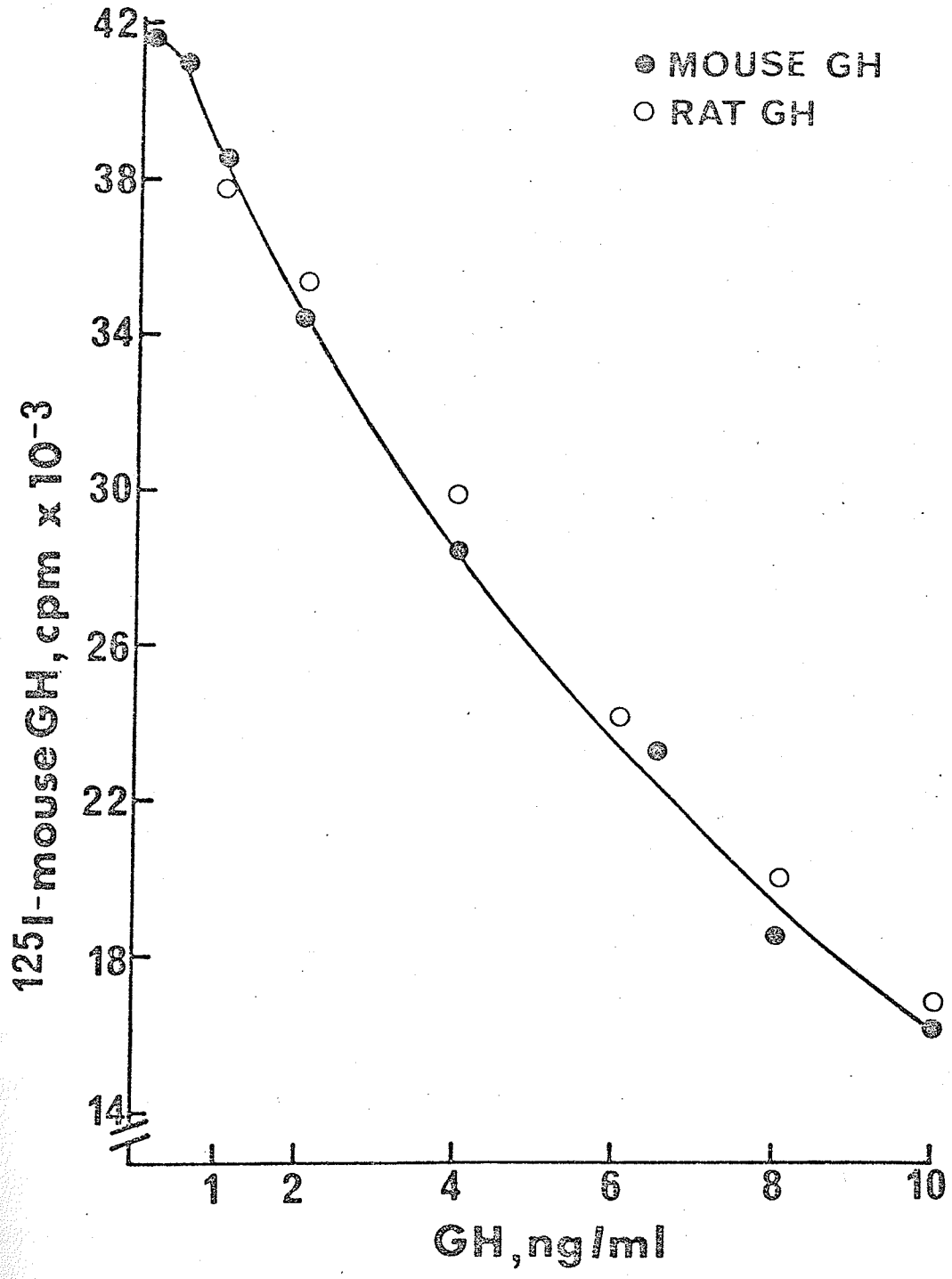
Sephadex gel filtration

All columns were equilibrated with a volume of buffer equal to 3 bed volumes of the column. The void volume (V_o) of each column was determined using blue dextran as a marker. Fractionations were carried out at 10 C. Eluates were collected by an LKB Ultrorac 7000 fraction collector.

Rapid method for detection of GH-binding factor

To a 12 x 75 mm glass test tube the reagents were added in the following order: a) 0.3 ml 0.025M tri-HCl buffer, pH 7.6 containing 1% BSA b) 0.1 ml purified GH solution (0.5 - 50 ug/ml) c) 0.1 ml ^{125}I-hGH (60,000 cpm) d) 0.1 ml pregnant mouse serum diluted 1:5 or 1:10 with assay buffer. Two controls were used. In one pair of tubes reagents a, b, c and an extra 0.1 ml of buffer were reacted. In another pair of tubes a, b, c, plus 0.1 ml of diluted non-pregnant female mouse serum were reacted. The tubes were shaken vigorously and allowed to stand at room temperature for 30

Figure 2. Standard curve obtained in a radioimmunoassay for mouse GH. The curve indicates the progressive displacement of ^{125}I-mGH from antiserum to rat GH by the addition of increasing amounts of mouse GH(●) and rat GH(o). In the absence of any hormone 54% of the total ^{125}I-mGH is bound to antibody.


MOUSE GH
RAT GH
42
38
34
30
26
22
18
14
^{125}I-mouse GH, cpm x 10^{-3}
1
2
4
6
8
10
GH, ng/ml

minutes. Components of the reaction were separated by adding 0.6 ml of dextran coated charcoal (31). The contents of the tubes were mixed on a Vortex mixer and were allowed to stand for 10 minutes before centrifugation at 760 x g for 10 minutes. The unreacted ^{125}I-hGH was adsorbed to the charcoal leaving the ^{125}I-hGH bound to the GH-binding factor in the supernatant. The supernatant was decanted into a clean tube and counted in an automatic gamma counter. Dextran coated charcoal was made up as follows: 1.25 g of dextran T-250 (Pharmacia) was dissolved in 1 litre 0.05 M sodium phosphate buffer pH 7.5 to which was then added 12.5 g charcoal (Norit A).

The appearance and disappearance of GH-LA in serum

a) Seven pregnant mice were bled daily between 1200 and 1400 hours from day 10 of pregnancy to term. All sera were examined in the same assay after the completion of the experiment. The presence of the GH-binding factor was indicated by the values obtained in the GH-RRA. Because of the small volume of sample available, after completion of the GH-RRA, sera obtained on the same day were pooled and assayed for lactogenic activity.

b) To determine the effect of removing the gravid uterus on circulating levels of GH-LA, 4 animals were hysterectomized on day 17 and 5 on day 18 of pregnancy. A serum sample was obtained the day before surgery. Blood was drawn 2 hours after excising the uterus and at intervals

during the succeeding 36 hours.

Binding of ^{125}I-hGH to a GH-binding factor

a) To demonstrate binding in vitro 25 ul of ^{125}I-hGH containing 50,000 to 100,000 cpm were added to 0.2 ml of pooled serum obtained at day 18 of pregnancy. These were allowed to react at room temperature for 5 minutes before being applied to a G 100 Sephadex column (0.9 x 95 cm). The sample was eluted with 0.025M tris-HCl pH 7.6 at 10 C. To discount possible buffer effects on the fractionation, similar samples were eluted with 0.05 M and 0.10 M ammonium bicarbonate pH 8.2, 0.05 M sodium phosphate buffer pH 7.0, 8.0 and 9.0, and unbuffered 0.15 M sodium chloride.

The immunoreactivity of the eluted ^{125}I-hGH was determined by adding antiserum to hGH in a quantity sufficient to bind more than 90% of the ^{125}I-hGH in the original sample. The ^{125}I-hGH bound to the antibody was separated by the addition of a second antibody directed against γ-globulin in a concentration sufficient to precipitate the ^{125}I-hGH-antibody complex.

b) Iodinated hGH and pregnant mouse serum were also incubated with 50, 500, 5000, 25,000 ng of unlabelled hGH. At the end of the reaction time each sample was fractionated on a Sephadex G 100 column and the distribution of ^{125}I-hGH in the eluates was determined by counting. Additional samples

which were examined in a similar manner and served as controls were; ^{125}I-hGH alone, ^{125}I-hGH in the presence of term pregnant mouse serum and 25,000 ng oPRL, ^{125}I-oPRL in the presence of term pregnant mouse serum. Tissue extracts prepared as described and sera from rat, guinea pig, hamster, rabbit, rhesus monkey and human, pregnant and non-pregnant, were similarly reacted with ^{125}I-hGH and fractionated on Sephadex G 100 columns.

Interference of the GH-binding factor in the radioreceptor assay

a) Term pregnant mouse serum and ^{125}I-hGH were reacted with solubilized rabbit liver receptor for 3 hours at room temperature according to the standard assay procedure (29). At the end of the incubation, the entire reaction mixture was applied to a Sephadex G 100 column (1 x 45 cm) and eluted with 0.025M tris-HCl buffer pH 7.6 containing 0.1% Triton X-100. The resulting 0.5 ml eluates were counted to determine the distribution of ^{125}I-hGH. The following additional samples were analyzed similarly and served as controls; receptor and ^{125}I-hGH reacted together and in the presence of either non-pregnant mouse serum plus 25 ug hGH or pregnant mouse serum plus 25 ug oPRL. The initial test sample of ^{125}I-hGH, soluble receptor, and pregnant mouse serum was also fractionated after only a 5 minute incubation period.

b) Mouse serum samples taken on different days of pregnancy and different volumes all from the same day of pregnancy were assayed in the presence of increasing amounts of receptor. In this analogy of the classical model of enzyme kinetics Receptor = Substrate, ^{125}I-hGH = Enzyme, Serum = Inhibitor, and Specific Binding = Velocity of Reaction. A Lineweaver-Burk plot of the resulting data was drawn (32).

Estimate of the molecular weight of the GH-binding factor

A Sephadex G 200 column (0.9 x 105 cm) was equilibrated at 10 C using 0.025M tris-HCl buffer pH 7.6. The column was calibrated using blue dextran, human gamma globulin, bovine serum albumin, ovalbumin, ^{125}I-hGH, cytochrome C, and free ^{125}Iodine. The experimental sample was 1.0 ml of day 18 mouse serum reacted for 5 minutes with 100,000 cpm of ^{125}I-hGH. Non-pregnant mouse serum incubated with ^{125}I-hGH was fractionated in the same way. The flow rate was adjusted to 2 ml/hr and the eluates were collected in volumes of 2 ml/tube. Marker proteins were detected by optical density readings at the appropriate wavelengths. The elution positions of the reference preparations were plotted against the logarithm of their molecular weight (33).

Binding to serum factor after injection of unlabelled hGH

Ten micrograms of unlabelled hGH in a volume of 0.1 ml were injected into the left jugular vein of day 18 pregnant

mice. The inferior vena cava was immediately exposed and the animal was exsanguinated. The resulting plasma sample was fractionated on a Sephadex G 100 column (0.9 x 95 cm) as described earlier. Aliquots of the resulting eluates were assayed for hGH in a double antibody radioimmunoassay. Unlabelled hGH was also injected into non-pregnant mice. Ovine PRL was injected into pregnant and non-pregnant animals. The resulting plasmas were also fractionated on Sephadex G 100 columns. In addition, fresh plasma obtained at day 18 of pregnancy was fractionated and the eluates were assayed to ascertain any interference of the GH-binding factor in the RIA.

Distribution of endogenous GH after gel filtration of mouse serum

One ml of fresh mouse serum obtained on day 17 of pregnancy to which had been added 40,000 cpm ^{125}I-hGH was fractionated on a Sephadex G 200 filtration column similar to the one used for determination of the molecular weight of the GH-binding factor. The eluates were counted, assayed for "GH-LA" by radioreceptor assay and for mouse GH using the heterologous mouse GH-RIA. One ml of non-pregnant female mouse serum was similarly processed and acted as a control.

Serum GH concentrations during pregnancy

Starting at day 10 of pregnancy, 18 mice were bled on alternate days until term. Sera were frozen and assayed in a single RIA for mouse GH. Blood was taken from 4 non-pregnant females on a similar schedule. In addition, 1 pool of day 18 serum (N=32), 1 pool of non-pregnant female serum (N=59) and 1 pool of normal male serum (N=32) were also assayed.

Half-time disappearance of ^{125}I-hGH from the circulation

Four day 18 pregnant mice were injected with 2 - 4 x 10^6 cpm ^{125}I-hGH into the left jugular vein. For the next 5 to 10 minutes samples were taken from the orbital sinus using calibrated 30 ul heparinized micro-hematocrit tubes. The blood was then lysed by dispensing into 0.5 ml distilled water in which the hematocrit tube was extensively rinsed. The entire lysate was counted. The immunoreactivity of these counts was determined by reacting the lysate with antiserum to hGH as described above. Samples obtained from 4 non-pregnant mice were treated similarly.

To determine the disappearance rate of the ^{125}I-hGH, the immunoprecipitable counts were used to determine an exponential curve which best fitted the data for each individual series of points. The slopes of the lines obtained for the 4 animals within a group were analysed to determine that they were not significantly different. Student's t-test

was used to compare the half-time disappearance rates observed in the pregnant and non-pregnant animals (34). The half-time disappearance rates of ^{125}I-oPRL from pregnant and non-pregnant animals were similarly determined and analysed.

RESULTS

Terminology: GH-LA and the GH-binding factor in serum from pregnant mice

As indicated in the Statement of Purpose, this study was originally undertaken to characterize what appeared to be a growth hormone-like factor detected by radioreceptor assay in the serum of pregnant mice. Later experiments demonstrated that a GH-binding factor present in the serum competed with the receptor in the RRA producing a false indication of growth hormone-like activity. Nevertheless, studies which characterized this competition showed that the amount of interference in the RRA, as expressed by the concentration of "GH-LA" was a reliable index of the activity of GH-binding factor in the mouse serum. Therefore, the results of initial studies designed to show the time course of appearance of this "GH-LA", the effect of hysterectomy on serum concentrations of the activity, and the distribution of "GH-LA" after fractionation of pregnant mouse serum by Sephadex gel filtration were expressed in terms of the values obtained by radioreceptor assay.

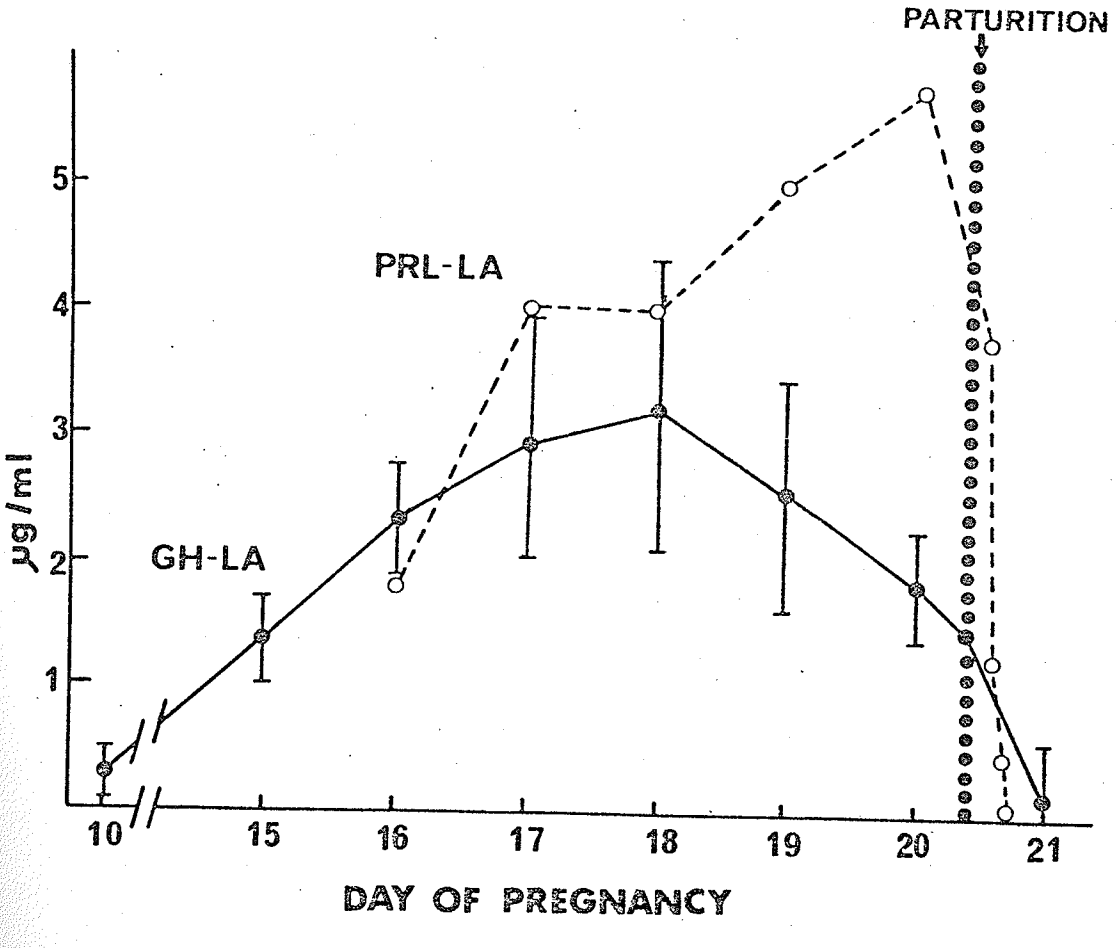
The appearance and disappearance of the GH-LA in serum

a) Radioreceptor assays for lactogens (PRL-LA) and for growth hormone-like factors indicatedthat the two activities did not appear or disappear at the same rate in the sera of

pregnant mice (Figure 3). GH-LA appears at mid-pregnancy and continues to increase until day 18 of pregnancy after which serum concentrations decline slowly and finally disappear 24 hours after parturition. Of samples obtained from 5 other animals in the immediate postpartum period, all showed GH-LA levels of more than 1 ug/ml more than 10 hours after birth of the pups. In the serum samples examined, the peak GH-LA and PRL-LA did not coincide temporally. In addition, the PRL-LA decreases much more rapidly at the time of delivery. In pools of sera obtained from foetuses at day 18 of gestation and from neonates no GH-LA or PRL-LA was detectable.

b) Four animals were hysterectomized on day 17 of pregnancy and serum samples obtained at intervals after surgery were assayed by both RRA's. Results of these assays are shown in Figure 4. The apparant GH-LA was still detectable 30 hours after removal of the gravid uterus, whereas lactogenic activity had almost disappeared 2 hours after surgery. The same relative rates of disappearance were detected when another 5 mice were hysterectomized on day 18 of pregnancy (data not shown). Extraction of placental tissue obtained from mice on day 18 of pregnancy reveals a lactogenic content of 50-100 ug (PRL equivalents) per gram of wet tissue as determined by RRA. GH-LA was undetectable in these same extracts.

Figure 3. The concentrations of GH-LA(●—●) and PRL-LA(o---o) in the sera of pregnant mice during the second half of pregnancy. The GH-LA concentration for each day represents the mean ± S.D. for N=4. The PRL-LA is represented by a single value obtained from a pool of the samples assayed for GH-LA. The dotted bar on the right indicates the time of parturition.



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GH-LA.
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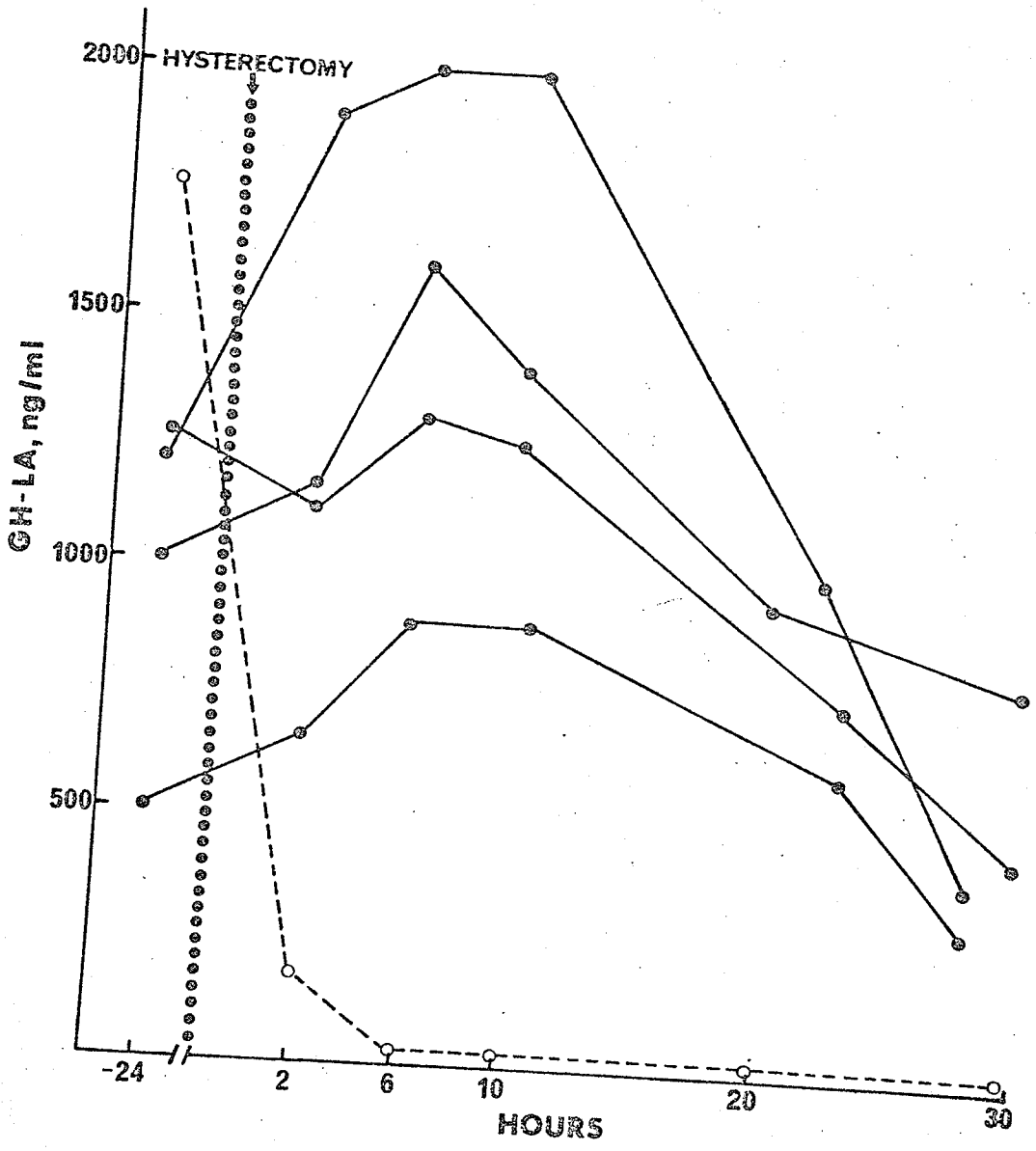
Figure 4. The effect of hysterectomy at day 17 of pregnancy on serum concentrations of GH-LA (●—●) and PRL-LA (o---o). Individual sera obtained from 4 animals at different intervals after surgery were assayed for GH-LA. Then samples were pooled and assayed for PRL-LA. The dotted bar on the left indicates the time of hysterectomy.


2000
HYSTERECTOMY
1500
1000
500
GH-LA, ng/ml
-24
2
6
10
20
30
HOURS

Apparent parallelism of mouse serum GH-LA and hGH in the GH-RRA

A sample of mouse serum obtained at day 18 of pregnancy was serially diluted and assayed in the GH-RRA. As shown by Figure 5, the inhibition curve generated by the diluted serum samples appeared to parallel that obtained with the purified hGH suggesting some similarity between the serum factor and GH standard. A demonstration of parallelism of this kind is frequently used to validate the nature of an unknown material (22).

Fractionation of serum on Sephadex G 100; distribution of GH-LA and PRL-LA in the eluates

Mouse serum obtained on day 18 of pregnancy was fractionated on a Sephadex G 100 column (Figure 6A). RRA of the resulting eluates showed that the GH-LA appeared in fractions shortly after the void volume of the column with $V_e/V_o = 1.2$, while PRL-LA appeared in fractions eluting with $V_e/V_o = 2.0$.

Binding of ^{125}I-hGH to a serum factor

a) Immediately before gel filtration of the serum, ^{125}I-hGH was added to the sample as a molecular weight marker. Figure 6B illustrates that, when fractionated in the presence of serum obtained on day 18 of pregnancy, ^{125}I-hGH appeared in fractions that corresponded to those in which GH-LA was detected by RRA, as shown in Figure 6A. This same elution profile was observed when the mixture of ^{125}I-hGH and serum obtained at day 18 of pregnancy were eluted from columns with either 0.05M

Figure 5. Parallelism of serial dilutions of mouse serum obtained on day 18 of pregnancy (●—●) and of purified hGH (o---o) in the particulate GH-RRA.

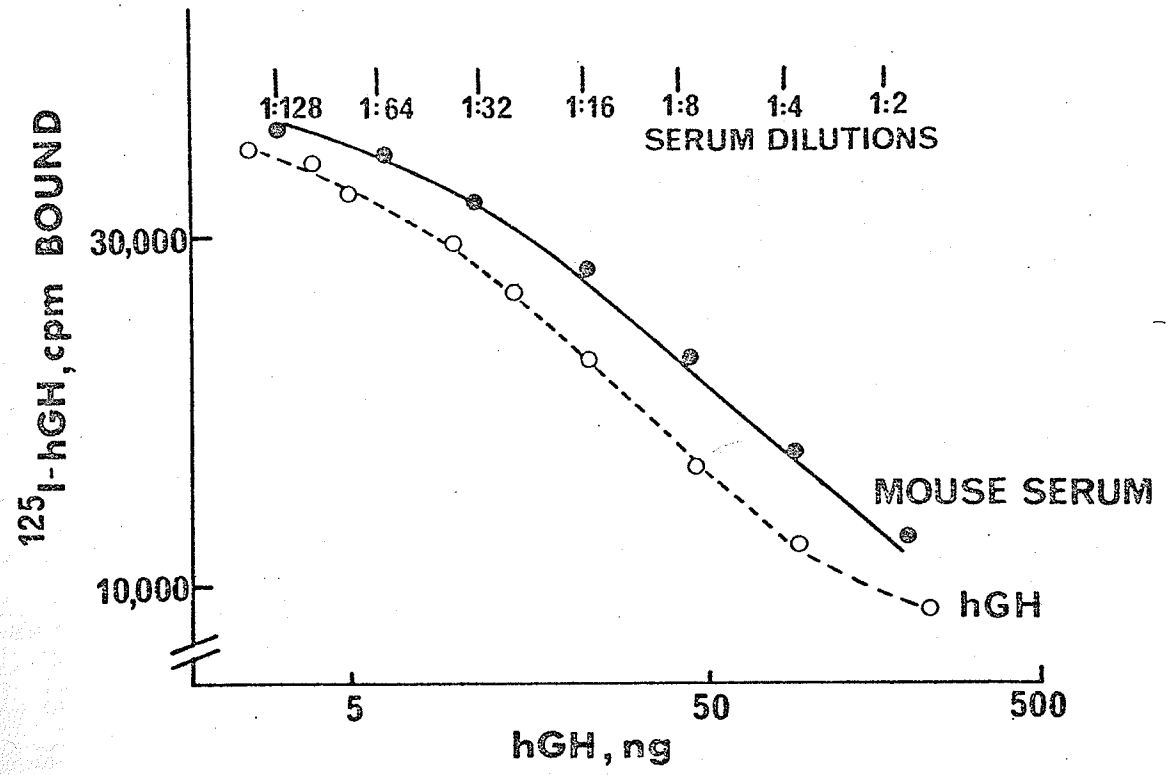
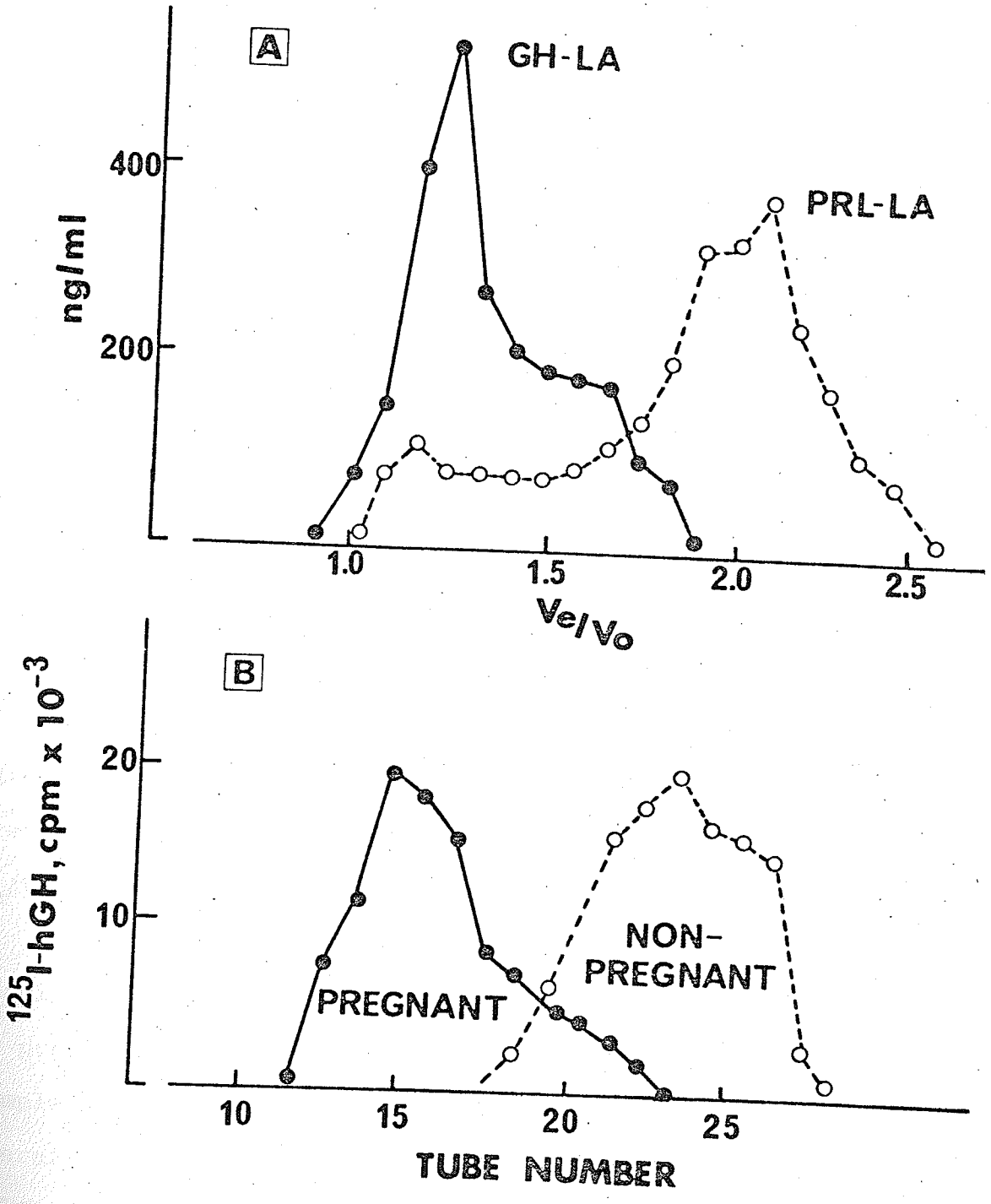

1:128
1:64
1:32
1:16
1:8
1:4
1:2
SERUM DILUTIONS
^{125}I-hGH, cpm BOUND
30,000
10,000
MOUSE SERUM
hGH
5
50
500
hGH, ng

Figure 6A. Distribution of GH-LA (●—●) and PRL-LA (o---o) in eluates of a Sephadex G 100 column (0.9 x 90 cm). The sample was 1.0 ml of mouse serum obtained at day 18 of pregnancy. The eluant is 0.025M tris-HCl pH 7.6. Eluate volume = 2ml/tube. Fractionation is carried out at 10C. The abscissa indicates the relative eluation volume (V_e/V_o). More than 85% of the GH-LA and PRL-LA applied in the sample is accounted for in the eluates.

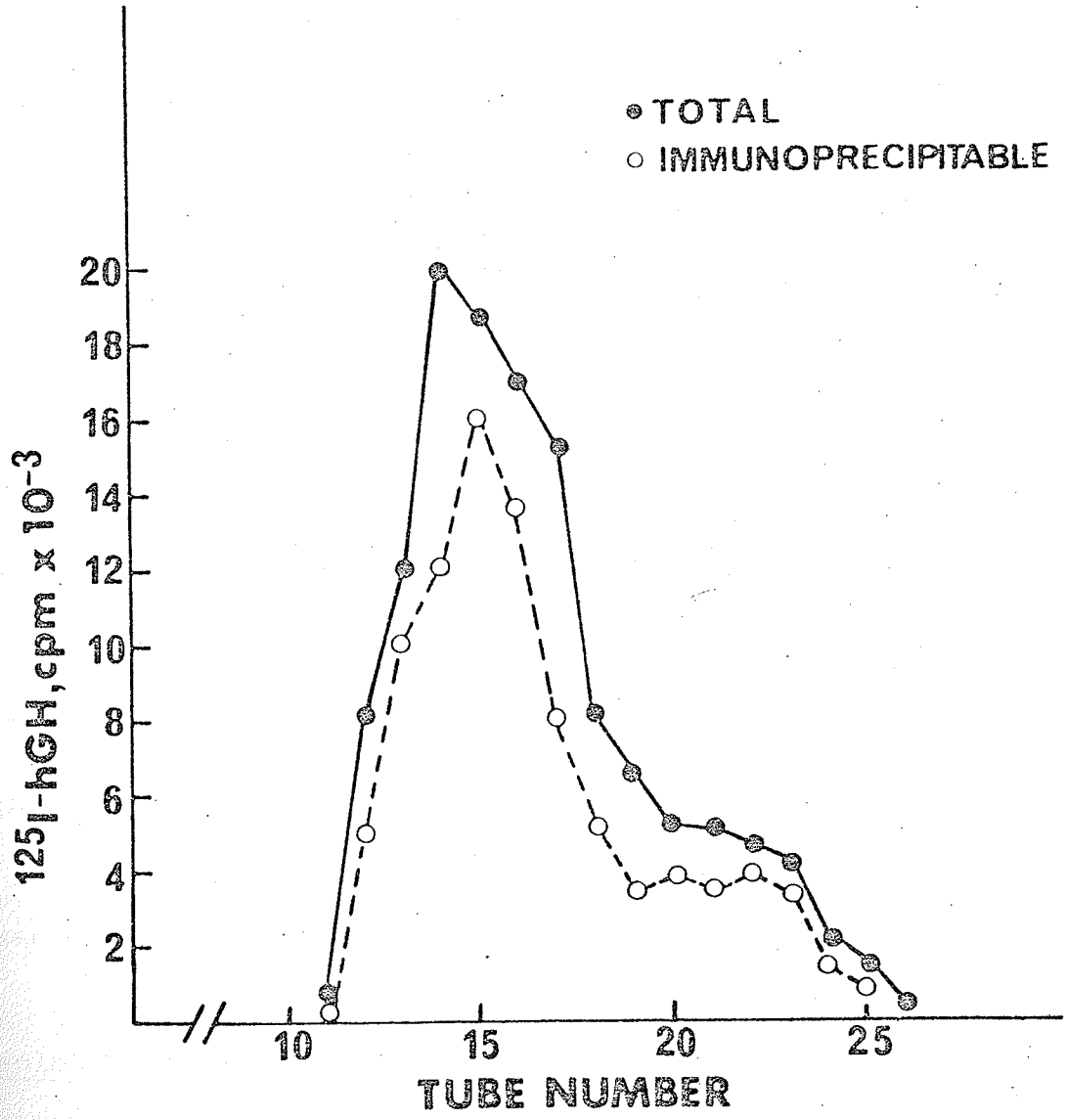
Figure 6B. Distribution of ^{125}I-hGH after fractionation on the same column of a mixture of ^{125}I-hGH and 1.0 ml of mouse serum obtained on day 18 of pregnancy (●—●) and of ^{125}I-hGH and serum from a non-pregnant mouse (o---o). The abscissa indicates the tube numbers which correspond to the V_e/V_o on the abscissa of the upper panel.


A
GH-LA
PRL-LA
400
200
ng/ml
1.0
1.5
2.0
2.5
Ve/Vo
B
^{125}I-hGH, cpm x 10^{-3}
20
10
PREGNANT
NON-
PREGNANT
10
15
20
25
TUBE NUMBER

or 0.10 M ammonium bicarbonate pH 8.2, 0.05 M sodium phosphate pH 7.0, 8.0, and 9.0, or with 0.15 M sodium chloride, the ^{125}I-hGH still appeared in fractions shortly after the void volume of the column with V_e/V_o = 1.2. When ^{125}I-hGH alone was fractionated, the radioactive hormone eluted with a relative elution volume equal to 2.0. When ^{125}I-hGH and serum from non-pregnant mice were fractionated together in a similar manner, the ^{125}I-hGH again eluted with a position represented by V_e/V_o = 2.0. ^{125}I-oPRL whether fractionated alone or in the presence of serum from pregnant or non-pregnant mice also eluted with V_e/V_o = 2. ^{125}I-rFSH when fractionated alone or with serum from pregnant or non-pregnant mice eluted with a V_e/V_o ratio equivalent to 1.7 consistent with the slightly larger molecular weight of rFSH. However, ^{125}I-bGH after fractionation revealed an elution profile similar to ^{125}I-hGH. In the presence of pregnant mouse serum ^{125}I-bGH eluted close to the void with V_e/V_o = 1.2. Alone, or in the presence of serum from non-pregnant mice, ^{125}I-bGH eluted with V_e/V_o = 2.0.

The ^{125}I-hGH in fractions shown in Figure 6B was precipitated by incubation with sufficient antiserum to hGH to precipitate more than 90% of the ^{125}I-hGH in the original sample applied to the column. The results are shown in Figure 7. Overall, 75% of the ^{125}I-hGH was recovered in the large molecular weight fractions (tube #1-18). Of the total counts in these same fractions, 68% was precipitated by excess antiserum to hGH.

Figure 7. Immunoprecipitability of ^{125}I-hGH in the eluates obtained after fractionation of ^{125}I-hGH and serum from a pregnant mouse as described for Figure 6B. Of the total ^{125}I-hGH in the eluates (⊙—⊙) 68% is precipitated (o---o) by excess antiserum to hGH.


• TOTAL
○ IMMUNOPRECIPITABLE
^{125}I-hGH, cpm x 10^{-3}
20
18
16
14
12
10
8
6
4
2
10
15
20
25
TUBE NUMBER

Binding of ^{125}I-hGH to extracts of tissues obtained from mice

Tissues obtained from pregnant and non-pregnant mice at sacrifice were extracted as described in the Materials and Methods. ^{125}I-hGH was reacted with 0.2 ml of extract at room temperature and the reaction mixture was fractionated on a Sephadex G 100 column as described above. In all cases, the ^{125}I-hGH was recovered in fractions with mean V_e/V_o = 2.0 corresponding to the fractions in which the radioactivity was recovered when ^{125}I-hGH alone was fractionated.

Binding of ^{125}I-hGH to sera from species other than the mouse

Sera from rat, guinea pig, hamster, rabbit, rhesus monkey and human, obtained from pregnant and non-pregnant donors, were incubated with ^{125}I-hGH before being fractionated on a Sephadex G 100 column as described above. In the presence of rat serum, up to 50% of the recovered ^{125}I-hGH eluted in the void volume. (data not illustrated) When reacted with any of the other sera, the ^{125}I-hGH eluted, as it did in the absence of any sera whatsoever, with V_e/V_o = 2.0.

Displacement of ^{125}I-hGH from GH-binding factor by unlabelled GH

To ascertain further the specificity of the binding factor, increasing quantities of unlabelled hormone were added to a mixture of 25 ul ^{125}I-hGH and 0.2 ml of serum obtained from mice. The mixture was incubated at room temperature for 5 minutes before being applied to a Sephadex G 100 column. The results are summarized in Table 1.

TABLE 1

Binding of ^{125}I-hGH to a GH binding factor

Serum 0.2 ml	Hormone Added ng	Percent ^{125}I-hGH 'bound' (V_e/V_o = 1.2)
-	-	11, 10*
non-pregnant female	-	17
male	-	12
pregnant, day 18	-	64, 62*
"	hGH, 50	65
"	250	64
"	2500	51
"	25,000	29, 19*
"	oPRL 25,000	68
"	bGH 25,000	34

* duplicate fractionation

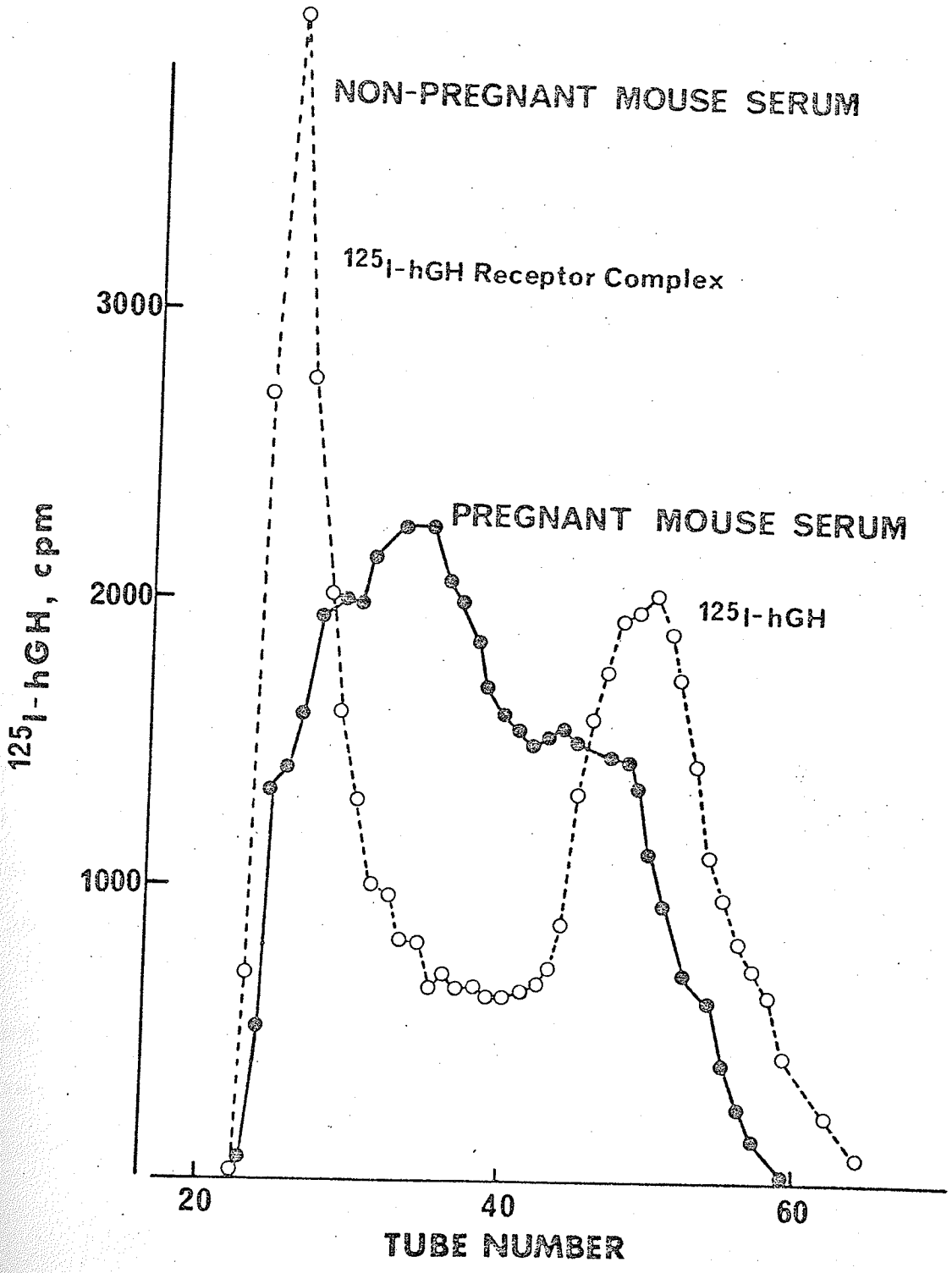
Percent ^{125}I-hGH "bound" refers to the radioactivity recovered in the equivalent of tubes #1-18 of Figure 7 as a percent of the total radioactivity recovered after fractionation.

Interference of the GH-binding factor in the RRA

It now became important to substantiate the idea that the GH-LA detected by the radioreceptor assay was an artefact caused by a GH-binding factor in the sera of pregnant mice which was capable of binding ^{125}I-hGH under assay conditions.

Mouse serum was again assayed by RRA this time using a solubilized rabbit liver receptor preparation. At the end of the standard incubation period, the entire reaction mixture was fractionated on a Sephadex G 100 column. The results are shown in Figure 8. When receptor and ^{125}I-hGH, alone or in the presence of non-pregnant mouse serum were fractionated, the ^{125}I-hGH was recovered in 2 major peaks. The receptor is known to have a molecular weight of more than 200,000 daltons and this first peak represents the complex of tracer and receptor (29). The remaining ^{125}I-hGH is recovered as unreacted monomeric ^{125}I-hGH. When 500 ng of unlabelled hGH was incubated with the ^{125}I-hGH and receptor and then fractionated, 73% of the radioactivity was recovered as monomeric ^{125}I-hGH. When serum obtained from a pregnant mouse was included in the assay, the distribution of radioactivity after fractionation of the sample shows ^{125}I-hGH clearly associated with proteins of an intermediate molecular weight. This same pattern was observed after gel filtration regardless of whether serum obtained from pregnant mice was reacted with receptor and ^{125}I-hGH for 5 minutes or for 3 hours and corresponds to the binding pattern observed when only ^{125}I-hGH and serum from pregnant mice, in the absence of receptor, were reacted before fractionation on the same column.

Figure 8. Distribution of ^{125}I-hGH after fractionation of the incubation mixture of soluble radioreceptor assays on a Sephadex G 100 column (1 x 45 cm). The RRA was carried out with serum from non-pregnant mice (o---o) and from day 18 pregnant mice (●—●) before fractionation. Volume of reaction mixture applied = 0.5 ml. The eluant is 0.025M tris-HCl pH 7.6 containing 0.1% Triton X-100. Eluate volume = 0.5 ml.


NON-PREGNANT MOUSE SERUM
125I-hGH Receptor Complex
PREGNANT MOUSE SERUM
125I-hGH
3000
2000
1000
125I-hGH, cpm
20
40
60
TUBE NUMBER

Inhibition of the ^{125}I-hGH binding to receptor by sera from pregnant mice

To define more clearly the inhibition of ^{125}I-hGH binding to receptor caused by serum from pregnant mice, assays were set up analogous to those used to study the kinetics of inhibitory enzymes. Fixed amounts of serum and ^{125}I-hGH were reacted with increasing amounts of receptor. This is analogous to the enzyme model with the following equivalences assumed: Substrate = Receptor, Enzyme = ^{125}I-hGH, Inhibitor = Serum, Velocity of reaction = Specific binding. The data that resulted are plotted in Figure 9. It can be seen that in the presence of increasing amounts of serum (inhibitor) more and more receptor is required to achieve maximal binding. A double reciprocal plot of these data is seen in Figure 10. The lines were fitted by regression analysis. The common intercept on the ordinate indicates that the reaction is truly a competitive inhibition of binding of ^{125}I-hGH to liver GH receptor by serum obtained from pregnant mice. By definition, this type of interaction is also reversible. Serum obtained from non-pregnant mice also inhibits the interaction of ^{125}I-hGH and receptor but to a lesser degree. A similar progressive inhibition of binding of ^{125}I-hGH to receptor was revealed when 1.25 ul of serum from a single mouse obtained on days 11, 14 and 16 of pregnancy were assayed in the same way. (data not shown)

Figure 9. Percent specific binding of ^{125}I-hGH to increasing amounts of receptor. The addition of increasing amounts of serum from a pregnant mouse (2.5, 5, 10 ul) produced a progressive inhibition of specific binding of ^{125}I-hGH to each concentration of receptor in the routine RRA. The amount of receptor is expressed as the protein concentration of the particulate liver membrane preparation.

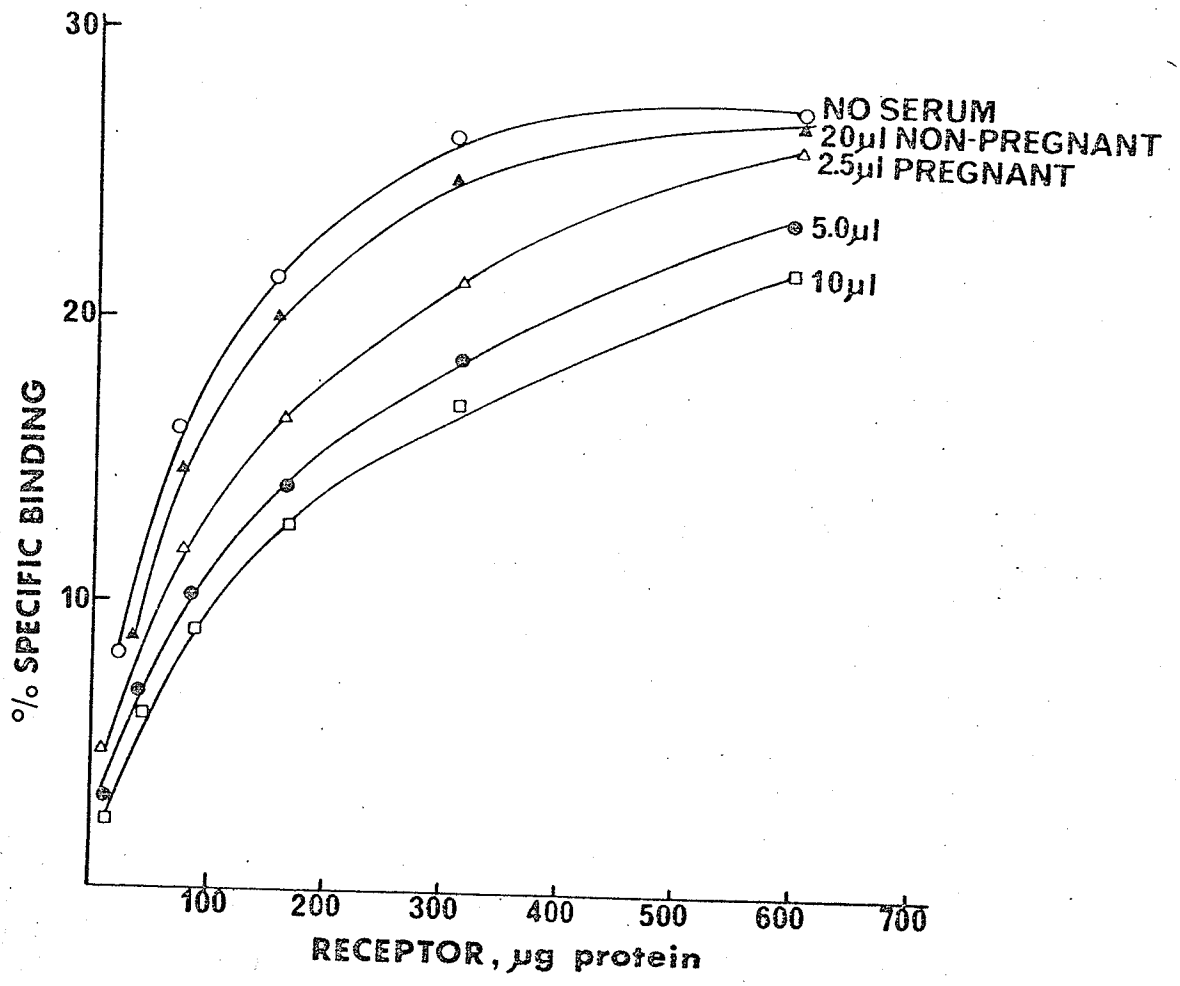
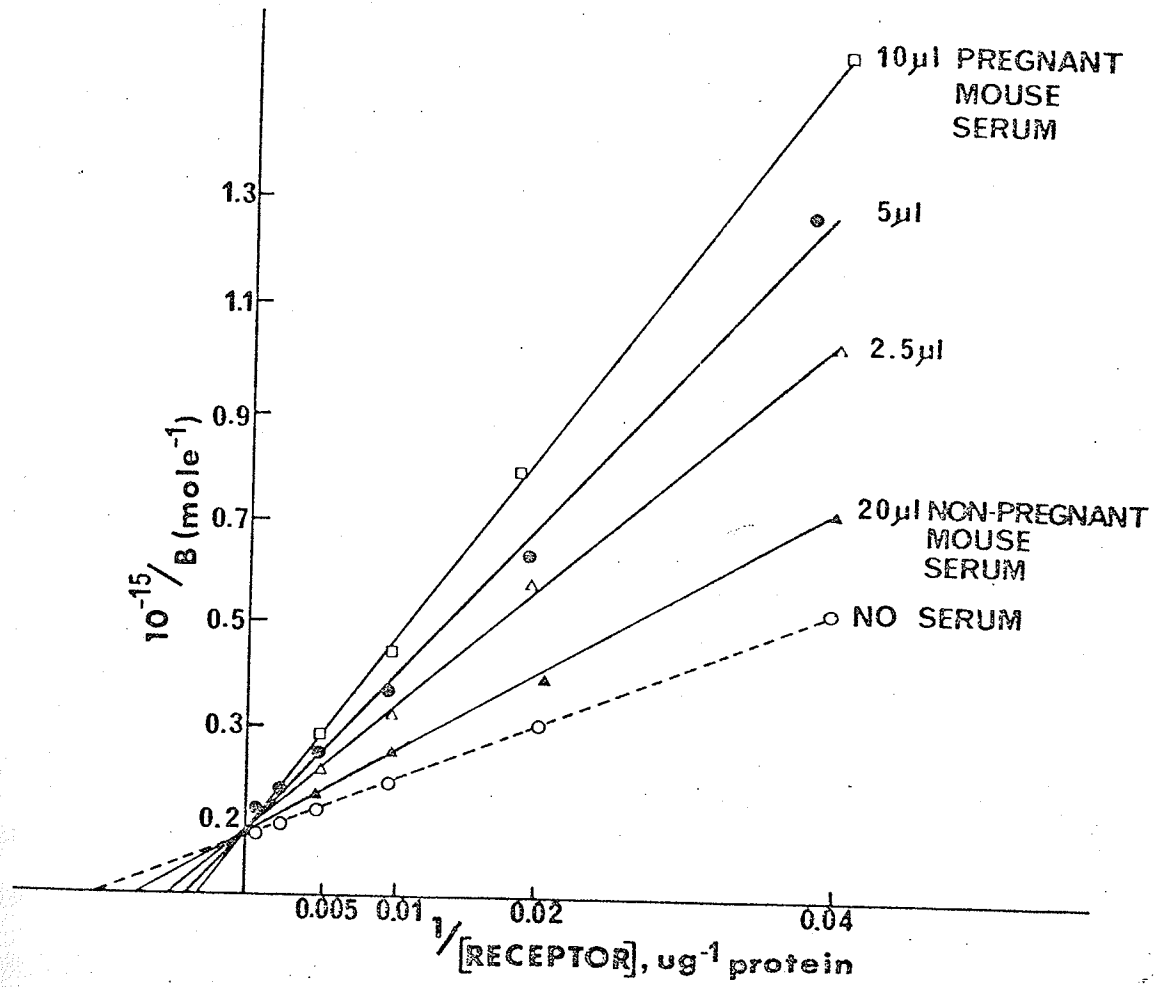

30
20
10
% SPECIFIC BINDING
NO SERUM
20µl NON-PREGNANT
2.5µl PREGNANT
5.0µl
10µl
100
200
300
400
500
600
700
RECEPTOR, µg protein

Figure 10. A Lineweaver-Burk plot of data shown in Figure 9.


10µl PREGNANT MOUSE SERUM
5µl
2.5µl
20µl NON-PREGNANT MOUSE SERUM
NO SERUM
1.3
1.1
0.9
0.7
0.5
0.3
0.2
10^-15/B (mole^-1)
0.005
0.01
0.02
0.04
1/[RECEPTOR], ug^-1 protein

Molecular weight of the GH-binding factor in serum

The molecular weight of the GH binding factor was estimated from its relative mobility (K_{AV}) when fractionated on a Sephadex G 200 column (Figure 11). The complex of ^{125}I-hGH and GH-binding factor has a molecular weight of 80,000 daltons. Assuming a monovalent reaction, subtraction of the molecular weight of hGH leaves the GH-binding factor with an estimated molecular weight of 60,000 daltons.

Binding of hormone to GH-binding factor after in vivo injection of unlabelled hormone

Ten micrograms of unlabelled hGH or oPRL were injected intravenously into pregnant and non-pregnant mice. The distribution of immunoreactive hormone recovered after fractionation on Sephadex G 100 of the resulting fresh serum is shown in Figure 12. When injected into pregnant mice IR-hGH was recovered in a relative elution volume of 1.2 while IR-oPRL persisted as the monomeric form of the molecule. To eliminate the time required to allow blood to clot, experiments were also carried out where blood was drawn into heparinized tubes. The plasma was separated by centrifugation and applied to a Sephadex G 100 column within 15 minutes of the injection. Fractionation of these plasma samples produced the same distribution of IR-hGH and IR-oPRL as were observed for serum samples.

When oPRL or hGH were injected into non-pregnant females matched for age, the immunoreactive hormones recovered after

Figure 11. Estimation of the molecular weight of the GH-binding factor complexed to ^{125}I-hGH (*). A column of Sephadex G 200 was calibrated with molecular weight markers as described in the Materials and Methods. The experimental sample was a mixture of ^{125}I-hGH and 1.0 ml of mouse serum obtained at day 18 of pregnancy.

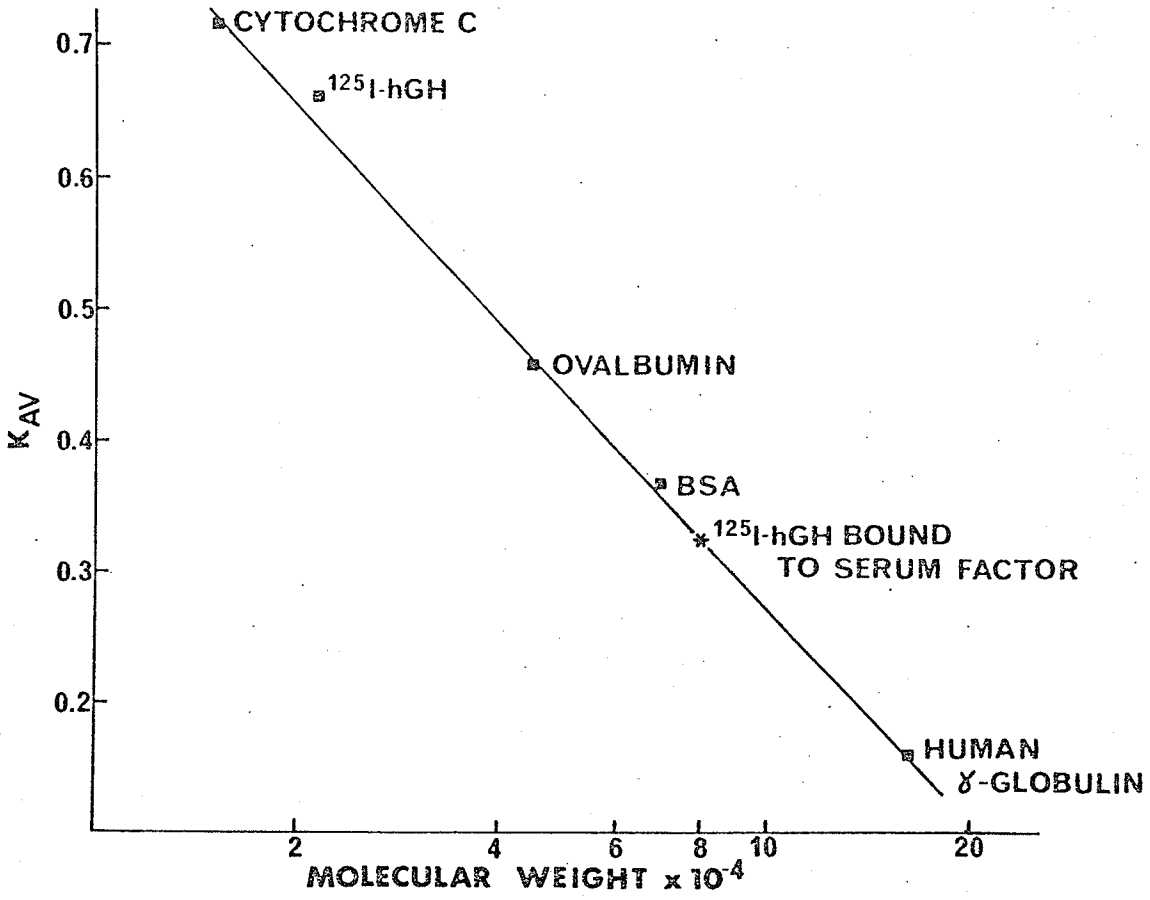
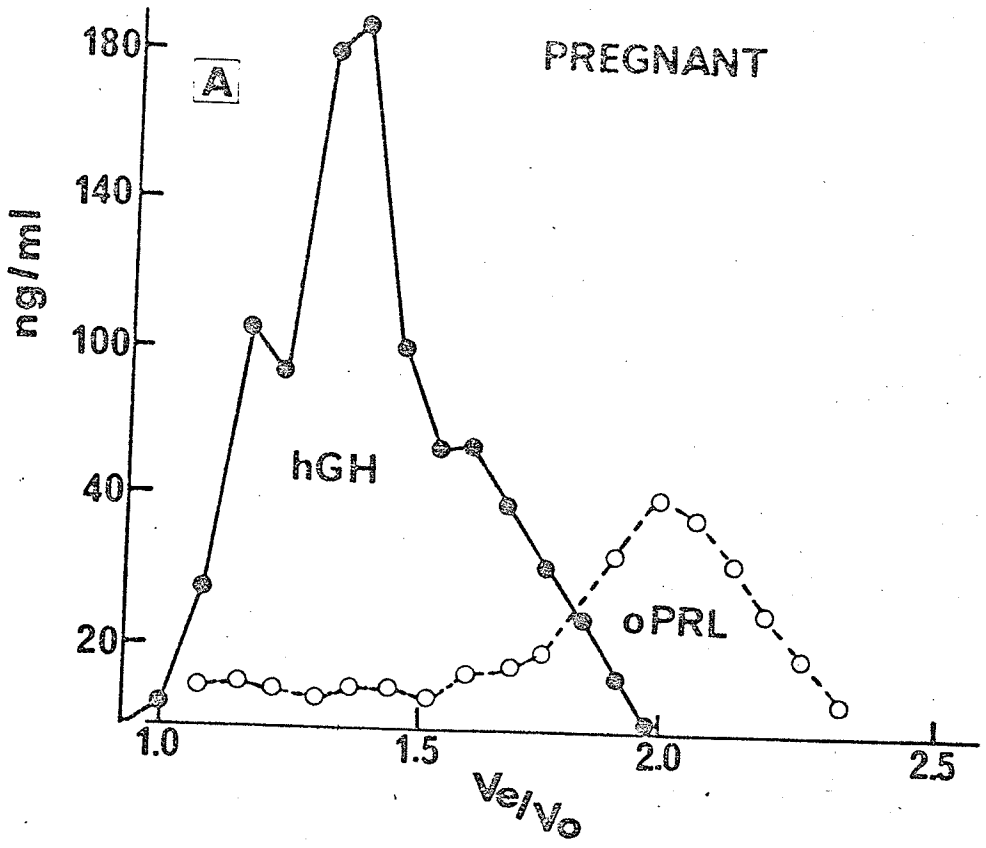
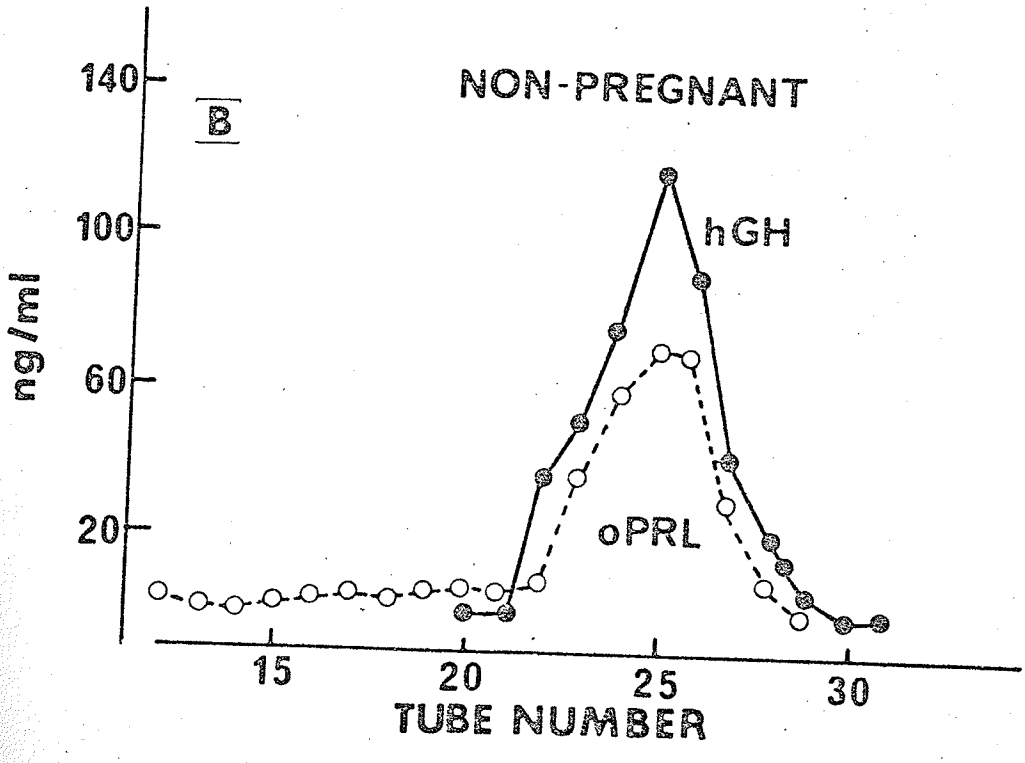

CYTOCHROME C
^{125}I-hGH
OVALBUMIN
BSA
^{125}I-hGH BOUND
TO SERUM FACTOR
HUMAN
γ-GLOBULIN
0.7
0.6
0.5
0.4
0.3
0.2
K_{AV}
2
4
6
8
10
20
MOLECULAR WEIGHT x 10^{-4}

Figure 12A. Distribution of hGH (◎—◎) and oPRL (o---o) as detected by radioimmunoassay of aliquots of eluates of Sephadex G 100 columns. Plasma recovered within 5 minutes of injection of 10 ug of oPRL or hGH into pregnant mice was fractionated as described for Figure 6A.

Figure 12B. Plasma recovered from non-pregnant mice after injection of unlabelled hormone was similarly fractionated and assayed.


A
PREGNANT
180
140
100
40
20
ng/ml
hGH
oPRL
1.0
1.5
2.0
2.5
Ve/Vo


B
NON-PREGNANT
140
100
60
20
ng/ml
hGH
oPRL
15
20
25
30
TUBE NUMBER

fractionation and assay of the eluates had a relative elution volume of 2.0. No hGH or oPRL activity was detected by radioimmunoassay of eluates obtained after sera from untreated pregnant mice were fractionated alone. Hence, IR-hGH injected intravenously into pregnant mice was recovered after gel filtration in a relative elution volume of 1.2 corresponding to the fractions in which ^{125}I-hGH was recovered after reaction of the ^{125}I-hGH and serum *in vitro*. It is worth noting that in the small volume of serum recovered after injection, 0.4 ml , almost one microgram of hormone appears bound.

Binding of endogenous mouse growth hormone

To examine the possibility that endogenous mouse GH was bound in a manner similar to the exogenously introduced hGH 1.0 ml of fresh serum obtained on day 18 of pregnancy was fractionated on a Sephadex G 200 column similar to the one used to determine the molecular weight of the GH-binding factor. A small amount of ^{125}I-hGH was added to the serum 5 minutes before applying the sample to the column. Protein distribution in the eluates was determined by absorbance at 280 nm. The eluates were assayed by RRA to detect "GH-LA" and by RIA to locate immunoreactive GH. As a control 1.0 ml of serum from non-pregnant mice was fractionated and assayed

in a similar manner. The results are shown in Figure 13. When serum from a non-pregnant mouse was fractionated (upper panel) the ^{125}I-hGH eluted in the same fractions that it did when fractionated in the absence of serum. No "GH-LA" was detected by RRA. Although 19 ng of IR-GH were applied, the amounts recovered were at the limits of sensitivity of the RIA. The validity of the distribution shown by the stippled areas is therefore open to question.

When serum from pregnant mice was fractionated (lower panel), the ^{125}I-hGH, "GH-LA" and IR-GH were all recovered in the same fractions, corresponding to a molecular weight of 80,000 daltons. A peak value of 400 ng/ml of "GH-LA" was detected by RRA. Mouse IR-GH was recovered in concentrations easily detected by the RIA. No IR-GH was detectable in fractions corresponding to the expected position of monomeric GH.

IR-GH in the sera of mice

Sera were obtained from a group of mice on alternate days of pregnancy and were assayed for GH by RIA. Because individual serum samples were sometimes used for other purposes and because of the limited volume of sample available, the number of samples for each group varies. In addition, pools of sera obtained by exsanguinating large numbers of animals were also assayed. Results are shown in Table 2.

Figure 13A. 1.0 ml of serum from a non-pregnant mouse was fractionated on a Sephadex G 200 column as described in the Materials and Methods. The eluates were monitored for protein concentration (Δ---Δ), ^{125}I-hGH (◎—◎), IR-GH (stippled) and GH-LA (---). No GH-LA was detected.

Figure 13B. Elution pattern after fractionation of 1.0 ml of serum from a day 18 pregnant mouse as described for Figure 13A.

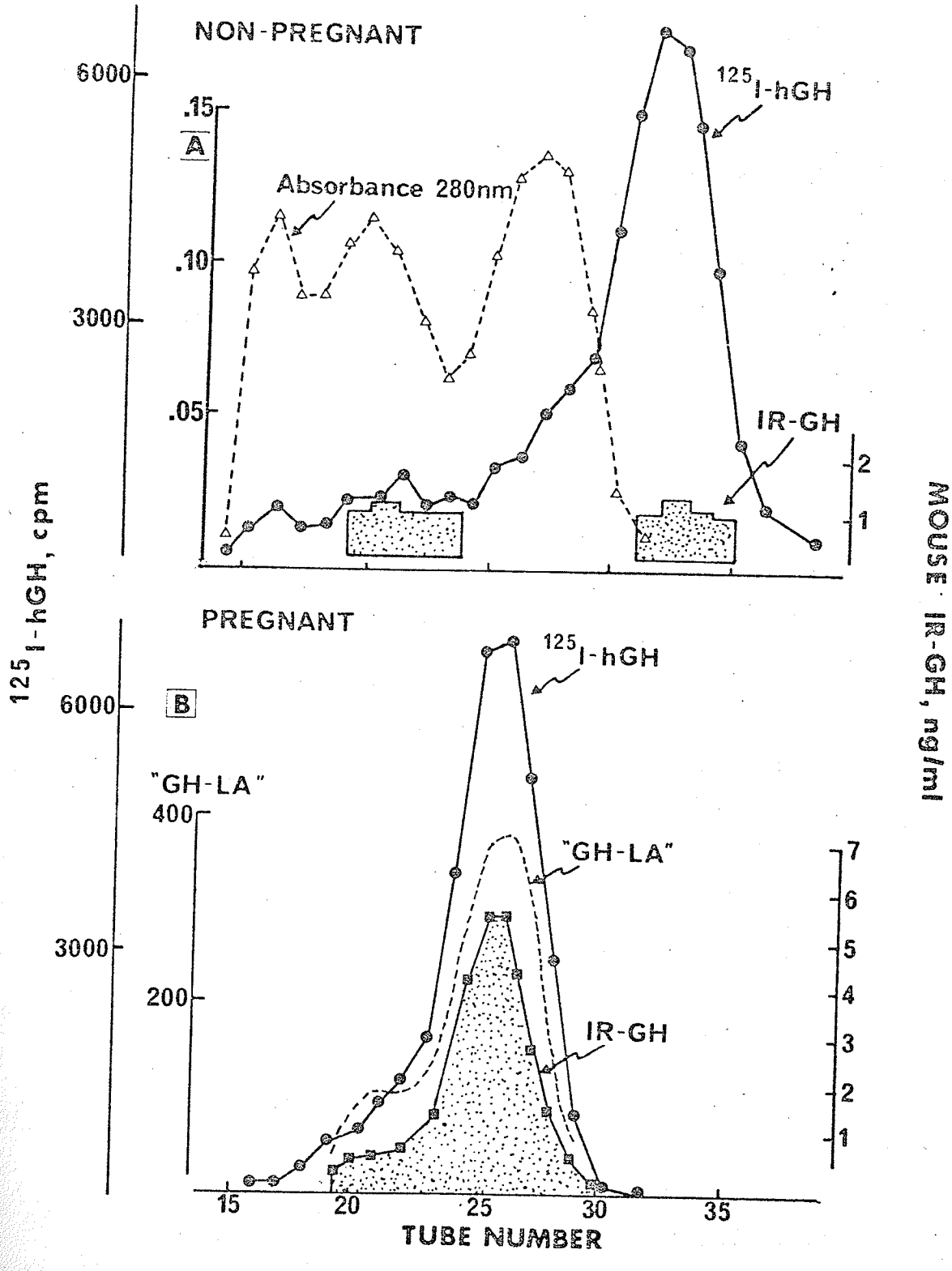

NON-PREGNANT
A
6000
3000
.15
.10
.05
Absorbance 280nm
125I-hGH
IR-GH
2
1
PREGNANT
B
6000
3000
"GH-LA"
400
200
125I-hGH
"GH-LA"
IR-GH
7
6
5
4
3
2
1
15
20
25
30
35
TUBE NUMBER
125I-hGH, cpm
Mouse IR-GH, ng/ml

TABLE 2

Serum Immunoreactive GH in Mice

Day of Pregnancy	IR-GH ng/ml ± S.D.	Number of Mice
11	26±4	13
13	43±6	15
15	60±8	16
17	60±5	9
Pooled sera		
non-pregnant female	20	32
normal male	22	59
day 18 of pregnancy	49	33

There appeared to be an increase of serum IR-GH during the latter half of pregnancy. The peak values were 2-3 times greater than those of sera obtained from non-pregnant female or male mice.

Half-time disappearance (T1/2) of ^{125}I-hGH from the circulation

The T1/2 of ^{125}I-hGH and ^{125}I-oPRL in the circulation of pregnant and non-pregnant mice were determined as described in the Materials and Methods. Results are illustrated in

Figure 14. Disappearance of immunoprecipitable ^{125}I-hGH (top) and ^{125}oPRL from the circulation of pregnant and non-pregnant mice. Each panel illustrates the data obtained from one representative animal of a group of 4. Lines were fitted by regression analysis.

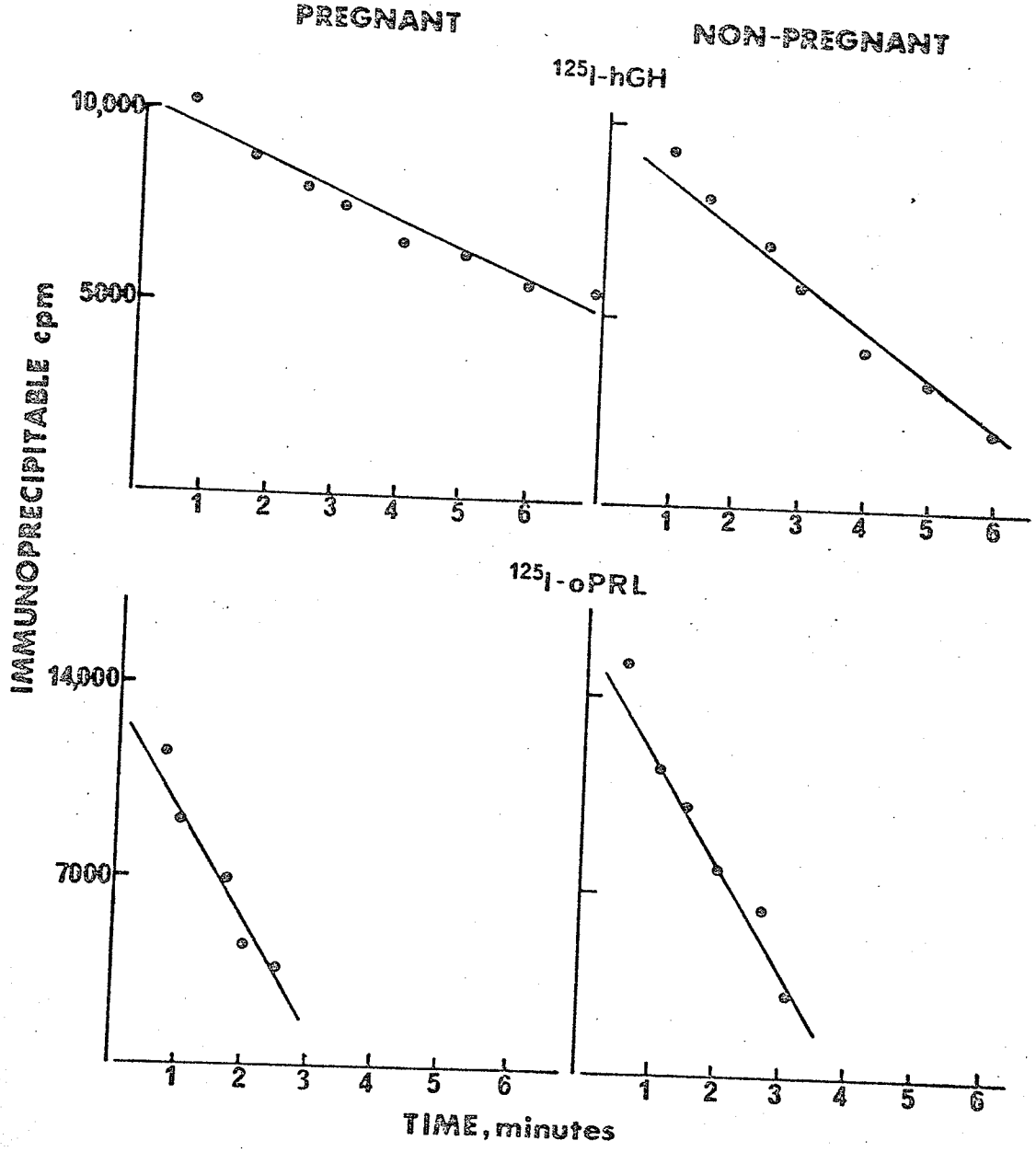

PREGNANT
NON-PREGNANT
^{125}I-hGH
10,000
5000
IMMUNOPRECIPITABLE cpm
1 2 3 4 5 6
^{125}I-oPRL
14,000
7000
TIME, minutes

Figure 14 and summarized in Table 3.

TABLE 3

Tl/2 of ^{125}I-hormone from the circulation

	Tl/2 ± S.D., Minutes		Significance
	Non-pregnant	Pregnant	Student's t-test
^{125}I-oPRL	2.3 ± 0.7	2.6 ± 0.5	$p < .247$
^{125}I-hGH	4.1 ± 0.5	6.3 ± 1.1	$p < .005$

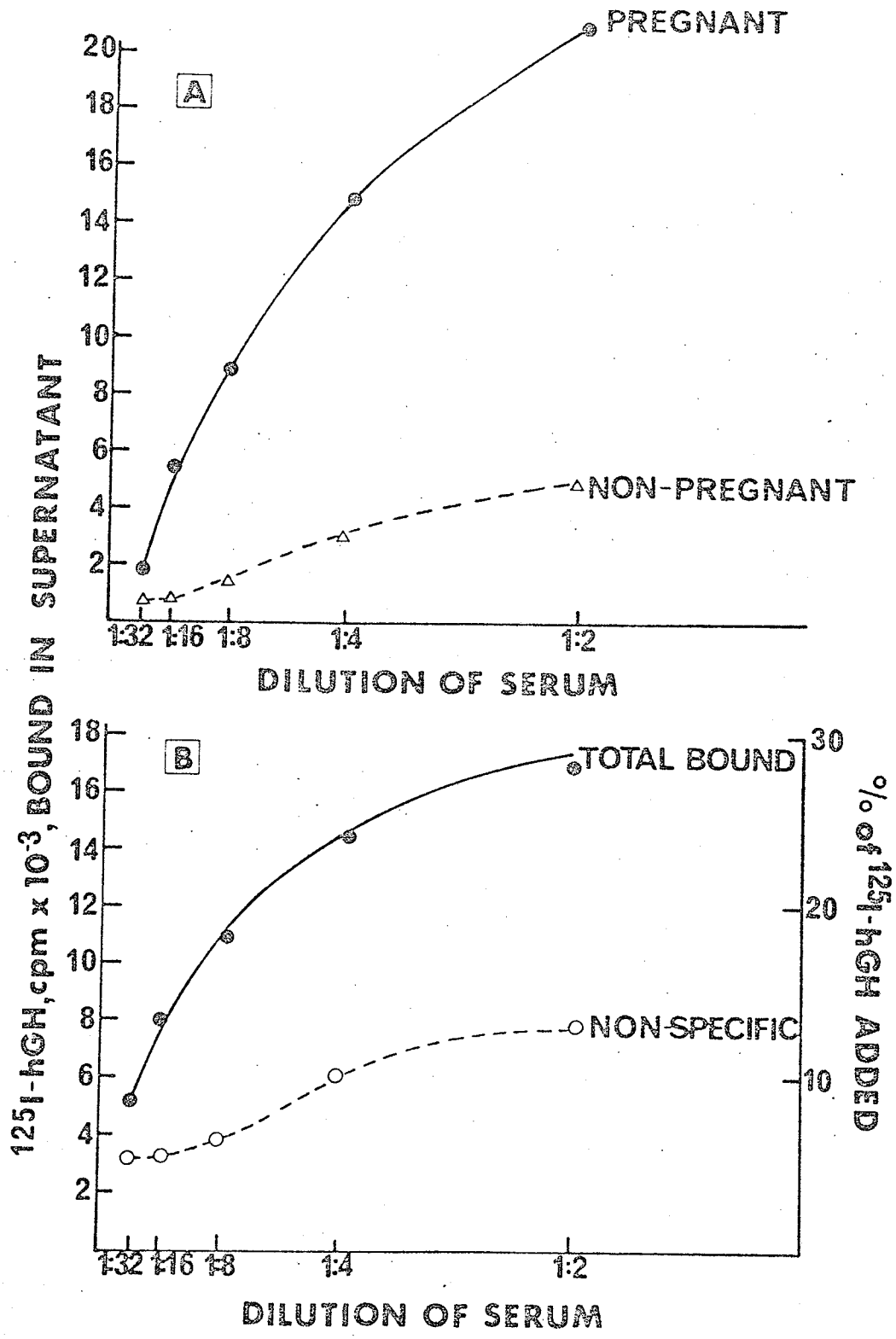
The Tl/2 of ^{125}I-hGH was significantly longer in the pregnant animal than in the non-pregnant control. The Tl/2 of ^{125}oPRL was similar in pregnancy and non-pregnancy.

Rapid detection of GH-binding factor in serum

Reagents employed for this determination are outlined in the Materials and Methods. The results of incubating different dilutions of a serum pool from pregnant and non-pregnant mice with a fixed amount of ^{125}I-hGH are shown in Figure 15A. Serum from non-pregnant mice no longer showed any activity when diluted more than 1:8. The binding of ^{125}I-hGH recovered in the supernatant after incubation of ^{125}I-hGH with different dilutions of serum from pregnant mice is illustrated in Figure 15B. Dilutions of 1:5 and 1:10 were most frequently within the linear part of the curve and were, therefore, used most often. The effect of varying the incubation

Figure 15A. ^{125}I-hGH and dilutions of serum from pregnant or non-pregnant mice were incubated as described in the Materials and Methods. The ^{125}I-hGH remaining in the supernatant after the addition of charcoal is on the ordinate.

15B. Serial dilutions of serum from pregnant mice were incubated with ^{125}I-hGH + 100 ug of hGH. 'Total bound' indicates the ^{125}I-hGH bound to the GH-binding factor in the absence of unlabelled hormone. 'Non-specific bound' indicates the counts remaining in the supernatant when incubated in the presence of excess hGH. The difference between the 2 curves represents the ^{125}I-hGH specifically bound to a GH-binding factor in serum. The ordinate on the right is the ^{125}I-hGH bound expressed as a percent of the iodinated hormone added to the assay tubes.


A
PREGNANT
NON-PREGNANT
20
18
16
14
12
10
8
6
4
2
1:32
1:16
1:8
1:4
1:2
DILUTION OF SERUM
^{125}I-hGH, cpm x 10^{-3}, BOUND IN SUPERNATANT
B
TOTAL BOUND
NON-SPECIFIC
30
20
10
% of ^{125}I-hGH ADDED
18
16
14
12
10
8
6
4
2
1:32
1:16
1:8
1:4
1:2
DILUTION OF SERUM

time of ^{125}I-hGH and serum are shown in Figure 16. Within 30 seconds of mixing the 2 reagents ^{125}I-hGH and the GH-binding factor are associated. Because binding reaches a plateau after 20 minutes of incubation, a reaction period of 30 minutes was chosen for the standardized procedure.

Using this simple technique, it was possible to further verify the specificity of the binding. Serum from pregnant mice and ^{125}I-hGH were reacted with increasing amounts of different hormone preparations. The results are seen in Figure 17. The diminished ability of rat GH to displace ^{125}I-hGH from the binding factor was found to correspond to its reduced activity in the radioreceptor assay (Figure 18).

Figure 16. Specific binding of ^{125}I-hGH to a GH-binding factor. A 1:10 dilution of serum from day 18 pregnant mice was incubated with ^{125}I-hGH for different time intervals at 22C before the addition of dextran coated charcoal.

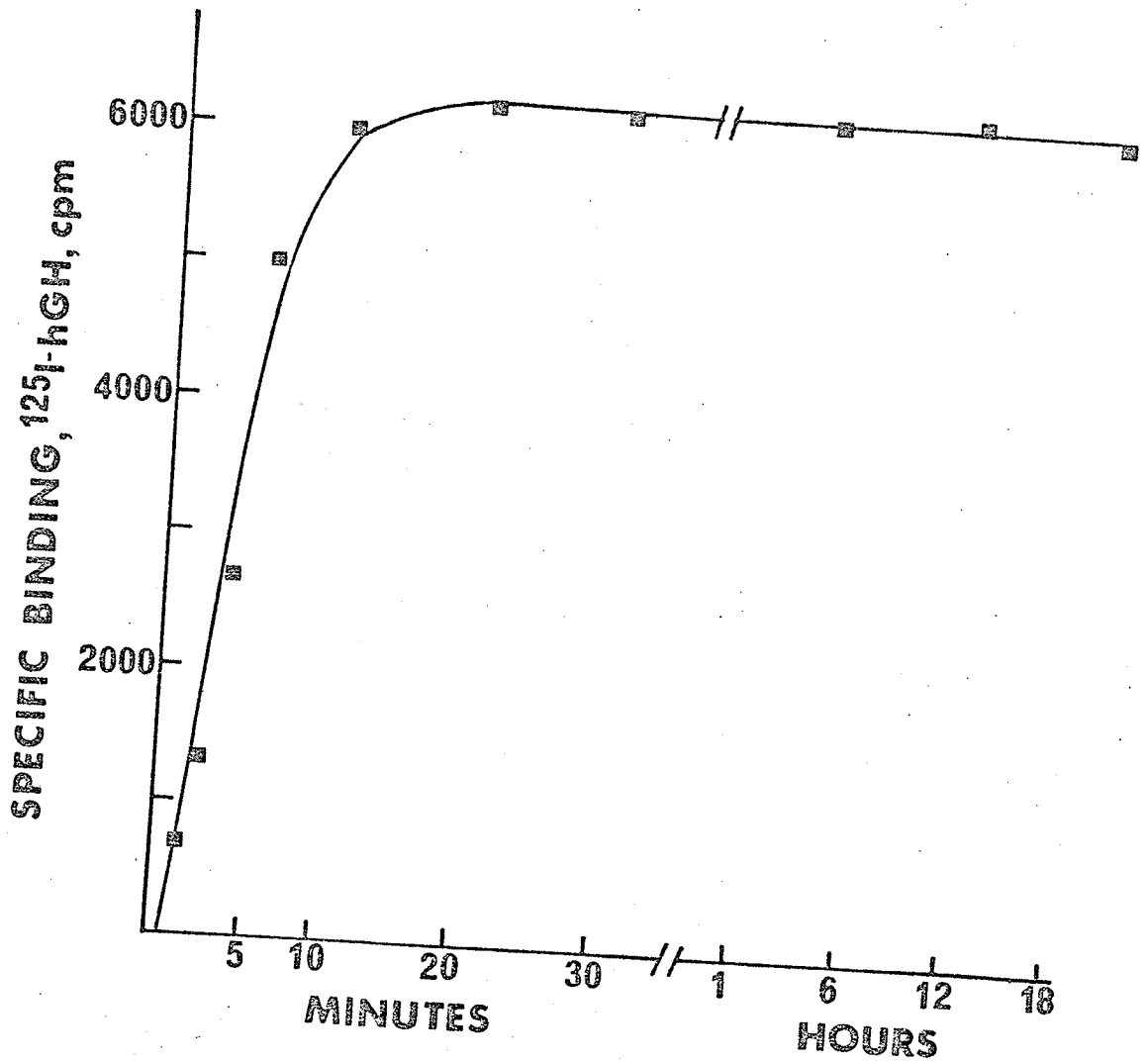

SPECIFIC BINDING, ^{125}I-hGH, cpm
6000
4000
2000
5
10
20
30
1
6
12
18
MINUTES
HOURS

Figure 17. Progressive displacement of ^{125}I-hGH from GH-binding factor in the serum of pregnant mice by the addition of increasing quantities of hormone.

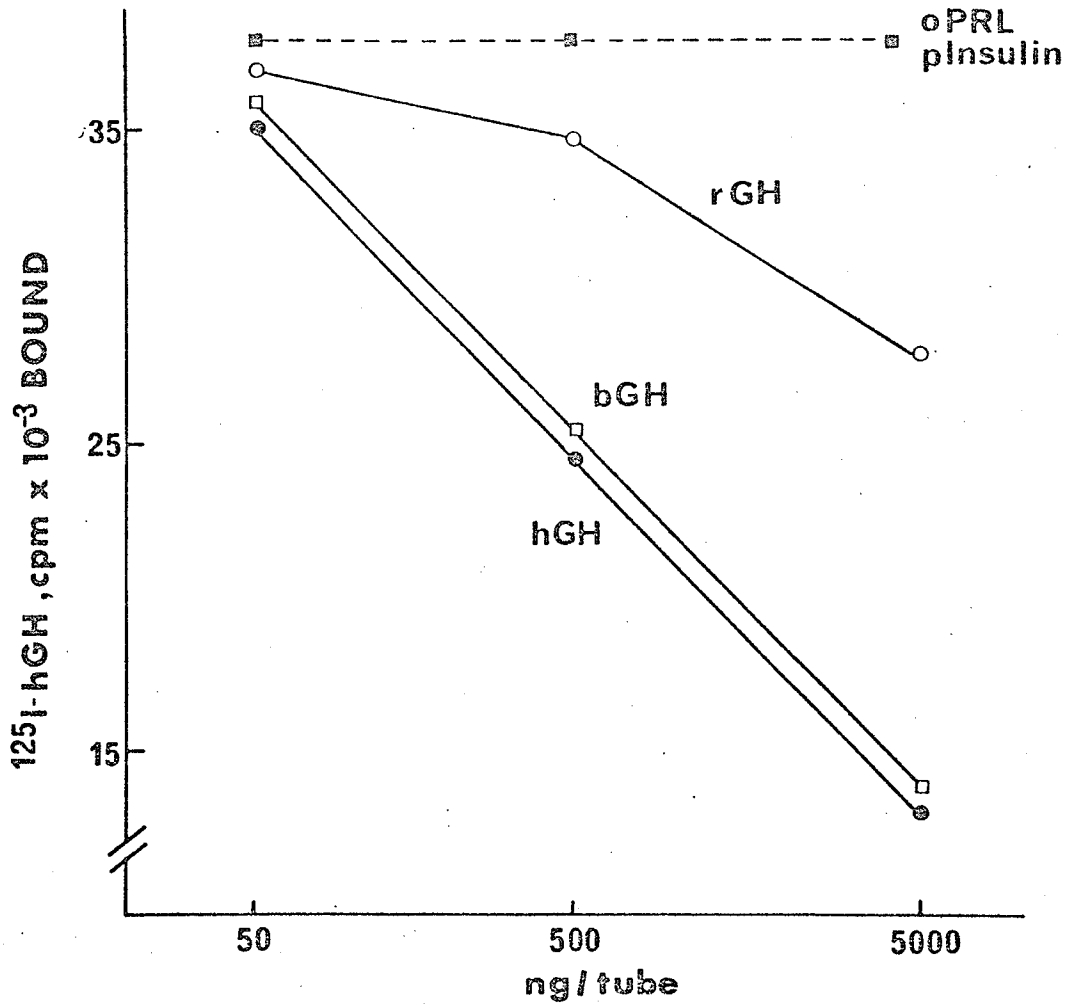
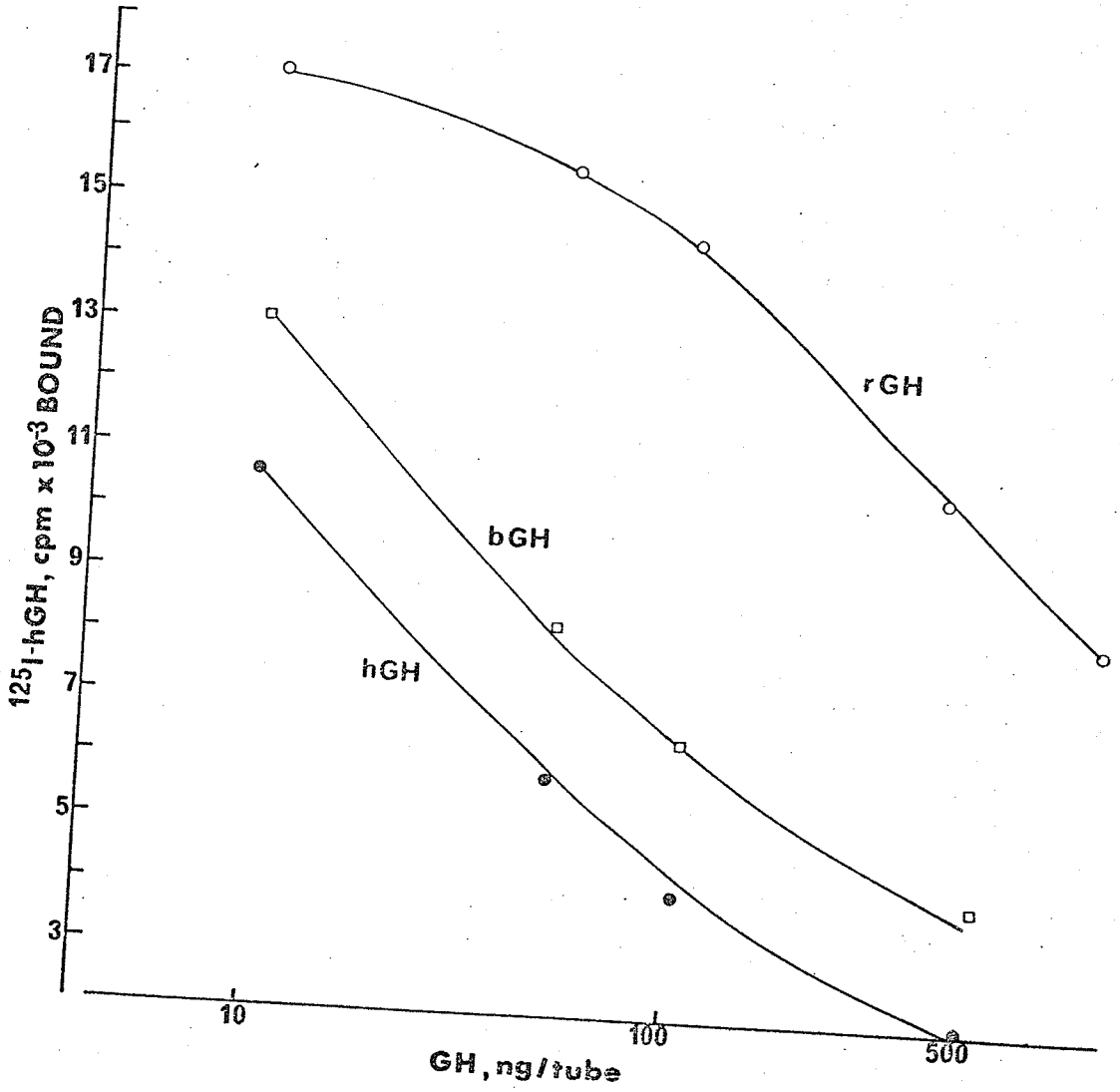

oPRL
pInsulin
rGH
bGH
hGH
35
25
15
^{125}I-hGH, cpm x 10^{-3} BOUND
50
500
5000
ng / tube

Figure 18. Progressive displacement of ^{125}I-hGH from rabbit liver receptor by increasing quantities of the 3 growth hormone preparations used in the experiment illustrated in Figure 17.


17
15
13
11
9
7
5
3
^{125}I-hGH, cpm x 10^{-3} BOUND
rGH
bGH
hGH
10
100
500
GH, ng/tube

DISCUSSION

Evidence from the literature for the existence of a GH-binding factor

For two decades evidence has accumulated that growth hormone, whether in purified pituitary preparations, in the circulation or as a biosynthetic product of pituitary culture, is represented by a heterogeneous population of molecules. Electrophoretic analyses of animal pituitary growth hormones gave the first indications of polymorphism (35,36). With the development of radioimmunoassays, it became possible to assess the immunoreactivity of physiological concentrations of hormone. This meant that circulating forms of the hormone could be examined (37,38,39). The activities of these growth hormones of different sizes have also been assessed by radioreceptor assay. Generally, the monomeric form of GH is found to be the most active in this system (47,48,49). Many studies that demonstrate the heterogeneity of growth hormone indicate that it may exist in close association with other proteins particularly in the circulation.

Gemzell in 1959 reported that TCA precipitation and ethanol fractionation of human serum concentrated endogenous growth promoting activity in an albumin rich fraction (40). Touber and Maingay showed that when ^{131}I-hGH was incubated with human serum, 11% of the radioactivity was recovered after gel filtration in the void volume of the fractionation

column (41). Immunoelectrophoresis showed that in this peak radioactivity was associated with α_2 and β_2 macroglobulins. In 1964 Hadden and Prout reported the binding of ^{125}I-hGH to the α_2 macroglobulin fraction of human serum (42). The ^{125}I-hGH was incubated with human serum for 4-8 days before being subjected to immunoelectrophoresis (IEP). Autoradiography of the IEP slide revealed the ^{125}I-hGH associated with a single band that showed a reaction of non-identity with the band formed by ^{125}I-hGH alone. The addition of 100 ug of unlabelled hGH to the incubation mixture prior to electrophoresis resulted in the displacement of the ^{125}I-hGH from the macroglobulin fraction. When antiserum to hGH was added to the reaction mixture prior to electrophoresis, the ^{125}I-hGH appeared in the γ globulin position indicating that the labelled hormone was still immunoreactive. The binding of ^{125}I-hGH to macroglobulins persisted in the presence of a peptidase inhibitor reducing the argument that enzyme degradation products were responsible for the initial observation.

In 1964 and 1966 Collipp et al. using acetylated (^{14}C)-hGH and paper electrophoresis to separate the components of their system showed that after incubation of labelled hGH with either human, rabbit, or rat serum the ^{14}C-hGH migrated to positions different from that observed when ^{14}C-hGH alone was electrophoresed (43,44). In 1970 Bala, Ferguson, and Beck found

that on gel filtration of plasma from normal and acromegalic human subjects more than 50% of the immunoreactive GH eluted before or with albumin (37). In 1972 Goodman, Tannenbaum and Rabinowitz reported that after gel filtration 14-28% of endogenous immunoreactive growth hormone was recovered in a fraction containing protein of molecular weight twice that of monomeric GH (38). Interestingly, in a footnote the authors comment that ^{125}I-hGH used as a molecular weight marker was in some cases associated with the albumin peak. Gorden, Hendricks and Roth also identified a hGH component with a molecular weight of 40,000 - 45,000 daltons (39).

Antoniades et al. have recently reported that the injection of monomeric iodinated human GH into rats results in the rapid binding of ^{125}I-hGH to serum proteins of 70,000 - 150,000 daltons (48). Within 5 minutes of injection more than 25% of the recovered ^{125}I-hGH was bound to these large molecular weight proteins. The authors reported a decreased immunoreactivity of the "bound" ^{125}I-hGH when compared to the unbound ^{125}I-hGH. This observation was similar to the decreased immunoprecipitability of ^{125}I-hGH after incubation with serum from a pregnant mouse. (Figure 7). Unlike the data reported in this thesis for mouse and rat serum, Antoniades et al. did not observe any binding of ^{125}I-hGH to rat serum when incubated *in vitro* for 2 hours. Similar observations on the binding of iodinated insulin to plasma proteins on

injection into cats and on the binding of insulin and proinsulin to proteins in the circulation of rats have been reported (49,50,51). Zapf et al. have recently reported evidence for the existence of a specific carrier protein for soluble non-suppressible insulin-like activity (NSILA-S) (52).

The evidence presented for the existence of a heterogenous population of growth hormones can be subject to a number of interpretations. 1) The possibility that the experimental procedures can induce artefacts of binding must be considered. Changes in protein conformations due to various extraction procedures, oxidation reactions occurring on exposure to air, type and duration of storage of samples, spontaneous polymerization, or incorporation into the hormone of a radioactive label, may all introduce modifications of molecular behavior (53,54,38,39,54,56). 2) The presence of large molecular weight GH in plasma may reflect the in vivo synthesis of 2 or more forms of GH. Recent biosynthetic experiments have established the existence of a GH-nucleic acid complex as an expected intermediate in the production of the hormone (57,58). Frohman and Stachura have established that when rat and human fetal pituitaries are cultured, there is, with time, a decrease in the amount of large molecular weight GH recovered in the media with a corresponding increase in monomeric GH (59,60). It has not been determined

if these forms of GH can be identified in the circulation. 3) The third possibility is that there exists in serum a specific carrier protein for GH. Evidence is put forward in this thesis for the existence of such a binding factor in the serum of pregnant mice.

The GH-binding factor as "GH-LA" detected by radioreceptor assay

Evidence has been presented that the GH-LA detected by RRA in the serum of pregnant mice is an artefact due to the activity of a GH-binding factor in the serum. This same RRA also detects GH-LA in the sera of pregnant hamster, rat, guinea pig, goat, sheep, and monkey (23). In these species, with the exception of the rat, both GH-LA and PRL-LA appear simultaneously in the serum at mid-pregnancy. Also, the relative concentrations of GH-LA and PRL-LA in guinea pig, goat, sheep and monkey serum seem to be constant during the last half of pregnancy. In addition, GH-LA is found in placental extracts from each of these animals. Although there is some question of whether one or two hormone molecules exist in the circulation of these last 4 species, the ovine placental lactogen isolated and characterized by Chan et al. indicates strongly the existence of one molecule with two biological activities (25).

In retrospect, the "GH-LA" that was detected in the serum of pregnant mice differs in 3 ways from the activity revealed in the sera of the other species examined. 1) The

rates of appearance and disappearance of GH-LA in serum from pregnant mice do not coincide with those of the PRL-LA. 2) After gel filtration of serum "GH-LA is recovered as a larger molecular weight hormone clearly separate from the PRL-LA. 3) No "GH-LA" is detectable in placental extracts that contain appreciable quantities of PRL-LA. On re-examining the work of Kelly et al. in the light of these three factors, 2 species other than the mouse stand out. (Figure 1, Ref. 23) In the hamster GH-LA and PRL-LA appear simultaneously in the serum, but after gel filtration the GH-LA is found in fractions in the void volume clearly separate from the PRL-LA. Extracts of hamster placental tissue contain little or no GH-LA. Admittedly, very low concentrations of activity are difficult to assess in any tissue extract for 2 reasons. Firstly, the high protein concentration of tissue extracts appears to interfere with both radioreceptor assays suppressing the binding of the radiolabelled hormone to the receptor. Secondly, it is difficult to avoid some contamination of the extract with blood. Unfortunately, there is no suitable control organ for the placenta.

In the rat, 2 peaks of PRL-LA are detected, one at day 12 of pregnancy, the other at day 17 (21). Growth hormone-like activity appears only with the later peak. After gel filtration of serum obtained at day 17 of pregnancy, the GH-LA is again recovered as a large molecule in the void volume of the fractionation column. In extracts of rat placenta, the

GH-LA, if present, exists in concentrations below the limit of sensitivity of the assay.

When serum from a pregnant hamster was incubated with ^{125}I-hGH and fractionated on a Sephadex G 100 column the ^{125}I-hGH was recovered with a relative elution volume (V_e/V_o) = 2 as expected for the monomeric hormone Under the conditions employed there is, therefore, no obvious binding of ^{125}I-hGH to larger proteins in hamster serum, although a systematic study remains to be done.

However, a different series of observations resulted when ^{125}I-hGH was incubated with rat serum prior to gel filtration. In a number of instances ^{125}I-hGH was recovered in the void volume fractions (V_e/V_o = 1) of the Sephadex G 100 columns. Results are summarized in Table 4:

TABLE 4

Binding of ^{125}I-hormone to a factor in rat serum

^{125}I-hormone 200,000 cpm, 25 ul	Serum 0.2 ml	Hormone Added ug, 25 ul	Percent ^{125}I-hGH 'bound' (V_e/V_o=1.0)
^{125}I-bGH	-	-	6
	male	-	36
	"	bGH, 25	13
	"	oPRL, 25	38
	female	-	44
	"	bGH, 25	26
	"	oPRL, 25	51
^{125}I-hGH	-	-	10
	female	-	15
	female, day 12	-	27
	female, day 12	hGH, 25	17
	male	-	53
	female, hypox	-	26
	female, hypox	bGH, 25	30
^{125}I-oPRL	-	-	6
	male	-	38
	female	-	8

It must be emphasized that the results shown in Table 4 are the positive observations. There were an equal number of samples from both male and female rats which did not show any ability to bind ^{125}I-GH. To simplify the presentation of Table 4 these negative results were not included.

In the rat the ability to bind ^{125}I-GH is not restricted to serum obtained from pregnant animals as seems to be the case for the GH-binding factor in the serum of pregnant mice. Although all samples were obtained from Sprague Dawley rats, they were not matched for age of the donor, anaesthetic used, site of sampling, anticoagulant, or storage time. However, when ^{125}I-hGH was incubated with fresh matched serum and plasma samples from a male rat, about 40% of the ^{125}I-hGH in both cases was recovered in fractions in the void volume of the fractionation column. This contrasts with the report of Antoniades in which ^{125}I-hGH binds to large molecular weight proteins only when injected *in vivo* but not when incubated *in vitro*.

During the last three years in this laboratory, it has been well recognized that serum "interferes" in the routine RRA suppressing the binding of radioligand to receptor (29). This is attributed to the high protein concentration of the serum sample and is usually regarded as a "non-specific" phenomenon. Indeed, a similar suppression of binding can be produced by using a solution of 8% BSA as a sample. However,

when sera obtained from normal and hypophysectomized rats are used as samples "GH-LA" concentrations of 500 - 1000 ng/ml are detected. This far exceeds the effect of an equivalent solution of BSA protein.

The "interference" of rat serum in the GH-RRA, absence of GH-LA in placental extracts, and its ability to bind ^{125}I-GH when incubated prior to gel filtration and the report of Antoniades and co-workers argue for the existence of a GH-binding factor in the sera of rats. Unlike the mouse, however, this GH-binding factor is also evident in the male and non-pregnant female rat.

Source of the GH-binding factor

The rise of apparent "GH-LA" in the circulation 2-6 hours after hysterectomy would indicate that the placenta is an unlikely source of the activity. (Figure 4) In addition, when placental extracts and ^{125}I-hGH were incubated and subjected to gel filtration, the ^{125}I-hGH was recovered with a relative elution volume (V_e/V_o) = 2.0 which is the expected elution position of monomeric ^{125}I-hGH. When similarly tested, extracts of liver, kidney, adrenal, spleen, uterus, ovary, heart, submaxillary gland, pituitary and brain did not show any capacity to bind ^{125}I-hGH. At the moment it is not known why, in the extract of an organ such as the liver where some blood contamination can be expected, binding

activity which might be attributed to this contamination is not evident. There is the possibility that the overnight extraction method used may have permitted enzymatic degradation of the binding factor. This possibility should be examined by doing extractions over a shorter period of time and/or including enzyme inhibitors in the tissue homogenates. There is, of course, the possibility that the GH-binding factor may arise in some other organ or in the circulation itself.

Specificity of the binding factor

The possibility that the binding of ^{125}I-hGH to a factor in serum from pregnant mice was a peculiarity of an iodinated hormone or the result of the fractionation procedure was examined. The binding of ^{125}I-hGH to a serum factor persisted when the mixture was fractionated with 4 different eluants, including non-buffered sodium chloride, at 5 different pH's. In addition, when unlabelled hGH and oPRL were injected into pregnant mice, fractionation of the resulting plasma showed that only IR-hGH was recovered associated with large molecular weight proteins. Table 1 shows that the addition of increasing amounts of hGH or bGH to the incubation mixture of ^{125}I-hGH and serum from pregnant mice resulted in a progressive decrease in the amount of ^{125}I-hGH bound to the serum factor. The addition of oPRL did not affect the binding of ^{125}I-hGH to the serum factor. This specificity is

again evident when dextran coated charcoal, rather than gel filtration is used to separate ^{125}I-hGH bound to the serum factor from unreacted ^{125}I-hGH. (Figure 17) Rat GH is least able to displace ^{125}I-hGH from the GH-binding factor. Initially, this was a little dismaying because the phylogenetic closeness of rat and mouse led us to expect that rat GH might show the greatest activity in this system. However, when the same preparations of GH were tested for their ability to displace ^{125}I-hGH from the rabbit liver receptor, rat GH was again the least effective displacing hormone. (Figure 18) Since in many cases RRA's are known to detect hormonal activity to a degree proportional to the potency of the hormone itself, this observation may be a preliminary indication that the binding factor in mouse serum exerts a similar selectivity. It will be necessary to test GH preparations of differing potencies in order to validate this suggestion.

A very rough index of the capacity of serum from pregnant mice to bind GH is the ability of 0.4 ml of serum recovered after injection of unlabelled hormone to bind almost 1 ug of immunoreactive hGH (Figure 12A). Assuming a blood volume of 3 ml, it would be possible for 3-4 ug of GH to circulate in the bound form at any one time. The speed of the binding reaction (within 30 seconds <u>in vitro</u>), its occurence in the presence of very low concentrations of GH (e.g. 1 ng of ^{125}I-hGH) and its detection in the mouse only during pregnancy argue against the possibility that the observed binding results from a non-specific polymerization.

The binding of endogenous GH

As shown in Figure 13B after fractionation of fresh mouse serum obtained on day 18 of pregnancy, immunoreactive mouse GH is recovered in a single peak. There is no indication that IR-GH is present in fractions that correspond to the expected elution position of monomeric GH (tubes 30-35). There is also an indication of immunoreactive GH in tubes #20-25. This activity is recovered after fractionation of sera from both pregnant and non-pregnant mice. The concentrations of GH indicated in these fractions (less than 1 ng/ml) are at the limits of sensitivity of the RIA and their significance is, therefore, open to question. It should be noted that the distribution of GH-LA in column eluates after gel filtration observed by Kelly et al. is represented by 2 peaks (Figure 1). This elution profile could be reproduced by freezing and thawing serum from pregnant mice prior to its application to a Sephadex G 100 column. However, when fresh serum or plasma from pregnant mice was the sample, a single discrete peak of GH-LA was recovered after fractionation. (Figure 13B) It has not yet been determined if the freezing and thawing similarly affects the distribution of IR-GH.

The possibility that the GH-binding factor interferes in the radioimmunoassay must be considered. The bound ^{125}I-hGH recovered after gel filtration of a mixture of ^{125}I-hGH and serum obtained from a pregnant mouse was 68% immunoprecipitable (Figure 7). This indicates that the serum may interfere

with the immunoreactivity of the hGH or that it may affect the antiserum itself. These possibilities cannot be discounted at this point but it was demonstrated that serum obtained from a mouse at day 18 of pregnancy did not show any "IR-GH activity" when assayed in the hGH-RIA, although it had been demonstrated that the factor was capable of binding ^{125}I-hGH. The IR-GH concentrations in the serum of mice listed in Table 3 must be examined in light of the artefacts that a GH-binding factor may introduce in the RIA.

Possible significance of the binding factor

Although there is no direct evidence that the increased half-time disappearance rate of ^{125}I-hGH in the circulation of pregnant mice is caused by the GH-binding factor, the data did suggest this possibility. An increase in the disappearance rate of ^{125}I-insulin from the circulation in cats was thought by Gjedde to be due to the presence of a specific carrier protein (49). The presence of thyroid hormone binding globulin is known to increase the half-life of thyroxine in the circulation (61). The metabolic consequences of such a GH-binding factor are open to speculation. It could serve to bind the hormone thereby reducing its metabolic activity during pregnancy where the maternal system is already in a "diabetogenic" state (13). Given the requirement of growth hormone to develop the mammary glands of the mouse, the GH-binding factor may act to establish a reserve pool of hormone which would guarantee

successful maturation of the gland thereby insuring an adequate lactation (62). Arguing along similar lines, it may serve as a buffer against sudden changes in circulating GH levels. As such it would serve as an extrapituitary control of the circulating concentrations of metabolically active GH.

Given the changes induced by pregnancy in the capacity of the liver to bind GH, it is tempting to speculate that the GH-binding factor may act as a modulator of receptor-hormone interaction at this level (63). Posner has reported the development of growth hormone receptors in the liver of the mouse during pregnancy (64). Interestingly, the pattern of appearance of these receptors in the liver is very similar to the appearance of the GH-binding factor in the circulation. Both peak near day 18 of pregnancy and start to decrease before parturition. When liver from pregnant mice was solubilized and fractionated on a Sephadex G 200 column, the GH receptor was recovered in fractions corresponding to a molecular weight greater than 200,000. This contrasts with the estimated molecular weight of 60,000 for the GH-binding factor. No binding of ^{125}I-hGH to the soluble GH receptor could be demonstrated after a 5 minute incubation which is sufficient for the serum GH-binding factor and ^{125}I-hGH to interact. As previously indicated, no binding of ^{125}I-hGH to extracts of liver could be detected. At this stage the relationship between the appearance of GH receptors in the liver and the detection of a circulating GH-binding factor remains to be explored.

SUMMARY

A growth hormone binding factor appears in the circulation of Swiss Webster mice during pregnancy. Mouse GH complexed to the binding factor appears to have a molecular weight of approximately 80,000 daltons. The GH-binding factor interferes with a routine radioreceptor assay for growth hormones leading to false estimates of GH-LA. It has been observed that during pregnancy the half-time disappearance rate of ^{125}I-hGH from the circulation is significantly prolonged and that the serum concentrations of immunoreactive growth hormone are elevated threefold over those observed in non-pregnant mice. The relationship of these last two observations to the existence of a growth hormone binding factor remains to be established.

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