Biochem. J. (1980) **187**, 479–492
Printed in Great Britain

Characteristics of Solubilized Human-Somatotropin-Binding Protein from the Liver of Pregnant Rabbits

Toshio TSUSHIMA,*† Norio SASAKI,† Yasuo IMAI,† Fukashi, MATSUZAKI,† and Henry G. FRIESEN‡

†*Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan, and* ‡*Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada*

(*Received 22 October 1979*)

A specific binding site for somatotropin was solubilized by 1% (v/v) Triton X-100 from a crude particulate membrane fraction of pregnant rabbit liver, partially purified and characterized. The solubilized binding site retained many of the charactertistics observed in the original particulate fraction, indicating that extraction of the binding site with Triton X-100 does not cause any major changes in its properties. The binding of human ^{125}I-labelled-somatotropin to the solubilized binding site is a saturable and reversible process, depending on temperature, incubation time, pH and ionic environment. Analysis of the kinetic data revealed a finite number of binding sites with an affinity constant of $0.32 \times 10^{10}\,M^{-1}$. The binding activity for human ^{125}I-labelled-somatotropin was adsorbed to a concanavalin-A–Sepharose column and was dissociated from the column with α-methyl-D-glucoside, suggesting that the binding protein may be a glycoprotein. Using affinity chromatography on concanavalin-A–Sepharose, ion-exchange chromatography on DEAE-cellulose and gel filtration on Sepharose 6B, the binding protein was purified 1000–4000-fold from the original liver homogenate. When the partially purified preparation was chromatographed on Sepharose 6B, the binding protein eluted as a molecule with an apparent molecular weight of 200000, with a Stokes' radius of 4.9 nm. Sucrose-density-gradient centrifugation of the preparation showed that the sedimentation coefficient of the binding protein was 7.2S. Isoelectric focusing experiments revealed that a major part of the protein has an acidic pI (4.2–4.5). Exposure of the protein to trypsin decreased the binding activity for human ^{125}I-labelled-somatotropin or bovine ^{125}I-labelled-somatotropin, whereas ribonuclease, deoxyribonuclease, phospholipase C or neuraminidase had little or no effect.

It is generally accepted that the initial step in the action of polypeptide hormone resides in the binding of the hormone to its specific receptor site on the plasma membrane of the target tissue.

A specific receptor site for human growth hormone (somatotropin) was identified in lines of cultured human lymphocytes (Lesniak *et al.*, 1973, 1974) and we developed a sensitive radio-labelled receptor assay for growth hormone using a particulate membrane fraction from the liver of pregnant rabbits (Tsushima & Friesen, 1973). Specific binding sites for somatotropin were also demonstrated in human liver (Carr & Friesen, 1976) and pregnant mouse (Posner, 1976).

The liver is one possible site of action of somatotropin, because McConaghey & Sledge (1970) as well as Phillips *et al.* (1976) reported that rat liver perfused with somatotropin generated somatomedin, a factor which is believed to mediate the action of somatotropin. However, the nature of the hepatic somatotropin-binding site or of the intracellular events that follow the binding is not clear at present, and direct evidence is lacking that the binding site for somatotropin is really the receptor that is linked to the action of somatotropin, for example in the production of somatomedins.

Recently, receptors for a variety of polypeptide hormones have been solubilized and partially puri-

* Present address: Department of Internal Medicine, Tokyo Women's Medical College, Kawada-cho 10, Ichigaya, Shinju-ku, Tokyo, Japan

0306-3275/80/050479-14$01.50/1

fied (Shiu & Friesen, 1974*b*; Dufau *et al.*, 1975; Jacobs *et al.*, 1977). Herington & Veith (1977) also reported solubilization of somatotropin-specific receptor from rabbit liver, and more recently Waters & Friesen (1979) described purification of the receptor.

In the present study we describe a partial purification and discuss the characteristics of the hepatic somatotropin-binding site obtained from pregnant rabbit liver.

Materials and Methods

Hormones and chemicals

Human somatotropin (NIH-HS 1652 C, 2.0 units/mg), bovine somatotropin (NIH-B 1003A, 1.9 units/mg), ovine somatotropin (NIH-S9, 1.0 units/mg), ovine prolactin (NIH-PS-12, 35 i.u./mg) and human prolactin (NIH-hPr VLS-3, 30 i.u./mg) were gifts from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A. Lactoperoxidase was obtained from Calbiochem, La Jolla, CA, U.S.A. Phospholipase C (*Clostridium perfringens*) and ribonuclease A were purchased from Worthington, Freehold, NJ, U.S.A. Trypsin (bovine pancreas), soya bean trypsin inhibitor, deoxyribonuclease (bovine pancreas) and neuraminidase (*Clostridium perfringens*) were obtained from Sigma, St. Louis, MO, U.S.A. Poly(ethylene glycol) 6000 was from Nakarai Chemicals, Tokyo, Japan and Triton X-100 was a product of Eastman Organic Chemicals, NY, U.S.A. Carrier-free $Na^{125}I$ was purchased from New England Nuclear, Boston, MA, U.S.A. and H_2O_2 (30%) was obtained from Fisher Scientific, Fair Lawn, NJ, U.S.A. Sephadex and concanavalin-A–Sepharose were from Pharmacia (Uppsula, Sweden).

Solubilization of the somatotropin-binding site

A crude membrane fraction containing somatotropin-binding sites was prepared from the liver of pregnant rabbits (New Zealand White, 25–29 days pregnant) as described previously (Tsushima & Friesen, 1973). The resultant 100000 *g* pellet, which is referred to as particulate receptor, was solubilized according to Shiu & Friesen (1974*b*). The 100000 *g* pellet was suspended and homogenized in 25 mM-Tris/HCl buffer, pH 7.4. The protein concentration of the suspension was adjusted to 5 mg/ml and Triton X-100 was added to a final concentration of 1% (v/v). The mixture was stirred for 1 h at 25°C, and then centrifuged for 3 h at 4°C and 200000 *g* in a Hitachi model 65P ultracentrifuge with an RP-50 rotor. The supernatant, which contained solubilized somatotropin-binding protein as well as other proteins, was stored at −20°C until use.

Iodination of hormones

Purified human somatotropin (NIH-HS 1652 C) and bovine somatotropin (NIH-B-1003A) were iodinated by the lactoperoxidase method of Thorell & Johansson (1971). Hormone (5 μg) in 25 μl of 50 mM-phosphate buffer, pH 7.4, 1 mCi of $Na^{125}I$ in 20 μl of phosphate buffer (0.4 M, pH 7.4) and 1 μg of lactoperoxidase in 4 μl of 50 mM-phosphate buffer, pH 7.4 were successively added to a reaction tube. Radioiodination was initiated by addition of 40 ng of H_2O_2. After incubation for 2 min, 2 ml of ice-cold phosphate buffer (50 mM, pH 7.4) was added, and the whole mixture was immediately filtered on a Sephadex G-100 column (1.5 cm × 40 cm) in 25 mM-Tris/HCl buffer, in order to separate labelled hormone from unreacted iodine and damaged aggregate. The few fractions corresponding to the peak of labelled hormone were selected for binding studies. The specific activity was 50–130 Ci/g.

Binding studies

The diluent for labelled hormones or the Triton extract was 50 mM-Tris/HCl buffer, pH 7.4 containing 0.1% (w/v) bovine serum albumin (Armour, Phoenix AZ, U.S.A., fraction V) and 10 mM-$MgCl_2$. In most studies 0.1 ml of labelled hormone (30000–50000 c.p.m.) was incubated with solubilized somatotropin-binding site in a final volume of 0.5 ml in a glass tube (1.0 cm × 8.0 cm) at room temperature (21–25°C) for 3 h. Poly(ethylene glycol) 6000 [final concentration 12.5% (w/v)] was used to precipitate the bound hormone as described (Cuatrecasas, 1972; Shiu & Friesen, 1974*b*). In all studies, specific binding was calculated by subtracting the non-specific hormone bound from the total labelled hormone bound. Non-specific hormone bound is that which is bound in the presence of an excess of unlabelled hormone (1 μg/tube). To assess the validity of the separation of bound and free hormone by poly(ethylene glycol), gel filtration was used in some experiments. A 0.1 ml aliquot containing solubilized somatotropin-binding sites was incubated with human ^{125}I-labelled-somatotropin in the presence or absence of excess unlabelled human somatotropin (1 μg/tube). At the end of the incubation, the total incubation mixture (1 ml) was fractionated at 4°C on a Sephadex G-100 column (1.5 cm × 40 cm) previously equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and 0.1% bovine serum albumin. Fractions (1 ml) were collected and the radioactivity of aliquots was measured in an automatic gamma counter.

Affinity chromatography

Triton extract (30 ml) from the 100000 *g* pellet of rabbit liver was diluted with an equal volume of

25mM-Tris/HCl buffer, pH 7.4, containing 10mM-$MgCl_2$, and the fine precipitate formed was removed by centrifugation for 30min at 4°C and 100000*g*. The supernatant was chromatographed at 4°C on a column packed with concanavalin-A–Sepharose that had been extensively washed with 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. The flow rate was adjusted to 20ml/h and fractions (5ml) were collected. The column was washed with the same buffer until no protein was eluted. Elution of the somatotropin-binding activity was achieved with 2 bed volumes of 0.1M-α-methyl-D-glucoside in 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100. After extensively washing the column with the buffer, a second elution was carried out using 0.1M-α-methyl-D-mannoside in the same buffer. The binding activity in the eluate was assessed using human ^{125}I-labelled-somatotropin, bovine ^{125}I-labelled-somatotropin and porcine ^{125}I-labelled-insulin. The active fractions were pooled and concentrated in an Amicon cell with a PM-30 filter.

Protein concentration in the eluates were determined by the method of Lowry *et al.* (1951). Because high concentrations of Triton X-100 (over 0.5%) interfered with the Lowry test, a more sensitive method using fluorescamine (Udenfriend *et al.*, 1972) was used in some experiments.

Ion-exchange chromatography

The protein concentration of the active fractions from the affinity column (glucoside-eluted fraction) was adjusted to approximately 1mg/ml, and aliquots were chromatographed on a column of DEAE-cellulose (DE-52; Whatman, London, U.K.) that had been equilibrated with 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100. The column was further washed with 10 bed volumes of the buffer, and elution was achieved with a linear gradient of NaCl from 0 to 0.5M in Tris/HCl buffer (25mM, pH 7.4) containing 0.1% Triton X-100. The active fractions were pooled and concentrated in an Amicon cell with a PM-30 membrane, which excludes molecules having a mol.wt. less than 30000. Further purification was carried out using gel filtration on a column of Sepharose 6B. The column was equilibrated with 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100, and the gel filtration was performed at 4°C.

Molecular size determination

A column of Sepharose 6B (1cm × 86cm) equilibrated with 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100 was used to determine the molecular size of the somatotropin-binding protein. The partially purified preparation was applied to the column and chromatographed at a flow rate of 5ml/h at 4°C. Fractions (1ml) were collected and aliquots were taken for the determination of the binding activity for human ^{125}I-labelled-somatotropin and bovine ^{125}I-labelled-somatotropin. Alternatively, an aliquot of the partially purified binding protein was incubated with human ^{125}I-labelled-somatotropin for 3h at 25°C in a final volume of 0.5ml, and the whole mixture was fractionated on the column. The radioactivity of the fractions was measured. The column was calibrated with several marker proteins including thyroglobulin, apoferritin, catalase, bovine serum albumin and human ^{125}I-labelled-somatotropin. The void volume (V_0) and the total liquid volume (V_t) were determined by the elution of Blue Dextran 2000 (Pharmacia) and Na^{125}I respectively. The chromatographic behaviour was expressed in terms of $K_{av.}$ according to Laurent & Killander (1964) as:

$$K_{av.} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume of the protein. The Stokes' radius was calculated according to Siegel & Monty (1966).

Density-gradient centrifugation

Density-gradient centrifugation on 5–20% (w/v) sucrose linear gradients was performed by the procedures described by Martin & Ames (1961) in Hitachi model 65P ultracentrifuge, employing an RPS 65TA rotor. Gradients were prepared with reservoirs containing 5% or 20% sucrose in 25mM-Tris/HCl buffer, pH 7.4, and 0.1% Triton X-100. A solution of the sample (up to 0.2ml) containing solubilized somatotropin-binding protein was applied. The sample had been dialysed for 24h at 4°C against the buffer used to prepare the sucrose solution. After centrifugation at 110000*g* for 16h at 2°C, fractions (0.17ml) were collected through a capillary. Several marker proteins including thyroglobulin, apoferritin, bovine serum albumin and human ^{125}I-labelled-somatotropin were dissolved in the same buffer (5mg/ml) and processed in the same manner. The position of protein was determined by measurement of absorbance at 280nm and peaks of labelled material were identified by radioactivity.

Isoelectric focusing

Isoelectric focusing between pH 3.5 and pH 10.0 was carried out using preparative flat-bed gel electrofocusing in a granulated gel with an LKB 2117 Multiphor (LKB-Produkter AB, Bromma, Sweden), according to LKB application note 198. Triton X-100-solubilized extract (10ml; 2mg of protein/ml) was diluted to 95ml with distilled water, and 5ml of Ampholine carrier ampholytes (LKB 1809-101, pH 3.5–10.0) was added. The concentration of Triton X-100 was adjusted to 0.1% (v/v). Sephadex G-75 superfine grade (5g; Ultrodex, LKB) was slowly added to the mixture. The suspension was placed on the tray, and was evaporated using a

gentle stream of air until fine cracks were visible on the surface. Focusing was carried out at 4°C for 12h at 8W constant power. At the end of electrophoresis the gel bed was sectioned with the aid of the fractioning grid, and each gel was transferred to a small column using a spatula. The column was then eluted with 2ml of distilled water. The eluate was assayed for binding activity to human and bovine ^{125}I-labelled-somatotropin.

Results

Solubilization

Approximately 70% of the total protein of the starting 100000*g* pellet and 95% of the binding capacity for human and bovine ^{125}I-labelled-somatotropin, as judged by the analysis of Scatchard, were solubilized with 1% Triton X-100 and remained in the 200000*g* supernatant. Efficiency of solubilization did not improve with higher concentrations (up to 5%) of Triton X-100. The binding activity for somatotropin was stable for at least 6 months when stored at −20°C. Repeated thawing and freezing did not result in a significant loss of the binding activity.

Binding studies

The Triton-solubilized extract was incubated with human ^{125}I-labelled-somatotropin in the presence or absence of unlabelled somatotropin, and the reaction mixture was subjected to gel filtration (Fig. 1). In the absence of unlabelled human somatotropin, 60% of the total radioactivity was detected in the void-volume fractions. On the other hand, in the presence of excess human somatotropin most of the labelled hormone eluted with proteins of mol.wt. 20000, indicating that the labelled hormone had been almost completely displaced from the large complex. Binding activity in Triton-solubilized extract was also demonstrated using poly(ethylene glycol) precipitation. A final concentration of 12.5% (w/v) poly(ethylene glycol) was sufficient to precipitate the hormone–receptor complex. Non-specific precipitation of unbound human ^{125}I-labelled-somatotropin was less than 6% of the total radioactivity and Triton X-100 (up to 0.2% final concentration) had no effect on the specific or non-specific binding of labelled hormone. The extent of specific binding obtained by the poly(ethylene glycol) assay was in good agreement with that determined by gel filtration on Sephadex G-100, and hence the former method was employed in this study because of its simplicity.

Bovine ^{125}I-labelled-somatotropin was re-purified immediately before use, because it aggregated readily under storage, resulting in a significant precipitation by 12.5% poly(ethylene glycol) in the absence of binding protein.

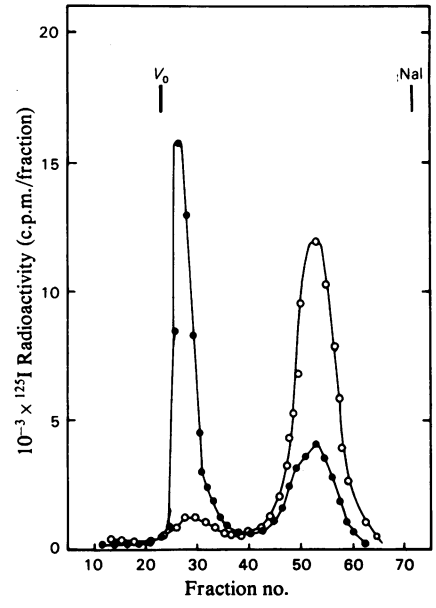

Fig. 1. *Gel filtration pattern demonstrating specific binding of human ^{125}I-labelled-somatotropin to the solubilized 100000g pellet of rabbit liver*

Approx. 100000c.p.m. of human ^{125}I-labelled-somatotropin was incubated with Triton extract (1mg protein) in 1ml of 25mM-Tris/HCl buffer, pH7.4, containing 0.1% bovine serum albumin and 10mM-$MgCl_2$ at 25°C for 3h in the presence (○) or absence (●) of 1μg/ml of unlabelled human somatotropin. The incubation mixture was applied to a column (1.5cm × 40cm) of Sephadex G-100, and the radioactivity of 1ml fractions was measured.

Specific binding of human or bovine ^{125}I-labelled-somatotropin to the solubilized binding site increased in a linear manner with protein concentrations up to 100μg/tube. The binding of human ^{125}I-labelled-somatotropin was dependent on time and temperature (Fig. 2). At 25°C, a steady state was attained within 3h, whereas the rate of association was much slower at 4°C. Though not shown in the Figure, non-specific binding also increased with the period of incubation. However, the magnitude of non-specific binding of human ^{125}I-labelled-somatotropin and bovine ^{125}I-labelled-somatotropin did not exceed 8% and 11% of the total labelled hormone respectively after incubation for 3–6h at 25°C.

The dissociation of bound human ^{125}I-labelled somatotropin was also time- and temperature-dependent. The kinetics of dissociation indicated a first-order reaction with a half-time of 6h at 25°C, whereas dissociation at 4°C is considerably slower and only 25–30% of labelled hormone was

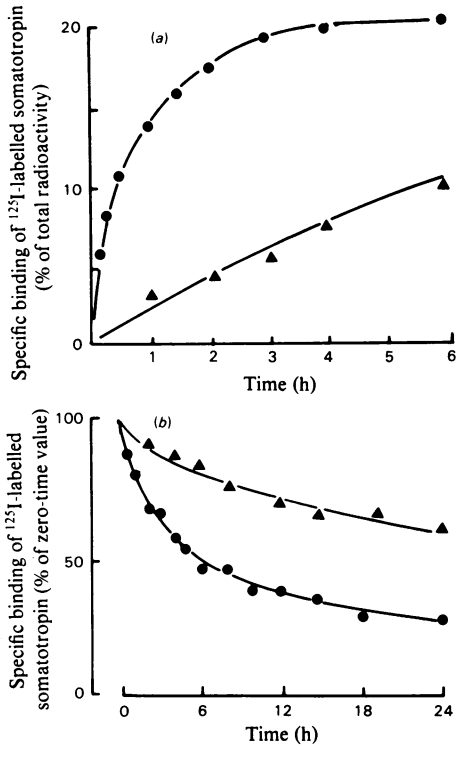

Fig. 2. *Effect of incubation period and temperature on the binding and dissociation of human ^{125}I-labelled-somatotropin*

(*a*) Triton extract (50μg protein) was incubated with 100000c.p.m. (0.5ng) of human ^{125}I-labelled-somatotropin at 4°C or 25°C and the specific binding of human ^{125}I-labelled-somatotropin (% of total radioactivity) was calculated. ▲, 4°C; ●, 25°C. (*b*) Shows the time course of dissociation. Triton extract containing 50μg protein was incubated with human ^{125}I-labelled-somatotropin in the presence or absence of 1μg of unlabelled human somatotropin. After 3h incubation (indicated as zero time), 0.1ml of human somatotropin solution (10μg/ml) was added to those tubes that did not contain it, and 0.1ml of 25mM-Tris/HCl buffer, pH7.4, containing 0.1% bovine serum albumin and 10mM-$MgCl_2$, was added to the tubes that already contained unlabelled hormone. The tubes were incubated at the temperature indicated. After various periods the reaction was terminated and specific binding of human ^{125}I-labelled-somatotropin was calculated. The specific binding of human ^{125}I-somatotropin was plotted as percentage of binding at zero time. ▲, 4°C; ●, 25°C.

dissociated after the addition of excess human somatotropin.

The effect of pH and the ionic environment of the incubation is shown in Tables 1 and 2 respectively.

Table 1. *Effect of pH on specific binding of human ^{125}I-labelled-somatotropin to the solubilized binding site*

Triton extract was dialysed against the appropriate experimental buffer for 24h at 4°C; if a fine precipitate was formed, it was removed by centrifugation for 30min at 4°C and 100000*g*. The protein concentration of the supernatant was adjusted to 1mg/ml. An aliquot (0.1ml) was incubated with 100000 c.p.m. of human ^{125}I-labelled-somatotropin at 25°C for 3h in a volume of 0.5ml. Sodium acetate buffer (0.1M) was used for pH4.0–6.0, 0.1M-Tris/HCl buffer for pH7.0–8.0, and 0.1M-sodium bicarbonate buffer for pH9.0–10.0. All buffers contained 10mM-$MgCl_2$, 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100. The value of specific binding is the mean ± S.D. of three determinations. No effect of pH was observed on the non-specific binding of labelled human somatotropin.

pH of the incubation medium	Specific binding of human ^{125}I-labelled-somatotropin (% of total labelled hormone)
4.0	10.2 ± 1.2
5.2	22.4 ± 2.1
5.6	28.2 ± 2.3
6.0	28.7 ± 1.8
7.0	29.1 ± 2.2
7.0	28.0 ± 1.1
8.0	22.8 ± 2.9
9.0	17.3 ± 0.7
10.0	8.2 ± 0.6

Changes in pH between 5 and 8 have little effect on the specific binding of human ^{125}I-labelled-somatotropin, but at pH values outside this range the binding is considerably reduced.

In many receptor-hormone interactions, bivalent ions such as Ca^{2+} or Mg^{2+} have significant effects on the binding of hormones to the receptor. In the present study both Ca^{2+} and Mg^{2+} increased the binding of ^{125}I-labelled-somatotropin more than 2-fold compared to control values.

Univalent ions had less effect, and the addition of Na^+ or K^+ to an incubation containing Ca^{2+} or Mg^{2+} that gave maximum binding did not produce a further increase of specific binding. Higher concentrations of these cations (1.0–5.0M) strongly inhibited binding. The effect is similar to that observed in the binding of prolactin to receptor in the mammary gland (Shiu & Friesen, 1974*a*) but differs from the observation with human lymphocytes in which changes in concentration of Mg^{2+} or Ca^{2+} had little effect on the binding of human somatotropin (Lesniak *et al.*, 1974).

Table 2. *Effect of ions on specific binding of human ^{125}I-labelled-somatotropin to the solubilized binding site*
Triton extract (100 μl; 100 μg of protein) was incubated with 50000 c.p.m. of human ^{125}I-labelled-somatotropin in various environments. The buffer used was 25 mM-Tris/HCl, pH 7.4, containing 0.1% bovine serum albumin and 0.1% Triton X-100. The incubation was carried out at 25°C for 3 h. The values were expressed as percentage of specific binding in the control (mean of two experiments). For the control, the specific binding of human ^{125}I-labelled-somatotropin in the absence of Mg^{2+}, Ca^{2+}, Na^+ or K^+ was employed (10558 c.p.m.). These ions had no effect on the magnitude of non-specific binding.

Ion(s)	Concentration (mM)	Specific binding of human ^{125}I-labelled-somatotropin (% of control)
Control		100
Ca^{2+}	2	129
	5	190
	10	223
	20	240
	50	234
Mg^{2+}	2	132
	5	211
	10	235
Na^+	10	131
	100	142
K^+	10	126
	100	132
$Ca^{2+} + Mg^{2+}$	5 + 5	239
$Ca^{2+} + Na^+$	10 + 100	230

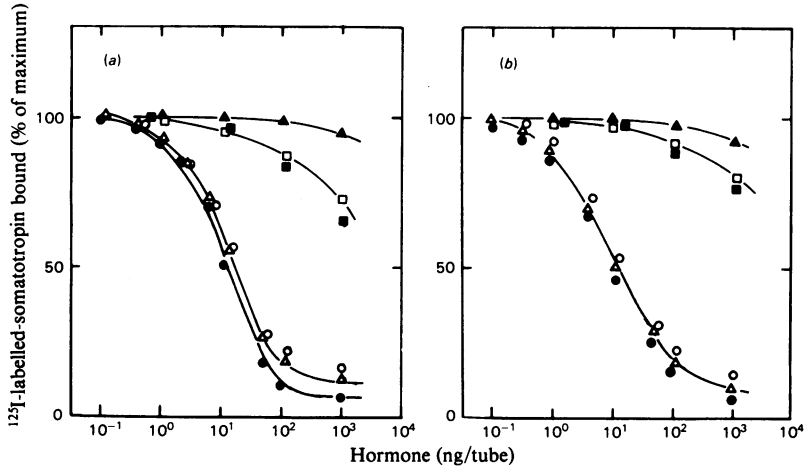

Fig. 3. *Hormonal specificity for the binding of (a) human and (b) bovine ^{125}I-labelled-somatotropin*
The specific binding of ^{125}I-labelled-hormone was plotted as a percentage of that obtained in the absence of unlabelled hormone. Protein from the Triton extract (100 μg) was incubated with 50000 c.p.m. of human or bovine ^{125}I-labelled-somatotropin in the presence of increasing amounts of human somatotropin (●), bovine somatotropin (△), ovine somatotropin (○), human placental lactogen (□), ovine prolactin (■), or prolactins from rat, pig and human (▲). Specific binding for the human and bovine ^{125}I-labelled-somatotropin was 28% and 19% respectively.

Specificity of the binding site

The hormonal specificity of the solubilized binding site is shown in Fig. 3. Using 100 μg of protein from the Triton extract, 25.2 ± 3.2% (mean ± S.D. of five experiments) of the human ^{125}I-labelled-somatotropin bound specifically to the solubilized binding site (non-specific binding, 7.4 ± 1.2%). As shown in Fig. 3, increasing amounts of unlabelled

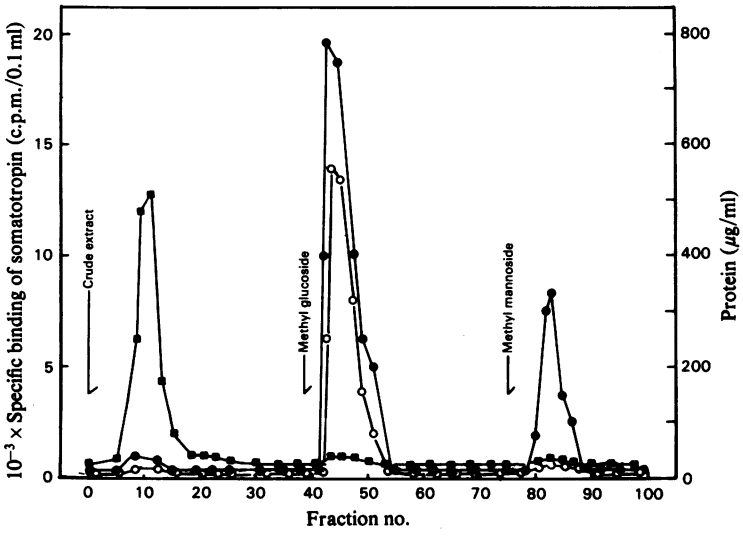

Fig. 4. *Affinity chromatography on concanavalin-A–Sepharose*
Triton extract (30ml) was diluted with an equal volume of 25mM-Tris/HCl buffer, pH 7.4, containing 10mM-$MgCl_2$. The fine precipitate was removed as described in the text and the supernatant (50ml; 0.5mg of protein/ml) was chromatographed on a column (2.5cm × 6.0cm) of concanavalin-A–Sepharose. The specific binding of human (●) and bovine (○) ^{125}I-labelled-somatotropin was determined using 100μl of the 5-ml fractions and 50000c.p.m. of labelled hormone. ■, Protein concentration.

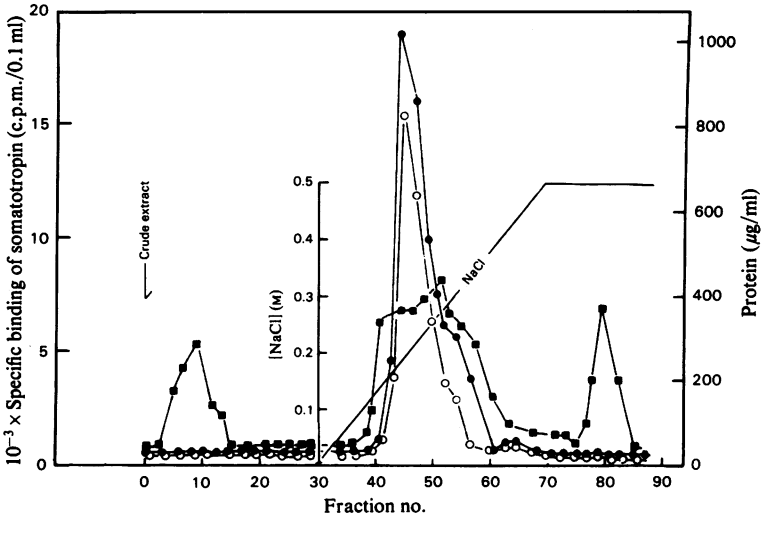

Fig. 5. *Ion-exchange chromatography on DEAE-cellulose*
Active fractions from the concanavalin-A–Sepharose column were pooled, concentrated and dialysed at 4°C against 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100 for 24h. The fine precipitate formed was removed by centrifugation at 4°C and 100000*g* for 30min. The supernatant (25ml; 1mg of protein/ml) was subjected to ion-exchange chromatography on a DEAE-cellulose column (2.0cm × 8.0cm) as described in the text. The flow rate was adjusted to 10ml/h. The binding activity of human (●) and bovine (○) ^{125}I-labelled-somatotropin was determined using 0.1ml of the 5-ml fractions and 50000c.p.m. of labelled hormone. ■, Protein concentration; ——, NaCl gradient.

somatotropin displaced the labelled hormone from the binding site with a minimum detectable level of 0.5 ng. As observed in the previous experiment using a particulate membrane fraction (Tsushima & Friesen, 1973), unlabelled monkey, bovine or ovine somatotropin displaced human ^{125}I-labelled-somatotropin in a similar manner. Ovine prolactin and human placental lactogen displaced human ^{125}I-labelled-somatotropin with 1% of the potency of human somatotropin. Prolactins from human, rat, monkey and pig were without effect except at very high concentrations. As described later, concanavalin A binds to the somatotropin-binding protein, but the plant lectin did not inhibit the binding of human ^{125}I-labelled-somatotropin in concentrations up to 100 μg/ml. Scatchard analysis of human ^{125}I-labelled-somatotropin binding revealed a finite number of binding sites (binding capacity 842 ± 46 fmol/mg protein; mean $\pm$ S.D. of five experiments) with an affinity of $(0.32 \pm 0.03) \times 10^{10}$ l/mol.

The specificity of bovine ^{125}I-labelled-somatotropin binding to the solubilized binding site was also studied (Fig. 3*b*). The specific binding of bovine ^{125}I-labelled-somatotropin to 100 μg of protein was $17.2 \pm 5.1\%$ (mean $\pm$ S.D. of six experiments) and non-specific binding was $11.4 \pm 1.8\%$. The binding capacity and affinity of the Triton-extracted receptor for bovine somatotropin were 653 ± 23 fmol/mg and $(0.23 \pm 0.03) \times 10^{10}$ l/mol (mean $\pm$ S.D. of five experiments) respectively. The values are higher than those reported by Herington & Veith (1977). The discrepancy may be ascribed to the difference in animals used. They used non-pregnant rabbits, while membrane fractions were prepared from late-pregnant rabbits in the present study.

Partial purification

Substantial purification of somatotropin-binding protein was achieved by affinity chromatography on concanavalin-A–Sepharose. As shown in Fig. 4, over 95% of the protein that lacked somatotropin-binding activity passed through the column. Of the total binding activity adsorbed to the column, approximately 60% was recovered in the fractions eluted with 0.1 M-α-methyl-D-glucoside. Another peak with somatotropin-binding activity was obtained by further washing the column with 0.1 M-α-methyl-D-mannoside. The activity recovered in the fractions eluted by mannoside was 5–10% of that adsorbed to the column. It is interesting to note that the specific binding activity for bovine ^{125}I-labelled-somatotropin is closely parallel to that for human ^{125}I-labelled-somatotropin in the fractions eluted by glucoside, whereas little or no binding of bovine ^{125}I-labelled-somatotropin was observed in the fractions eluted by mannoside. Although not shown in the Figure, the binding activity for pig ^{125}I-labelled-insulin was exclusively eluted by mannoside, which enabled us to separate the binding protein for somatotropin from that for insulin. With affinity chromatography on concanavalin-A–Sepharose, 20–50-fold purification was achieved with a satisfactory yield.

The active fractions eluted by α-methyl-D-glucoside were pooled, concentrated in an Amicon cell with a PM-30 membrane and subjected to ion-exchange chromatography on DEAE-cellulose (Fig. 5). The human somatotropin-binding protein was found to adsorb to the column at pH 7.4 and was eluted with a gradient of NaCl in 25 mM-Tris/HCl buffer containing 0.1% Triton X-100. The binding activity for bovine ^{125}I-labelled-somatotropin was exactly parallel to that for human ^{125}I-labelled-somatotropin. Recovery of human somatotropin-binding activity was approximately 60%. Although only a 2–3-fold purification was achieved by the procedure, this step is, like the concanavalin-A–Sepharose step, very useful for elimination of the concentrated Triton X-100 which passed through the column.

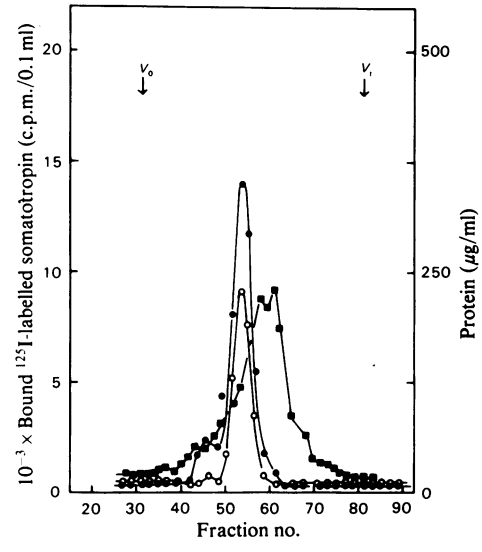

Fig. 6. *Purification by gel filtration*
The active fractions from the DEAE-cellulose column were pooled and concentrated. Part of the sample (10 ml; 1–2 mg of protein/ml) was filtered on a column of Sepharose 6B (2.5 cm × 85 cm), that had been equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100. Chromatography was carried out at 4°C at a flow rate of 25 ml/h. Fractions (5 ml) were collected and the binding activity for human (●) and bovine (○) ^{125}I-labelled-somatotropin was determined using 0.1 ml of the eluate and 40000 c.p.m. of labelled hormone. ■, Protein concentration.

The active fractions were again pooled, concentrated and chromatographed on Sepharose 6B which had been equilibrated with 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100 (Fig. 6). Approximately 90% of the binding activity for human ^{125}I-labelled-somatotropin was recovered in the fraction corresponding to a mol.wt. of 200000. The active fractions were pooled, concentrated and re-chromatographed on the same column and the few fractions corresponding to the peak (which is referred to as the partially purified preparation) were stored at 4°C until use. The binding protein was purified 10-fold by gel filtration.

By a combination of affinity chromatography, ion-exchange chromatography and gel filtration, the somatotropin-binding protein was purified 200–800-fold from the crude Triton extract (1000–4000-fold purification from the crude liver homogenate).

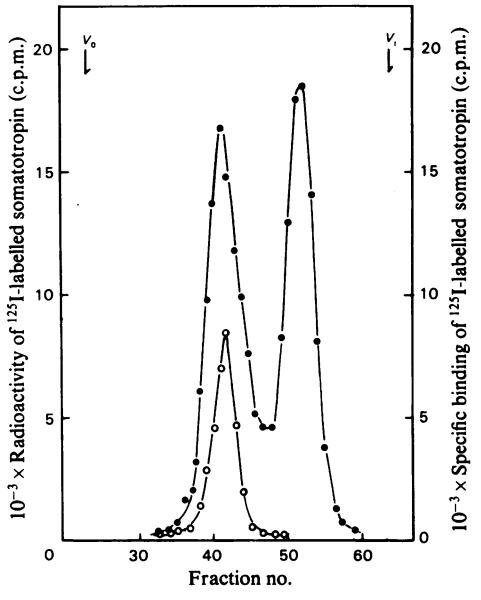

Fig. 7. *Chromatographic behaviour of somatotropin-binding protein on a Sepharose 6B column*

The partially purified human somatotropin-binding protein (10μg) in 25mM-Tris/HCl buffer, pH 7.4 containing 0.1% Triton X-100 and 0.1% bovine serum albumin was chromatographed on a column of Sepharose 6B (1cm × 86cm) equilibrated with the same buffer without albumin. Aliquots (0.1ml) of the 1-ml fractions were incubated with 60000c.p.m. of human ^{125}I-labelled-somatotropin to determine the binding activity (○). The elution profile of the hormone-binding protein complex on the same column is also shown (●). Part of the partially purified preparation (5μg of protein) was incubated with 100000c.p.m. of human ^{125}I-labelled-somatotropin for 3h at 25°C. The whole mixture (0.5ml) was then gel filtered on the column. Fractions (1ml) were collected and the radioactivity of an aliquot was measured.

Gel filtration and sucrose-density-gradient centrifugation

When the partially purified somatotropin-binding protein was incubated with human ^{125}I-labelled-somatotropin and then subjected to gel filtration on Sepharose 6B, two radioactive peaks were observed (Fig. 7). The excluded peak with $K_{av.} = 0.41$ represents the bound ^{125}I-labelled-somatotropin and the second peak is unbound hormone. The first peak was almost completely eliminated when the incubation was carried out in the presence of excess unlabelled human somatotropin, indicating that the bound hormone had been specifically displaced by the unlabelled hormone. When the partially purified preparation was filtered on the same column without prior incubation, the binding activity for human ^{125}I-labelled-somatotropin eluted with a $K_{av.}$ of 0.43, which is coincident with that for bovine ^{125}I-labelled-somatotropin binding activity (data not shown).

Fig. 8 shows a plot of $(-\log K_{av.})^{\frac{1}{2}}$ versus the Stokes' radius of the human somatotropin-binding protein and marker proteins that were run on the same Sepharose 6B column in the presence of 0.1% Triton X-100. The Stokes' radius of the somatotropin-binding protein was estimated to be 4.9nm.

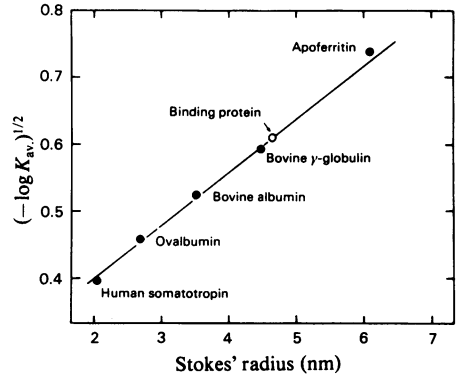

Fig. 8. *Determination of Stokes' radius of the somatotropin-binding protein*

The $K_{av.}$ values of somatotropin-binding protein and the marker proteins were determined from the chromatographic behaviour of the proteins on the Sepharose 6B column used in Fig. 7. Each marker protein was dissolved in 1ml of 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100, and was filtered separately on the column. Fractions (1ml) were collected and protein concentrations were determined by the method of Lowry *et al.* (1951).

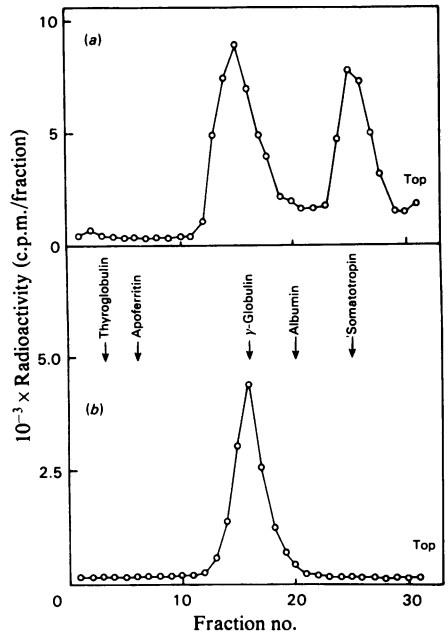

Fig. 9. *Sucrose-density-gradient centrifugation*
(*a*) Partially purified somatotropin-binding protein (10 μg) was incubated with 100000 c.p.m. of human ^{125}I-labelled-somatotropin for 3 h at 25°C in a volume of 0.5 ml. Part of the mixture (200 μl) was subjected to density-gradient centrifugation as described in the text. After the centrifugation, fractions (20 drops) were collected and the radioactivity was measured. (*b*) Sedimentation behaviour of free somatotropin-binding protein. Partially purified somatotropin-binding protein (10 μg) in 0.2 ml of 25 mM-Tris/HCl buffer was centrifuged in the same manner and the binding activity for human ^{125}I-labelled-somatotropin in the 20 μl-fractions was determined. Several marker proteins were processed in the same manner and the positions of protein peaks are indicated by arrows.

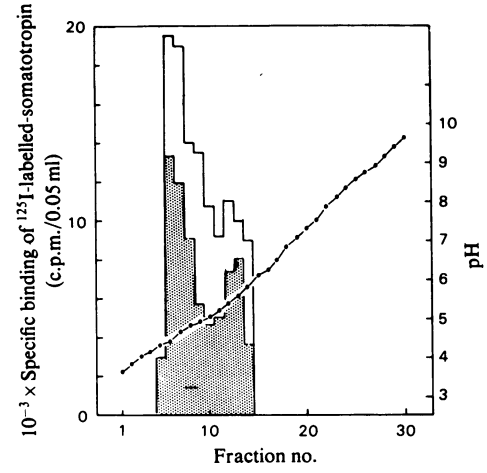

Fig. 10. *Isoelectric focusing*
Isoelectric focusing of the Triton extract (10 mg protein) was carried out as described under the Materials and Methods section. The eluate (50 μl) from each gel section was incubated with 50000 c.p.m. of human or bovine ^{125}I-labelled-somatotropin in 0.5 ml of 100 mM-Tris/HCl buffer, pH 7.4, containing 0.1% bovine serum albumin and 10 mM-$MgCl_2$, and the binding activity was determined. White columns, specific binding of human ^{125}I-labelled-somatotropin; shaded columns, specific binding of bovine ^{125}I-labelled-somatotropin; ●——●, pH.

Sucrose-density-gradient centrifugation of the somatotropin-binding protein gave the sedimentation pattern shown in Fig. 9, revealing a single peak of binding activity for human ^{125}I-labelled-somatotropin with a sedimentation coefficient of 7.2 S (mean of three experiments). The binding activity for bovine ^{125}I-labelled-somatotropin migrated at the same position (data not shown). The sedimentation coefficient of the hormone-binding protein complex was calculated to be 7.5 S by comparison with reference proteins. Such estimation of the sedimentation coefficient was considered valid because the sedimentation patterns of reference proteins on 5–20% sucrose gradients containing 0.1% Triton X-100 revealed that a linear relationship exists between migration distance of the protein and sedimentation coefficient.

Isoelectric focusing

A crude Triton extract was subjected to isoelectric focusing between pH 3.5 and 10.0 in a flat-bed gel (Fig. 10). The binding activity for human ^{125}I-labelled-somatotropin was focused between pH 4.1 and pH 5.4, with peak activity at pH 4.2–4.5. In most experiments, another small peak of activity was detected at pH 5.2–5.4. The binding activity for bovine ^{125}I-labelled-somatotropin was distributed in a similar manner with peak activity at pH 4.2.

Effect of enzymes

Treatment of the partially purified preparation with trypsin resulted in significant loss of the binding activity for human and bovine ^{125}I-labelled-somatotropin (Table 3). Phospholipase C and neuraminidase decreased the binding activity only slightly, suggesting that phospholipid and sialic acid have only a minor role in the binding process. The effect of these enzymes on the binding activity for

Table 3. *Effect of enzymes on the specific binding of human and bovine ^{125}I-labelled-somatotropin to the solubilized binding site*

Partially purified somatotropin-binding protein was incubated with different concentrations of enzymes at 37°C for 20min in 25mM-Tris/HCl buffer, pH 7.4. For trypsin digestion of the preparation, a 5-fold excess (over trypsin) of soya bean trypsin inhibitor was added immediately after the treatment. The treated medium (0.1ml) was incubated with 50000c.p.m. of human or bovine ^{125}I-labelled-somatotropin at 25°C for 3h to determine the binding activity. For controls, fractions that were not exposed to enzymes but were incubated in the same buffer and for the same periods were used. The experiment was carried out in duplicate and the value is the mean of duplicate determinations.

Enzyme	Concentration (μg/ml)	Specific binding (% of control)	
		Human somatotropin	Bovine somatotropin
Trypsin	80	8	5
	10	22	21
Ribonuclease	80	101	98
	10	100	97
Deoxyribonuclease	80	94	95
	10	103	100
Phospholipase C	80	82	79
	10	91	95
Neuraminidase	80	92	89
	10	98	94

Table 4. *Effect of various treatments on the binding of human and bovine ^{125}I-labelled-somatotropin to the solubilized binding site*

Partially purified preparations were exposed to the reagents described for various periods at 25°C. After exposure, the incubation mixtures that were treated with acid or alkali were neutralized. After treatment with the other regents, the contents of the tubes were dialysed against 25mM-Tris/HCl buffer, pH 7.4, containing 0.1% Triton X-100 for 16 at 4°C. The neutralized or dialysed medium (0.1ml) was tested for binding activity with ^{125}I-labelled hormones. For controls, preparation that were not exposed to the reagents, but incubated in the same manner and dialyzed for the same periods were used. The values are the means of triplicate determinations.

Treatment	Concentration	Time (min)	Specific binding (% of control)	
			Human somatotropin	Bovine somatotropin
HCl	0.1M	20	52	45
NaOH	0.1M	20	19	11
Urea	1.0M	30	95	97
Urea	5.0M	30	79	80
Guanidinium chloride	5.0M	30	56	50
Dithiothreitol	10mM	30	77	72
N-Ethylmaleimide	10mM	30	98	100
Dithiothreitol + *N*-ethylmaleimide	10mM + 10mM	30	25	17
Heating at 100°C		5	0	0
Heating at 50°C		10	18	15

human somatotropin was almost parallel to that on the binding activity for the bovine hormone.

Effect of various reagents

The binding activity for both human and bovine ^{125}I-labelled-somatotropin was sensitive to acid and alkaline conditions (Table 4). Treatment with a variety of protein denaturants resulted in a various degrees of decrease of the binding activity for the labelled hormone. The activity was significantly decreased by reduction and by alkylation with dithiothreitol and *N*-ethylmaleimide. Heating at 100°C for 5min completely destroyed the binding activity.

Discussion

Recently receptors for several peptide hormones have been solubilized (Shiu & Friesen, 1974*b*; Dufau *et al.*, 1975; Jacobs *et al.*, 1977). Herington & Veith

(1977) first reported solubilization of somatotropin receptor from rabbit liver using the non-ionic detergent Triton X-100, and Waters & Friesen (1979) described purification of the receptor. The properties of the hepatic somatotropin-binding site have not been completely clarified. In the present study the binding sites for somatotropin were solubilized with 1% (v/v) Triton X-100 and partially purified and characterized.

Similar to the receptors for other hormones, the solubilized human somatotropin-binding activity emerged as a large molecule on gel filtration on a Sephadex G-100 column. The binding characteristics of the detergent-solubilized site are quite similar to those of the original particulate membrane fraction (100000*g* pellet). The binding of human ^{125}I-labelled-somatotropin was rapid, reversible, saturable, dependent on time and temperature, and a function of concentration of protein. The affinity for human somatotropin is similar to that observed with the particulate fraction (Kelly *et al.*, 1974). The effects of ions, enzymes or various chemicals on the binding activity for human somatotropin are comparable to those observed with the particulate fraction (our unpublished work). These observations suggest that extraction of the site with Triton X-100 does not cause any major changes in properties of the molecule.

Bovine or ovine somatotropin displaced human ^{125}I-labelled-somatotropin in a similar manner as did the unlabelled human hormone, whereas lactogenic hormones such as rat, human or porcine prolactin were without effect except at very high concentrations. Ovine prolactin and human placental lactogen cross-reacted to an extent of 1%.

The specificity of human ^{125}I-labelled-somatotropin binding is different from that reported by Herington & Veith (1977) in that bovine or ovine somatotropin are less able to displace the labelled human somatotropin. This may be due to the difference in the source of liver membrane fractions (pregnant versus non-pregnant rabbit). Our results suggest that the human-somatotropin-binding protein from the liver of pregnant rabbit is largely specific for somatotropin. However, there is no doubt that rabbit liver has at least two types of receptors: a somatotropin receptor that binds bovine somatotropin with high affinity and ovine prolactin with a lower affinity, and a prolactin-specific receptor that binds ovine prolactin with high affinity, as shown by Posner (1974) and Waters & Friesen (1979). The latter also found that the prolactin-specific receptor, when solubilized with Triton, exhibits a 4-fold increase in its affinity. As compared with their observations, we found that ovine prolactin was much less potent in displacement of bovine ^{125}I-labelled-somatotropin from the solubilized binding protein. Also, solubilization with Triton X-100 did not result in an increase of the affinity of the binding site for human somatotropin. It is difficult, however, to provide an explanation for the discrepancy.

In affinity chromatography on concanavalin-A–Sepharose, the fractions eluted by α-methyl-D-glucoside contained binding activity for both human and bovine ^{125}I-labelled-somatotropin, but binding of bovine ^{125}I-labelled-somatotropin to the mannoside-eluted fraction was absent or quite low in spite of significant binding activity for human somatotropin. This observation also indicates the heterogeneity of human-somatotropin-binding sites.

Recently several hormone receptors have been partially purified using affinity chromatography (Shiu & Friesen, 1974*b*; Dufau *et al.*, 1975; Jacobs *et al.*, 1977). Shiu & Friesen (1974*b*) purified the prolactin receptor from rabbit mammary gland with human somatotropin coupled to the *N*-hydroxysuccinimide ester of 3,3′-diaminodipropylaminosuccinyl-Agarose (Affi-Gel 10). Very recently Waters & Friesen (1979) were able to separate somatotropin-specific receptor from prolactin-specific receptor using differential affinity chromatography. In the present study we have used affinity chromatography on concanavalin-A–Sepharose column as the initial step for purification because of its simplicity. As with the insulin receptor from rat-liver membrane (Cuatrecasas & Tell, 1973), the binding activity for human ^{125}I-labelled-somatotropin was adsorbed on the concanavalin-A–Sepharose column, and was effectively eluted from the column by α-methyl-D-glucoside. The specificity and affinity of human ^{125}I-labelled-somatotropin for the glucoside-eluted fraction was not significantly different from those for the crude Triton extract. On the other hand, the binding of human ^{125}I-labelled-somatotropin to the mannoside-eluted fraction was displaced less by bovine somatotropin than was the binding to the glucoside-eluted fraction (data not shown). Furthermore, the binding activity for bovine ^{125}I-labelled-somatotropin is significantly higher in the glucoside-eluted fractions. Unlike the human hormone, bovine somatotropin lacks lactogenic activity in the rabbit. Therefore these results suggest that the glucoside-eluted fraction contains more binding sites specific for somatotropin than does the mannoside-eluted fraction.

Using affinity chromatography on concanavalin-A–Sepharose, a 20–50-fold purification was achieved in a single step. The adsorption of the binding protein on the concanavalin-A–Sepharose column and the dissociation by specific sugars strongly suggests that the binding protein may be a glycoprotein.

Further purification was achieved by ion-exchange chromatography on DEAE-cellulose, followed by repeated gel filtration on Sepharose 6B.

The binding capacity for human somatotropin of the original Triton extract was approximately 0.8 pmol/mg protein, and the most highly purified preparation had a capacity of 0.6 nmol/mg. Assuming that the human somatotropin-binding protein has a mol.wt. of 200000, and that one receptor molecule has one binding site, the binding capacity of the completely purified preparation could be 5.0 nmol/mg. Polyacrylamide-disc-gel electrophoresis [7.5% (v/v) polyacrylamide gel] of the partially purified preparation revealed at least four stained bands (data not shown). Thus it is clear that further purification is needed.

Gel filtration of the human somatotropin-binding protein on Sepharose 6B demonstrated that the protein behaves as a molecule with an apparent mol.wt. of 200000, assuming that the binding protein and marker proteins have the same symmetry, since the chromatographic behaviour of macromolecules correlates with Stokes' radius rather than molecular weight (Siegel & Monty, 1966). The Stokes' radius of the human somatotropin-binding protein was estimated to be 4.9 nm. The apparent molecular weight (300000) and the Stokes' radius (6.2 nm) of the protein obtained by Waters & Friesen (1979) are slightly higher than the values we have found. In both cases, however, the value for the Stokes' radius is similar to that reported for the prolactin receptor (Shiu & Friesen, 1974*b*) or for the gonadotropin receptor (Dufau *et al.*, 1973). Our preliminary studies with sodium dodecyl sulphate/polyacrylamide gel electrophoresis showed that the binding activity for human somatotropin migrates as a molecule with mol.wt. 55000 (data not shown), which suggests that the somatotropin-binding protein is composed of subunits, as reported for the insulin receptor. The sedimentation behaviour on sucrose-density-gradient centrifugation of somatotropin-binding protein was also similar to that of the gonadotropin receptor from rat testis (Dufau *et al.*, 1973). Isoelectric focusing experiments revealed that the somatotropin-binding protein has an acidic pI, which is in agreement with the observation by Waters & Friesen (1979).

Treatment with trypsin resulted in a significant loss of the binding activity, indicating that polypeptide forms an important constituent of the binding site. Reduction and alkylation with dithiothreitol and *N*-ethylmaleimide also strongly decreased the binding activity for somatotropin, as reported for the gonadotropin receptor (Dufau & Catt, 1974). Intrachain disulphide bonds may be of some importance for the binding of somatotropin. Thus, somatotropin-binding proteins from rabbit liver seem to share several characteristics with receptors for other polypeptide hormones.

Although somatotropin has several biological effects on a variety of tissues, the liver may be one of the important sties of action. Administration of somatotropin to hypophysectomized rats increases protein and RNA synthesis and alters the activity of several enzymes in liver (Jänne & Raina, 1969; Kenny, 1967). Furthermore, the liver is believed to produce somatomedins, serum concentrations of which are dependent on somatotropin. However, it is not clear at present whether the hepatic somatotropin-binding protein is related to the action of somatotropin. It has been reported that antibodies directed against the prolactin receptor blocked the action of this hormone on its target tissue (Shiu & Friesen, 1976). Similar work with the somatotropin-binding protein may be very useful in answering the question whether the hepatic somatotropin-binding site really represents the 'somatotropin receptor'.

We thank Miss M. Yamazaki for technical assistance. The constant encouragement and help of Professor K. Kosaka is greatly appreciated. This research was supported by grants from the M.R.C. of Canada, the Child Health and Human Development Institute, NIH grant No. 07438-05, and grants from the Ministry of Education and Culture, Japan.

References

Carr, D. & Friesen, H. G. (1976) *J. Clin. Endocrinol. Metab.* **42**, 484–493

Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 318–322

Cuatrecasas, P. & Tell, G. P. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 485–489

Dufau, M. L. & Catt, K. J. (1974) *Biochim. Biophys. Acta* **343**, 417–422

Dufau, M. L., Charreau, E. H. & Catt, K. J. (1973) *J. Biol. Chem.* **248**, 6973–6982

Dufau, M. L., Ryan, D. W., Baukal, A. J. & Catt, K. J. (1975) *J. Biol. Chem.* **250**, 4822–4824

Herington, A. C. & Veith, N. M. (1977) *Endocrinology* **101**, 984–987

Jacobs, S., Schechter, T., Bissel, K. & Cuatrecasas, P. (1977) *Biochem. Biophys. Res. Commun.* **77**, 981–987

Jänne, J. & Raina, A. (1969) *Biochim. Biophys. Acta* **174**, 769–772

Kelly, P. A., Posner, B. I., Tsushima, T. & Friesen, H. G. (1974) *Endocrinology* **95**, 532–539

Kenny, F. T. (1967) *J. Biol. Chem.* **242**, 4376–4371

Laurent, T. C. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330

Lesniak, M. A., Roth, J., Gorden, P. & Gavin, J. R. III (1973) *Nature (London) New Biol.* **241**, 20–22

Lesniak, M. A., Gorden, P., Roth, J. & Gavin, J. R. III (1974) *J. Biol. Chem.* **249**, 1661–1667

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379

McConaghey, P. & Sledge, L. B. (1970) *Nature (London)* **225**, 1249–1250

Phillips, L. S., Herington, A. C., Karl, I. E. & Daughaday, W. H. (1976) *Endocrinology* **98**, 606–614
Posner, B. I., Kelly, P. A., Shiu, R. P. C. & Friesen, H. G. (1974) *Endocrinology* **95**, 521–531
Posner, B. I. (1976) *Endocrinology* **98**, 645–654
Shiu, R. P. C. & Friesen, H. G. (1974*a*) *Biochem. J.* **140**, 301–311
Shiu, R. P. C. & Friesen, H. G. (1974*b*) *J. Biol. Chem.* **240**, 7902–7911
Shiu, R. P. C. & Friesen, H. G. (1976) *Science* **192**, 259–261
Siegel, L. M. & Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346–362
Thorell, J. I. & Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369
Tsushima, T. & Friesen, H. G. (1973) *J. Clin. Endocrinol. Metab.* **37**, 334–337
Udenfriend, S., Stein, S., Böhlin, P., Dairman, W., Leimgruber, W. & Weigele, M. *Science* **178**, 871–872
Waters, M. J. & Friesen, H. G. (1979) *J. Biol. Chem.* **254**, 6815–6825