Characterization of the Binding of Human Growth Hormone to Microsomal Membranes from Rat Liver

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(Received 16 February 1976)

The binding of ^{125}I -labelled human growth hormone to the 100000g microsomal membrane fraction prepared from the livers of normal female rats was dependent on time, temperature, pH, membrane concentration and concentration of ^{125}I -labelled human growth hormone. At 22°C binding reached a steady state after 16 h, with the mean maximal specific binding being 20% of the tracer initially added. Dissociation of ^{125}I -labelled human growth hormone from the membranes, after addition of excess of unlabelled hormone, was relatively slow with a half-time greater than 24 h. Only minor degradation of the ^{125}I -labelled human growth hormone was observed during incubation with membranes for 16 or 25 h at 22°C. Similarly, no significant change in the ability of membranes to bind human growth hormone was evident after preincubation of the membranes for 16 or 25 h. Specificity studies showed that up to 90% of the ^{125}I -labelled human growth hormone bound could be displaced by 1 μg of unlabelled hormone. Ovine prolactin also showed considerable competition for the binding site. Non-primate growth-hormone preparations (ovine, bovine, porcine and rat) and non-related hormones (insulin, thyrotropin, lutropin and follitropin) all showed negligible competition. Scatchard analysis of the binding data was consistent with two classes of binding site with binding affinities of $0.64 \times 10^{10} \pm 0.2 \times 10^{10} \text{ M}^{-1}$ and $0.03 \times 10^{10} \pm 0.007 \times 10^{10} \text{ M}^{-1}$ and corresponding binding capacities of $98.4 \pm 10 \text{ fmol/mg}$ of protein and $314.6 \pm 46.3 \text{ fmol/mg}$ of protein. These studies provide data which, in general, are consistent with the criteria required for hormone-receptor interaction. However, proof of the thesis that the human-growth-hormone-binding sites in female rat liver represent physiological receptors must await the demonstration of a correlation between hormone binding and a biological response.

A physical interaction between a hormone molecule and a specific binding site (receptor) on the surface membrane of target cells is assumed to be the initial step in the mechanism of action of protein and polypeptide hormones (Roth, 1973). For growth hormone, however, there has been little direct evidence for this. Studies with ^{125}I -labelled human growth hormone have demonstrated the existence of specific binding sites for this hormone in cultured human lymphocytes (Lesniak *et al.*, 1973, 1974) and in microsomal membranes prepared from livers of rabbits (Tsushima & Friesen, 1973; Herington *et al.*, 1974) and rats (Posner *et al.*, 1974b; Herington *et al.*, 1976; Veith *et al.*, 1975). Additional studies have now been carried out on the physiological regulation of liver binding sites (Herington *et al.*, 1976; Posner *et al.*, 1974a), their ontogenesis (Kelly *et al.*, 1974) and species variation (Posner *et al.*, 1974b). It has been suggested on the basis of some of these studies (Posner *et al.*, 1974b) that the human-growth-hor-

mone-binding sites of rat liver may, in fact, represent lactogenic sites and not growth-hormone sites as such, the binding of human growth hormone being due to its inherent lactogenic activity rather than to its growth activity. The significance of this observation and indeed the physiological role, if any, of these binding sites is still unknown.

Despite these fairly extensive studies, the characteristics of the interaction between human growth hormone or prolactin and the 'lactogenic' binding sites of liver [a target tissue for both growth hormone (Korner, 1965; Janne *et al.*, 1968; Phillips *et al.*, 1976) and prolactin (Chen *et al.*, 1972; Richards, 1975; Francis & Hill, 1975)] have not been reported in detail.

As a first step in understanding the possible significance of the binding sites, characterization of the binding reaction is required. This paper therefore describes a number of the characteristics of binding of human growth hormone to microsomal membranes prepared from livers of normal female rats. Although the binding sites may recognize only lacto-

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genic hormones, the present studies were performed with ^{125}I -labelled human growth hormone rather than the purely lactogenic ^{125}I -labelled prolactin, because, first, most of the initial studies (Posner *et al.*, 1974a,b; Kelly *et al.*, 1974; Herington *et al.*, 1976) that prompted the present investigation had been performed by using ^{125}I -labelled human growth hormone, and secondly, in the studies of the ontogenesis (Kelly *et al.*, 1974) and physiological regulation (Posner *et al.*, 1974a) of binding of both human growth hormone and ovine prolactin, very similar results were obtained for the two hormones, suggesting that at least in this particular system the two hormones were indeed equivalent.

Materials and Methods

Materials

Human growth hormone (CSL 11.1) was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, and was purified by gel filtration on a column (75 cm $\times$ 2.8 cm) of Sephadex G-100 (medium grade: Pharmacia, Uppsala, Sweden). All other hormones used for the specificity studies (bovine, rat, porcine and ovine growth hormones and ovine prolactin) were purified preparations supplied by the National Pituitary Agency and the National Institutes of Arthritis, Metabolism and Digestive Disease, Bethesda, MD, U.S.A. Chloramine-T was obtained from Merck A.G., Darmstadt, Germany. Na^{125}I (carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Iodination of human growth hormone

Iodination of the hormone with ^{125}I to specific radioactivity of 80–100 $\mu\text{Ci}/\mu\text{g}$ was performed by a chloramine-T method (Greenwood *et al.*, 1963) modified as follows. Human growth hormone (10 μg), 0.5M-sodium phosphate buffer (pH 7.5) (12.5 μl), Na^{125}I (1 mCi) and chloramine-T (10–20 μg) were added sequentially and allowed to react for 30 s. The iodination reaction was terminated by the addition of 5 μl of sodium metabisulphite (10 mg/ml). Unchanged I^- and damaged hormone were removed by washing with 50 mM-sodium phosphate buffer (pH 7.4) through a cellulose (Whatman CF11) column (55 mm $\times$ 6 mm). The adsorbed ^{125}I -labelled human growth hormone was eluted with veronal buffer (0.02 M, pH 7.4) containing 20% (v/v) acetone and 5% (v/v) bovine serum albumin, stored at 4°C, and was repurified before use by gel filtration on Sephadex G-100 (fine) by using 0.025 M-Tris/HCl (pH 7.4) containing 10 mM- CaCl_2 , the buffer used for subsequent binding studies. The results of repurification were qualitatively similar to those described for porcine prolactin (Jacobs *et al.*, 1972).

Preparation of microsomal membranes from liver

For all binding studies, the 15000–100000 g microsomal-membrane fraction of rat liver was prepared as described previously (Herington *et al.*, 1976), based on the original method for pregnant-rabbit liver (Tsushima & Friesen, 1973). All preparatory steps were performed at 4°C. The livers were removed from normal adult female rats (Sprague-Dawley strain, 150 g), rinsed in 0.3 M-sucrose, weighed wet and minced with scissors. The liver mince was homogenized in 2–3 vol. of 0.3 M-sucrose in a Sorvall Omnimixer (setting 3, 20 s) and then centrifuged for 20 min at 1500 g in a fixed-angle head (SS 34) of a refrigerated Sorvall RC2-B centrifuge. The supernatant was then centrifuged serially at 15000 g (20 min, Sorvall SS 34 head) and then at 100000 g (90 min, 6 $\times$ 14 ml titanium swing-out rotor of an MSE 65 ultracentrifuge). The 100000 g pellet was resuspended in 0.025 M-Tris/HCl (pH 7.4) containing 10 mM- CaCl_2 , to a volume of 1 ml/g original wet weight of liver. Each membrane preparation was stored at -17°C in 1 or 2 ml portions, and the protein concentration was determined by the method of Lowry *et al.* (1951).

Human-growth-hormone-binding studies

All binding studies were carried out in triplicate by conventional competitive protein-binding techniques as described previously (Herington *et al.*, 1976). The reaction mixture (500 μl) consisted of 100 μl of membrane preparation (200 μg of membrane protein), 100 μl of ^{125}I -labelled human growth hormone (15000–25000 c.p.m.), 100 μl of unlabelled human growth hormone (standard) when required and 0.025 M-Tris/HCl (pH 7.4) containing 10 mM- CaCl_2 , 0.1% (v/v) bovine serum albumin and 0.02% (v/v) sodium azide.

The inclusion of calcium has been reported (Tsushima & Friesen, 1973; Posner *et al.*, 1974b) to increase binding of human growth hormone and to allow complete sedimentation during subsequent centrifugation of the hormone-membrane complex. Bovine serum albumin was included to minimize losses of hormone by adsorption to surfaces, and sodium azide was added as an anti-bacterial agent. The binding reaction was terminated by dilution with 2–3 ml of ice-cold 0.025 M-Tris/HCl (pH 7.4) containing 10 mM- CaCl_2 , 0.1% bovine serum albumin and 0.02% sodium azide. Separation of bound and free hormone was achieved by centrifugation at 2000 rev./min for 25 min in a refrigerated centrifuge. The supernatant was decanted and the ^{125}I -labelled human growth hormone bound to the membrane pellet was counted for radioactivity in a Nuclear-Chicago auto-gamma counter.

Total binding was measured in the absence of unlabelled hormone and non-specific binding was taken

as the ¹²⁵I remaining bound in the presence of 1 µg of unlabelled hormone. Specific binding was assessed by subtracting the non-specific from the total binding.

Results

Time- and temperature-dependence

The binding of ¹²⁵I-labelled human growth hormone to microsomal-membrane preparations from rat liver was both time- and temperature-dependent (Fig. 1). Specific binding at 37°C was initially somewhat more rapid than binding at 22°C, but began to decline after 15 h of incubation because of probable degradation of the hormone and/or binding site. Binding at 22°C, on the other hand, continued to rise and reached an apparent steady state (equilibrium) at about 16 h. The maximum binding observed in this experiment at 22°C was 11.3% and this was maintained until at least 30 h. For all experiments performed at 22°C the mean specific binding reached at equilibrium was 20.6 ± 1.4% (mean ± S.E.M.; n = 9) of the total ¹²⁵I-labelled human growth hormone added. Binding at 4°C, a temperature often used for hormone-binding studies because of negligible degradation problems, was very slow and somewhat more variable. As a result of these observations all subsequent incubations were carried out at 22°C for 16 h.

Degradation of ¹²⁵I-labelled human growth hormone and binding site

One of the problems in many hormone-binding systems is that of hormone or binding-site degradation during the incubation period. Possible degradation of ¹²⁵I-labelled human growth hormone during

Fig. 1. Effects of time and temperature on binding of ¹²⁵I-labelled human growth hormone to liver membranes from normal female rats

Specific binding was determined as described in the Materials and Methods section. Values shown are means ± S.E.M. of triplicate determinations and are expressed as a percentage of the total ¹²⁵I-labelled human growth hormone added per tube. Where no error bars are shown the S.E.M. was within the size of the point symbol. ○, 37°C; ●, 22°C; ▲, 4°C.

incubation with membranes at 22°C was tested in the following way. ¹²⁵I-labelled human growth hormone was incubated in the presence of liver membranes for 16 or 25 h. The incubation mixture was then spun at 4°C (2000 rev./min) and the integrity of the unbound or free ¹²⁵I-labelled human growth hormone remaining in the supernatant was tested in two ways. Samples were tested in triplicate for their ability (a) to rebind (during a 2 h incubation) to fresh microsomal membranes from rat liver and (b) to be precipitated by trichloroacetic acid. Each of these parameters was compared with results obtained with ¹²⁵I-labelled human growth hormone that had not been preincubated with membranes (control). Table 1 demonstrates that there was no significant difference in either parameter compared with controls (100%) at either 16 or 25 h. Although some loss of rebinding ability of ¹²⁵I-labelled human growth hormone was observed after 25 h incubation, this was not statistically significant.

Degradation of the binding site was tested by preincubating membranes at 22°C for 16 or 25 h before addition of fresh ¹²⁵I-labelled human growth hor-

Table 1. Effect of preincubation on degradation of ¹²⁵I-labelled human growth hormone and binding site

Hormone degradation was tested after preincubation of ¹²⁵I-labelled human growth hormone with rat liver membranes for either 16 or 25 h at 22°C. The incubation mixture was then spun, and the integrity of the unbound ¹²⁵I-labelled human growth hormone remaining in the supernatant was tested (in triplicate) for its ability (a) to rebind to fresh membranes during a 2 h incubation and (b) to be precipitated by 10% (w/v) trichloroacetic acid. Specific binding was determined in each case as described in the Materials and Methods section and is expressed as a percentage (mean ± S.E.M., n for n > 2) of the values obtained for ¹²⁵I-labelled human growth hormone that had not been preincubated with membranes (control; 100%). Compared with the control data, none of the groups showed a significant difference by Student's *t* test. Binding-site degradation was tested by preincubating membranes at 22°C for 16 or 25 h before addition of ¹²⁵I-labelled human growth hormone for a further 2 h. Specific binding is again expressed as a percentage of the value obtained for membranes that had not been preincubated (control, 100%).

	Percentage of control value	
	16h	25h
Degradation of ¹²⁵ I-labelled human growth hormone		
(a) Rebinding to fresh membranes	98 ± 7 (4)	83 ± 14 (3)
(b) Precipitation by trichloroacetate	93, 98 (2)	93, 97 (2)
Binding-site degradation		
Binding of fresh ¹²⁵ I-labelled human growth hormone	99 ± 9 (4)	111 ± 22 (4)

mone for a further 2h. Compared with control membranes (no preincubation, 100%) binding was essentially unchanged at both 16 and 25h, although some degree of scatter was observed in the data for the 25h preincubation.

Effects of pH, membrane concentration and ^{125}I -labelled human growth hormone concentration

Fig. 2 shows the effect of medium pH on binding of human growth hormone. A peak in specific binding occurred between pH 5.3 and 6.8; binding was dramatically decreased below pH 4.0 and above pH 8.0. The effect of pH values above 8.0 may not be simply an effect on binding, however, since membrane dissolution occurred during the prolonged 16h incubation at high pH values.

Fig. 3 shows the effect on binding of ^{125}I -labelled human growth hormone of increasing amounts of membrane protein. Specific binding appeared to be saturable at about 600 μg of protein. At low amounts of protein (<200 μg) binding was approximately linear and therefore 200 μg of membrane protein was chosen for all kinetic and equilibrium studies.

Binding as a function of increasing ^{125}I -labelled human growth hormone concentration is shown in Fig. 4. Specific binding was detected at the lowest concentration used (11 pM) and appeared to reach saturation by 0.36 nM, the highest concentration poss-

ible in this particular experiment. Non-specific binding (less than 5.0% of counts added) increased in a linear fashion.

Dissociation of bound ^{125}I -labelled human growth hormone

A requirement for all hormone-receptor binding systems is that binding should be reversible. Fig. 5 demonstrates the reversibility of binding of human growth hormone to the microsomal membranes. ^{125}I -labelled human growth hormone was bound in

Fig. 3. Effect of amount of membrane protein on binding of ^{125}I -labelled human growth hormone to rat liver membranes

Specific (●) and non-specific (○) binding were determined and are expressed as described for Fig. 2.

Fig. 2. Effect of pH on binding of ^{125}I -labelled human growth hormone to rat liver membranes

Specific (●) and non-specific (○) binding were determined as described in the Materials and Methods section. Values shown are the means $\pm$ S.E.M. of triplicate determinations and are expressed as a percentage of the total ^{125}I -labelled human growth hormone added per tube. Where no error bars are shown the S.E.M. was within the size of the point symbol. The pH of the incubation buffer (0.025 M-Tris) was adjusted with 1 M-HCl or 1 M-NaOH as required.

Fig. 4. Effect of ^{125}I -labelled human growth hormone concentration on binding to rat liver membranes

Specific (●) and non-specific (○) binding were determined as described in the Materials and Methods section. Values shown are means $\pm$ S.E.M. of triplicate values and are expressed as absolute counts bound or pmol bound. Where no error bars are shown the S.E.M. was within the size of the point symbol. Specific radioactivity of ^{125}I -labelled human growth hormone used in this experiment was 90 $\mu\text{Ci}/\mu\text{g}$.

Fig. 5. Effect of delayed addition of excess of unlabelled human growth hormone on dissociation of specifically bound ^{125}I -labelled human growth hormone

Specific binding was determined as described in the Materials and Methods section for tubes incubated without addition of unlabelled human growth hormone (●). Corresponding sets of tubes had an excess ($3.7\text{ }\mu\text{g}$) of unlabelled growth hormone added (without significant volume change) after incubation periods of 1 (○), 5 (Δ) and 16 h (□) (shown by arrows). The amount of ^{125}I -labelled human growth hormone remaining bound was followed for as long as 48 h in some cases. Non-specific binding has been subtracted in all cases. Values shown represent means $\pm$ S.E.M. of triplicates and are expressed as a percentage of the total ^{125}I -labelled human growth hormone added per tube. Where no error bars are shown the S.E.M. was within the size of the point symbol.

the absence of unlabelled human growth hormone for a period of 30 h. A typical time-course for specific binding was observed. The addition of a large excess of unlabelled human growth hormone at 1, 5 or 16 h led to an immediate dissociation of bound labelled hormone. The slope of the dissociation curves indicates that the half-time at 22°C for exchange of labelled human growth hormone by unlabelled human growth hormone was at least 24 h.

Hormone specificity

The specificity of binding of ^{125}I -labelled human growth hormone is shown in Fig. 6. Addition of increasing amounts of unlabelled human growth hormone resulted in a substantial decrease in binding of labelled tracer, with a more than 70% decrease produced by $1\text{ }\mu\text{g}$ of unlabelled hormone. In other experiments the non-displaceable bound tracer was often less than 10% of the total binding. Ovine prolactin also competed for binding of ^{125}I -labelled human growth hormone, but with a much diminished potency (between 25 and 5%) compared with human growth hormone itself. This potency range, which in this experiment is rather wide because of the observed non-parallelity of the two displacement curves, was estimated by comparing the amounts of unlabelled

Fig. 6. Hormonal specificity of binding of human growth hormone to rat liver membranes

Competition for binding of ^{125}I -labelled hormone (expressed as a percentage of binding in the absence of unlabelled hormone) was measured in the presence of increasing amounts of unlabelled human growth hormone (●), ovine prolactin (○), bovine growth hormone (■) or rat growth hormone (Δ). Insulin, human thyrotropin, human lutropin and human follitropin were all tested at a single dose ($1\text{ }\mu\text{g}/\text{tube}$) and are represented by the square bracket. Each point is the mean $\pm$ S.E.M. of triplicates; where no error bars are shown the S.E.M. was within the size of the point symbol.

hormone (human growth hormone or ovine prolactin) required to displace 30, 40 or 50% of the bound labelled human growth hormone. For example, the data of Fig. 6 show that about 20 times more ovine prolactin (250 ng) than human growth hormone (12 ng) was required to produce a 50% displacement of bound human growth hormone, indicating that at this level of displacement ovine prolactin had 5% of the potency of human growth hormone. Other growth-hormone preparations, however, [bovine and rat (shown in Fig. 6) and ovine and porcine (not shown, but which gave data identical with those for rat and bovine respectively)] showed minimal competition for binding of ^{125}I -labelled human growth hormone. Similarly, the non-related hormones (lutropin, follitropin, thyrotropin and insulin) all failed to compete for binding of human growth hormone.

Analysis of binding parameters

Fig. 7 shows a typical Scatchard plot (Scatchard, 1949) of data derived from a binding displacement curve similar to that shown for human growth hormone in Fig. 6. The curvilinear Scatchard plot obtained (closed circles) was analysed by a modification of the graphical method of Weder *et al.* (1974). Two classes of binding site (denoted by the two straight-line components) were revealed. The parent curve regenerated by these two components (dashed curve) represents a very close fit to the original experimental points. One preliminary experiment showed no evidence for site-site interactions of the negative co-operative type which have been proposed to explain the curvilinear Scatchard plots observed for insulin binding to human lymphocytes (de Meyts *et al.*, 1973). Assuming, therefore, two classes of binding site, the mean binding affinity and binding capacity for each of the sites was measured for a number of different membrane preparations from normal female rats (Table 2). The binding affinity of class I sites (0.64×10^{10} litre/mol) was approximately 20-fold greater than that for class II sites and is entirely com-

Fig. 7. Scatchard plot of the binding of ^{125}I -labelled human growth hormone to rat liver membranes

Scatchard analysis (specific binding only) was carried out on data obtained from a competition experiment similar to that shown in Fig. 6 for various concentrations of hormone. ●, Calculated values for the experimental data; ----, parent theoretical curve regenerated by the two straight-line components (—) derived from the experimental data by graphical analysis (Weder *et al.*, 1974). The negative of the slope of each of the component lines is a measure of the hormone-binding affinity, and the X intercept for each component is a measure of the hormone-binding capacity.

Table 2. Parameters of binding of ^{125}I -labelled human growth hormone to liver membranes from normal female rats

For each membrane preparation studied, Scatchard analyses were carried out in duplicate, by using two different batches of ^{125}I -labelled human growth hormone as tracer. The values shown are the means $\pm$ S.E.M. for four different membrane preparations. Scatchard analysis was carried out in each case as depicted and described in Fig. 7.

Binding site	Binding capacity (fmol/mg of protein)	$10^{-10} \times$ Binding affinity (litre/mol)
Class 1	98.5 ± 10	0.643 ± 0.175
Class 2	314.6 ± 46.3	0.030 ± 0.007

patible with the known circulating concentrations of growth hormone. There was an approximately 3-fold difference in the binding capacity of the two classes of site.

Discussion

Binding of human growth hormone to a number of different tissues in a variety of species has been described previously (Lesniak *et al.*, 1974; Posner *et al.*, 1974b; Herington *et al.*, 1974, 1976), but little was known of the fundamental characteristics of binding to rat liver in particular or whether or not the hormone-binding-site interaction was consistent with anticipated hormone-receptor characteristics. The studies reported here describe some of the basic details of the binding of human growth hormone to microsomal membranes from rat liver.

The use of the 15000–100000g microsomal-membrane fraction to study the binding of ^{125}I -labelled human growth hormone was based on earlier studies (Tsushima & Friesen, 1973; Herington *et al.*, 1976) indicating that this fraction exhibited the highest binding of human growth hormone, and bound ^{125}I -labelled human growth hormone to the same extent as 'purified' liver plasma membrane prepared by the method of Meldolesi *et al.* (1971).

Binding was studied only in microsomal membranes prepared from normal female rats, since earlier studies (Kelly *et al.*, 1974; Herington *et al.*, 1976) had shown a marked sex difference in the ability of rat liver to bind human growth hormone.

Binding of human growth hormone depended on several parameters including time, temperature, pH, tracer and membrane concentrations, as has been described for interactions of other protein and polypeptide hormones with their target-tissue binding sites (Cuatrecasas, 1971; Kahn *et al.*, 1974; Bhalla & Reichert, 1974; Lee *et al.*, 1973).

The kinetics observed in this particular system (both association and dissociation rates) are considerably slower than has been generally observed for other

hormone-binding sites studied. The time required for binding of human growth hormone to reach equilibrium at 22°C in this study (16h) is much longer than the 3–4h required, as described by Herington *et al.* (1976). An explanation for these differences is not readily apparent, although there were some modifications in the initial preparation of the membrane fractions. Kelly *et al.* (1973) have also studied the time-course of binding of human growth hormone, but only at 4°C, and observed a very long (36h) equilibration time.

The kinetics of dissociation (half-time >24h at 22°C) are also quite slow compared with other hormone systems, but are quite consistent with a value quoted by Posner *et al.* (1974b) for binding of human growth hormone to rat liver microsomal membranes and with reports from Catt *et al.* (1972) for human choriogonadotropin dissociation from the testis receptor, and by Shiu & Friesen (1974) for ovine prolactin dissociation from a mammary-gland receptor. The significance of such slow kinetics in physiological systems is uncertain. One possible explanation for the system described here is that since microsomal-membrane preparations have a tendency to form 'inside-out' vesicles (Meldolesi *et al.*, 1971), extravesicular hormone molecules might be effectively shielded from the binding sites. The observed kinetics may then be simply a reflexion of the rate of entry of hormone into the lumen of the vesicle. Differences in the extent of vesiculation, resulting perhaps from slight differences in preparative methods, might be a reason for the observed kinetic differences between the present study and that already reported by Herington *et al.* (1976).

More highly purified plasma membranes prepared by a method based on that described by Neville (1968) do not readily form inverted vesicles (Meldolesi *et al.*, 1971), and preliminary studies with such preparations indicate that binding of human growth hormone reaches equilibrium at 22°C by 5–6h (A. C. Herington, unpublished work). Although this is still a relatively long equilibration time, it is much shorter than the 16h required by the membrane preparations used in the present study. These data suggest that the extent of vesiculation, which may be significantly altered by apparently minor changes in preparative methods, might well play an important role in the observed kinetics of binding of human growth hormone. Whatever the cause (real or artifact) for the slow kinetics observed in this system, it is obviously a major factor to be considered in future binding studies of growth hormone, prolactin or indeed any other hormone, when membrane preparations that may form inverted vesicles are to be used.

Although interpretations of 'physiologic significance' become difficult with this system, it is noteworthy, in view of the kinetics observed, that although relatively rapid changes in circulating con-

centrations of growth hormone can occur under appropriate stimuli *in vivo*, a number of the measurable metabolic effects of growth hormone *in vivo* or *in vitro* show a significant lag period (from 1 to 3h) before being demonstrable (Daughaday & Parker, 1965; Raina & Holttä, 1972; Hjalmarson, 1968).

A second observation of some note relates to the studies on degradation of both ¹²⁵I-labelled human growth hormone and the binding site. In contrast with most other hormone-binding systems studied previously, there appears to be very little, if any, degradation of hormone during incubation with membranes at 22°C. There is an indication (Fig. 1) that at 37°C degradation may be more pronounced. It should be emphasized, however, that because of the prolonged half-time for dissociation of the hormone-binding-site complex (Fig. 5), it is doubtful that much of the 'free' hormone, which was tested for rebinding or trichloroacetate precipitability, had in fact participated in a binding reaction. No studies have yet been carried out on hormone that has been dissociated from the membrane complex, and it is quite possible that these hormone molecules might exhibit a greater loss of molecular integrity. The integrity of the binding site was also maintained and did not lose the ability to bind ¹²⁵I-labelled human growth hormone during preincubation at 22°C. A short-term (2h) degradation study by Posner *et al.* (1974b) revealed a small (12%) degree of degradation of ¹²⁵I-labelled human growth hormone during incubation with microsomal membranes from pregnant-rat liver. Although this is marginally greater than observed in the present study, the results are not strictly comparable, since Posner *et al.* (1974b) used pregnant, not normal, female rats for the initial degradation study, and they tested the ability of the human growth hormone to rebind to membranes prepared from rabbit liver and not from rat liver. The binding of ¹²⁵I-labelled human growth hormone to microsomal membranes from rabbit liver is known to exhibit several different properties (e.g. time-course, specificity) from binding of human growth hormone to microsomal membranes from rat liver (Posner *et al.*, 1974b), and may in part account for the observed differences.

It has been suggested that the binding sites under study in these particular membrane preparations are in fact lactogenic sites rather than growth-hormone sites as such (Posner *et al.*, 1974b). This was based on earlier reports of binding specificity (Posner *et al.*, 1974a; Herington *et al.*, 1976), where there was a lack of competition by growth hormone from species other than humans and a significant but variable competition by various prolactin preparations. The results of the rather limited survey reported in the present study (Fig. 6) are in good agreement with these earlier reports. The binding of human growth hormone to the lactogenic sites can be explained on

the basis of its inherent lactogenic properties, which are not possessed by growth-hormone molecules from other species.

The displacement curve for unlabelled human growth hormone shown in the specificity study is typical of those obtained as a routine. Scatchard analysis of these curves indicated, for the first time, that there may be two classes of human-growth-hormone-binding site in these particular membrane preparations. This is in contrast with previous reports (Kelly *et al.*, 1974; Herington *et al.*, 1976), where a straight-line Scatchard plot (one class of binding site) was generally observed. However, Kelly *et al.* (1974) did report observing a second class of low-affinity sites in some cases. The affinity and capacity of the class I sites in the present study compare quite favourably with those values reported for the single class of site. The affinity is slightly increased (with a correspondingly small decrease in binding capacity) owing to the removal of the contribution at all points, of the low-affinity higher-capacity site (class II).

The values obtained for the binding affinity of the high-affinity site are quite consistent with the normal circulating concentrations of prolactin or growth hormone (approx. 0.25 nM), and, together with the other binding characteristics reported, are in general consistent with recognized hormone-receptor interactions (Roth, 1973).

Further indirect evidence to support the notion that the binding sites of human growth hormone might be physiologically important comes from studies demonstrating a marked physiological regulation of the binding sites, marked increases in binding being induced by oestrogen administration, pregnancy and high prolactin and/or growth-hormone concentrations, and marked decreases being induced by hypophysectomy or testosterone administration (Herington *et al.*, 1975, 1976; Posner *et al.*, 1974a).

Although these studies are compatible with the hypothesis that these binding sites might have some biological significance (i.e. true hormone receptors), there is in fact no direct evidence to support this. Perhaps the most important evidence required is a demonstration of a direct link between hormone binding and a hormone-related metabolic event. This has not been achieved to date for growth hormone. Since it does not appear to function via the membrane-bound adenylate cyclase/cyclic AMP system and since there are no other growth-hormone-controlled metabolic events occurring in isolated membrane systems, it is not possible to approach this question by using the liver microsomal-membrane preparations. It is probable that studies utilizing liver slices or isolated hepatocytes will be required to provide definitive answers to the question of the biological importance of the hepatic binding sites for human growth hormone.

This work was supported by the National Health and Medical Research Council of Australia.

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