Characterization of human somatotropin binding to detergent-solubilized lactogenic receptors from rat liver

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Lactogenic receptors from rat liver microsomal fraction ('microsomes') were extracted by treatment with 1% (w/v) Triton X-100. Triton X-100 exerts an inhibitory effect on both the binding reaction and the separation of the free hormone from the complex. The association and dissociation of ^{125}I -labelled human somatotropin are time- and temperature-dependent processes. The association rate constant, k_1 , is $6.7 \times 10^6 \text{ mol} \cdot \text{litre}^{-1} \cdot \text{min}^{-1}$ at 25°C , and the dissociation rate constant, k_{-1} , is $1.1 \times 10^{-3} \text{ min}^{-1}$ at 25°C . Scatchard analysis of saturation data reveals the existence of a single class of receptors and that solubilization leads to a slight decrease in affinity and a sharp increase in binding capacity. The dissociation constant, K_d , of the solubilized preparation is 0.22 nM and the binding capacity 2900 fmol/mg of protein. Similar results were obtained from competition experiments. Binding of ^{125}I -labelled human somatotropin to the solubilized receptors is specifically inhibited by hormones with lactogenic activity. Incubation of the solubilized preparation with trypsin resulted in an 80% decrease in binding activity. The solubilized form of the receptor has a slightly increased sensitivity to the inactivation by trypsin, heat and extremes of pH, with respect to the membrane-bound form.

Two types of receptors present in rat liver cells are capable of binding human somatotropin; according to their specificity they have been named somatotropic or somatogenic and lactogenic (Ranke *et al.*, 1976a; Postel-Vinay, 1976). The binding of human somatotropin to the last named receptors is inhibited by the presence of lactogenic hormones like prolactins from various species, whereas non-primate somatotropins, which are devoid of lactogenic properties, only inhibit the binding of human somatotropin to the somatogenic receptors. The ability of human somatotropin to bind to both types of receptors is explained by the coexistence in this molecule of somatotropic (Evans *et al.*, 1943) and lactogenic (Kleinberg & Frantz, 1971) properties.

Female rat hepatocytes display both kinds of receptors (Ranke *et al.*, 1976a,b; Postel-Vinay & Desbuquois, 1977), but other biological preparations have only one of them. Thus in hepatocytes from male rats (Ranke *et al.*, 1976a,b; Postel-Vinay & Desbuquois, 1977) or hypophysectomized rats (Turyn *et al.*, 1976), in liver microsomal fraction ('microsomes') from male rats (Postel-Vinay, 1976) and in plasma membranes from female rats (Etzrodt *et al.*, 1976), only somatogenic receptors are found.

Alternatively, liver microsomes from female rats (Posner *et al.*, 1974; Herington *et al.*, 1976a,b) or from oestrogen-treated male rats (Dang Vu & Bénard, 1978) have almost exclusively lactogenic binding properties.

The role of lactogenic receptors in liver function is far from clear, although it is known that prolactin increases RNA synthesis in the liver as well as the formation of polyribosomes (Chen *et al.*, 1972) and the production of somatomedin (Francis & Hill, 1975). It also stimulates the activity of ornithine decarboxylase (Richards, 1975; Thomson & Richards, 1978) and probably decreases lipogenesis (Agius *et al.*, 1979).

Although many studies have been carried out on the characterization and regulation of these receptors no information exists on the nature and properties of the receptor molecule itself. To achieve this end the first step consists in its solubilization, and preliminary successful attempts have already been reported (Bonifacino *et al.*, 1978; Du Caju *et al.*, 1978).

The present work deals with the solubilization by Triton X-100 of the lactogenic receptors from female rat liver and includes a detailed charac-

terization of their interaction with ^{125}I -labelled human somatotropin. The use of ^{125}I -labelled prolactin was not favoured because in the presence of Triton X-100 it produces large-molecular-weight aggregates hampering the correct measurement of its binding to the solubilized receptors (Shiu & Friesen, 1974).

Materials and methods

Materials

Human somatotropin was prepared by the method of Mills *et al.* (1969) from glands collected in acetone. The method was followed up to the stage of the first precipitate of crude human somatotropin. This material was dissolved in 0.1 M-potassium phosphate buffer, pH 7.0, and filtered through Sephadex G-100. The human somatotropin used for labelling was submitted to a second Sephadex G-100 filtration under the same conditions. Bovine somatotropin was prepared by the method of Dellacha & Sonenberg (1964) and equine somatotropin by the method of Conde *et al.* (1973). The chemical purity and homogeneity of these hormones were established by amino-acid analysis, C-terminal determinations and polyacrylamide-gel electrophoresis. The biological activity was 3.0 i.u./mg, 1.0 i.u./mg and 1.0 i.u./mg for human, bovine and equine somatotropin respectively. Ovine prolactin (NIH-P-S-3; 15 i.u./mg), bovine prolactin (NIH-P-B-1; 13 i.u./mg), human prolactin (NIH-hPrL-VLS-3; 30 i.u./mg) and rat prolactin (NIAMD-rPrL-RP-1; 11 i.u./mg) were obtained from N.I.H., N.I.A.M.D.D. Hormone Distribution program, Bethesda, MD, U.S.A. Human chorionmammotropin was from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. Bovine serum albumin, bovine γ -globulin, trypsin from bovine pancreas, trypsin inhibitor from ovomucoid, neuraminidase from *Clostridium perfringens*, phospholipase C from *Clostridium welchii* and Triton X-100 were from Sigma, St. Louis, MO, U.S.A. Dr. J. C. Vidal generously donated phospholipase A_2 from *Bothrops neuwiedii*. Poly(ethylene glycol) (Carbowax 6000) was obtained from Union Carbide Corp., New York, NY, U.S.A.; Sephadex was from Pharmacia, Uppsala, Sweden, and Na^{125}I was from New England Nuclear, Boston, MA, U.S.A. All other chemicals were of A.R. quality.

Iodination of human somatotropin

^{125}I -labelled human somatotropin was prepared by the method of Greenwood *et al.* (1963) by using limiting amounts of chloramine-T to introduce no more than 1 g-atom of iodine/mol of hormone. Usually, 5 mCi of Na^{125}I was added to 20 μg of human somatotropin dissolved in 60 μl of 0.2 M-potassium phosphate buffer, pH 7.4, followed by

0.72 μg of chloramine-T dissolved in 10 μl of 0.05 M-potassium phosphate buffer, pH 7.4. The reaction was allowed to proceed for 5 min, at 20°C, when 500 μl of the elution buffer was added and the mixture was chromatographed at 4°C on a Sephadex G-100 column (5 mm $\times$ 600 mm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 1% (w/v) bovine serum albumin. Fractions (500 μl) were collected and their radioactivity was measured. The fractions containing the ^{125}I -labelled human somatotropin were pooled, diluted with 25 mM-Tris/HCl buffer, pH 7.5, to a final concentration of 0.18–0.25 ng/ μl and kept at 4°C. The mean specific activity $\pm$ s.d. obtained in 15 iodinations was 105 ± 19 $\mu\text{Ci}/\mu\text{g}$.

Crude liver microsomal preparation

The livers were obtained from female adult (210–250 g) rats of the Long-Evans strain during oestrous.

The animals were killed by cervical dislocation and the livers were quickly removed and homogenized for 1 min at 0°C in 10 vol. (v/w) of 0.3 M-sucrose/0.5 mM- CaCl_2 /5 mM-Tris/HCl buffer, pH 7.5, in a Sorvall Omni-Mixer, at a speed setting of 5.

The homogenate was centrifuged for 20 min at 10000 g (r_{av} 7.4 cm) at 4°C in a Sorvall RC2-B centrifuge with an SS34 rotor. The supernatant was centrifuged for 1 h at 100000 g (r_{av} 5.9 cm) at 4°C on a type 40 rotor of a Spinco model L ultracentrifuge and the pellet was suspended in 25 mM-Tris/HCl buffer, pH 7.5, using a Dounce-type homogenizer. A sample of the suspension was solubilized by heating for 30 min at 100°C in 1 M-NaOH and the protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The concentration of protein in the suspension was 70.4 ± 6.2 mg/ml (mean $\pm$ s.d. for 10 experiments).

The crude microsomal suspension was kept at -60°C until used.

Solubilization of the microsomes

The crude microsomal suspension was 10-fold diluted with 25 mM-Tris/HCl buffer, pH 7.5, plus the addition of Triton X-100 to a final concentration of 1% (w/v). The suspension was stirred during 30 min at 20°C by gentle inversions of the tube and then centrifuged at 150000 g (r_{av} 5.6 cm) during 4 h at 4°C in an MSE PrepSpin 50 ultracentrifuge. A thin layer of lipids present on the surface of the supernatant was carefully removed and the rest of the solution was pooled forming the 'microsomes extract'. The protein was measured by the method of Lowry *et al.* (1951) adapted by Chandrarajan & Klein (1975) to Triton X-100-containing samples. The mean ($\pm$ s.d.) protein concentration was $3.1 \pm$

0.3 mg/ml ($n = 10$). The extract was kept at -60°C until used. Under these conditions its binding activity remained unaltered for at least 4 months and was unaffected by up to eight freeze-thawing cycles.

Measurement of ^{125}I -labelled human somatotropin binding

Unless otherwise specified binding assays were performed by incubating for 15 h at 25°C 1.8–2.2 ng of ^{125}I -labelled human somatotropin (125 000–150 000 c.p.m.) with 15–30 μg of solubilized proteins or 40–90 μg of microsomal proteins in a total volume of 0.5 ml. Plastic tubes were used and the incubation medium consisted of 25 mM-Tris/HCl, pH 7.5, 10 mM- CaCl_2 and 0.08% (w/v) bovine serum albumin. Under these conditions the specific binding was found to be directly proportional to the concentration of proteins in the medium.

In each determination, tubes containing the same mixtures described plus 5 μg of unlabelled human somatotropin were incubated in parallel.

The binding to the microsomes was terminated by diluting with 4 ml of cold incubation medium and centrifuging at 1400 g (r_{av} , 15 cm) for 25 min at 4°C . The supernatant was discarded and the tubes were kept inverted on filter paper for at least 12 h at 4°C , before being counted for radioactivity.

The poly(ethylene glycol) method (Cuatrecasas, 1972) was used to precipitate the hormone-solubilized receptor complex. The procedure was as follows. The incubation was stopped by placing the tubes in crushed ice, adding 0.5 ml of cold 0.1% (w/v) bovine γ -globulin in 25 mM-Tris/HCl buffer, pH 7.5, followed by 1 ml of cold 24% (w/v) poly(ethylene glycol) in 25 mM-Tris/HCl buffer and mixing vigorously. After waiting 10 min to allow for

complete precipitation the tubes were centrifuged and handled as described for the microsomes.

All determinations were carried out in triplicate and the results are expressed as mean values. The standard deviation was always less than 3%. The percentage of specific binding was calculated as $(C_1 - C_2) \times 100 / A$ where C_1 is the amount of radioactivity bound in the absence of unlabelled human somatotropin, C_2 is the amount of radioactivity bound in the presence of 5 μg of unlabelled human somatotropin and A is the total amount of radioactivity initially placed in each tube.

Results

Quantitative studies of the solubilization procedure

Solubilization is minimal up to 0.1% (w/v) Triton X-100. Above this concentration the efficiency of the process increases to reach a maximum value at 0.75% (w/v). Consequently a Triton X-100 concentration of 1% (w/v) was routinely used.

Table 1 shows the specific and total receptor activities found with the same liver microsomal preparation (adult female rat), alone or in the presence of Triton X-100 and in the extracted proteins. The values obtained clearly show that the detergent causes a 366% increase in the total binding activity originally detectable in the microsomes and that 55% of this amount is recovered in solubilized form. The solubilization process increases the specific receptor activity of the preparation 4.6 times.

The binding activity of the insoluble pellet was 30–40% of that originally present in the microsomes. As it was very much lower than the activity found in the solubilized extract it was not subjected to further study.

The presence of 2 mM-EDTA in the extraction

Table 1. *Quantitative evaluation of the solubilization procedure using Triton X-100*

Three 1 ml samples of a suspension of microsomes from the liver of female rats (see the Materials and methods section), were treated as follows. One sample was diluted 1:10 with 25 mM-Tris/HCl buffer (pH 7.5); another, was similarly diluted, but Triton X-100 was added until its concentration was 1% (w/v). The mixture was kept stirred by gentle inversions for 30 min at 20°C . The third sample was treated as the second sample and an extract was obtained by centrifugation as indicated in the Materials and methods section. The protein concentration and the ^{125}I -labelled human somatotropin binding corresponding to each sample was measured. The binding measurement was carried out by incubating ^{125}I -labelled human somatotropin (86 fmol) with 90 μg (sample 1) or 23 μg (samples 2 and 3) of protein, as indicated in the Materials and methods section. Total activity is the amount of ^{125}I -labelled human somatotropin bound to the total mass of proteins in the sample. The values in parentheses are means $\pm$ s.d. obtained from ten independent experiments.

Material	Protein mass (mg)	Total activity (fmol)	Specific activity (fmol/mg)	Protein mass (%)	Total activity (%)	Ratio of specific activity of material/specific activity of microsomes
Microsomes	66.3	17 240	260	100	100	1.0
Microsomes + Triton X-100	66.3	59 466	896	100	345 (366 $\pm$ 106)	3.5 (3.7 $\pm$ 1.1)
Microsomes extract	29.8	41 539	1393	45 (44 $\pm$ 7)	241 (200 $\pm$ 6.5)	5.4 (4.6 $\pm$ 1.5)

medium decreases the recovery of total binding activity to 50% of the original value in the microsomes, confirming our previous finding (Bonifacino *et al.*, 1978). Low recovery values were also obtained when Triton X-100 was replaced by sodium deoxycholate. No activity was detectable in

Fig. 1. Inhibitory effects of Triton X-100 on specific binding measurements

The incubation mixture (see the Materials and methods section) contained ^{125}I -labelled human somatotropin (150 000 c.p.m.), 10 μl of the microsomal extract with 30 μg of proteins and various amounts of Triton X-100, which were added after (O) or before (●) the incubation at 25°C. The poly(ethylene glycol) precipitation method was applied afterwards.

the extracts made with Tween 80 and Brij 35, but Nonidet P40 was as effective as Triton X-100.

Liver microsomes from male rats do not bind ^{125}I -labelled human somatotropin (Posner *et al.*, 1974) and no activity appeared when they were treated with Triton X-100.

Replacement of ^{125}I -labelled human somatotropin by ^{125}I -labelled bovine somatotropin caused a decrease to zero of the binding activity detectable in the extracts of liver microsomes from female rats.

Inhibitory effects of Triton X-100 on the specific binding

Two kinds of inhibitory effects were detected by adding various amounts of Triton X-100 to the assay medium, after and before incubation, and both were found to be concentration-dependent (Fig. 1). The decrease in specific binding occurring when the detergent is added after the incubation reaches a stable value of 19% and can be attributed to interference with precipitation of the hormone-receptor complex by poly(ethylene glycol).

The most significant inhibitory effect was obtained when the detergent was added before the incubation period, indicating that it also interferes with the formation of the hormone-receptor complex. This inhibition reaches a 60% value at 1.5% (w/v) Triton X-100 concentration. Accordingly, all measurements referring to the characterization of the hormone-receptor interaction were done in the presence of low concentrations of Triton X-100 (0.01–0.02%, w/v).

Kinetics of association

The specific binding of ^{125}I -labelled human somatotropin to the solubilized receptors is time-

Fig. 2. Effect of time and temperature on the binding of ^{125}I -labelled human somatotropin by solubilized receptors (a) ^{125}I -labelled human somatotropin (82 fmol) was incubated for various times with 24 ng of solubilized proteins at the indicated temperatures. The specific binding was measured at 36°C (O), 25°C (●), 14°C (□) and 5°C (■). The binding capacity of the solubilized preparation, measured by Scatchard analysis of saturation curves at 25°C, was 2380 fmol/mg. (b) Analysis of the kinetics of association. Data from (a) at 25°C were plotted as $\ln(H_0 - C/R_0 - C)$ at different times, where H_0 and R_0 represent the concentrations of ^{125}I -labelled human somatotropin and of solubilized receptors respectively at time zero, and C the concentration of hormone-receptor complex at each time.

and temperature-dependent, as shown in Fig. 2(a). At 25°C maximal binding is obtained within 6 h and this value remains constant for at least 24 h. At 14°C equilibrium is reached in 15 h, whereas at 5°C longer incubation times are needed. At 36°C the association, which initially progresses faster than at 25°C, rapidly stops and then the binding decreases, probably due to inactivation or degradation of the receptor.

In Fig. 2(b) the association values obtained at 25°C are represented as the logarithm of the relationship of free hormone to free receptor, at different times. The curve obtained has an initial linear portion, suggesting that the association obeys second-order kinetics. The mean value of the association rate constant (k_1) at 25°C, obtained from two similar experiments, was 6.7×10^6 mol · litre⁻¹ · min⁻¹.

Kinetics of dissociation

Once formed, the hormone–receptor complex is dissociable in the presence of unlabelled hormone (2500-fold molar excess). Dissociation is slower than association and also dependent on the temperature (Fig. 3a). Total reversion of binding is obtained in 55 h at 25°C or at 36°C. In this time dissociation is only 57% at 14°C or 37% at 5°C. The occurrence of an inactivating process leading to dissociation of the labelled hormone due to the long times employed and to the seeming instability of the receptor at 36°C cannot be discarded.

In Fig. 3(b) we have represented dissociation data at 25°C, expressed as the variation of the logarithm of the concentration of hormone–receptor complex with time. The linear relationship indicates first-order

kinetics. The mean value of the dissociation rate constant (k_{-1}) obtained from two similar experiments was 1.1×10^{-3} min⁻¹.

The dissociation equilibrium constant (K_d) calculated as k_{-1}/k_1 has the value 0.16 nM.

Effect of hormone concentration on the specific binding of ¹²⁵I-labelled human somatotropin

Fig. 4(a) shows that the specific binding of ¹²⁵I-labelled human somatotropin, either to the microsomes or to the extract, is a saturable process. It is noteworthy that the amount of hormone bound, at each value of labelled hormone added, is always higher for the extract, in spite of its lower concentration of proteins, in comparison with the microsomal suspension.

The corresponding Scatchard (1949) plots were linear for both preparations (Fig. 4b).

The receptor in the microsomes has a $K_d = 0.13 \pm 0.05$ nM and a binding capacity of 440 ± 190 fmol/mg of proteins (mean $\pm$ s.d., $n = 5$). The corresponding values for the solubilized receptor were: $K_d = 0.22 \pm 0.08$ nM; binding capacity 2900 ± 580 fmol/mg of proteins (mean $\pm$ s.d.; $n = 7$). Statistical analysis (Student's t test) showed that the homologous parameters for both preparations were significantly different, indicating a slight decrease in affinity for the solubilized receptors coupled to a 6.7-fold increase in binding capacity.

Specificity of the interaction of human somatotropin-solubilized receptors

Fig. 5 shows the inhibition curves obtained when the specific binding of ¹²⁵I-labelled human somato-

Fig. 3. Effect of time and temperature on dissociation of ¹²⁵I-labelled human somatotropin from the hormone–receptor complex

(a) ¹²⁵I-labelled human somatotropin (150 000 c.p.m.) was incubated with 24 μ g of solubilized proteins during 15 h at 25°C when 5 μ g of unlabelled human somatotropin was added to each tube and the incubation resumed for a variety of time intervals at different temperatures. Symbols: ○, 36°C; ●, 25°C; □, 14°C; ■, 5°C. (b) Analysis of the kinetics of dissociation. Data from (a) at 25°C were plotted as ln of the picomolar concentration of the hormone–receptor complex (C) at different times.

Fig. 4. Specific binding of ${}^{125}\text{I}$ -labelled human somatotropin as a function of labelled hormone concentration (a) A constant amount of proteins in the form of microsomes ($40\mu\text{g}$; ●) or solubilized proteins ($16\mu\text{g}$; ○) were incubated with increasing amounts of ${}^{125}\text{I}$ -labelled human somatotropin for 15 h at 25°C . The specific binding was measured as indicated in the Materials and methods section. (b) Scatchard analysis of the binding data from the experiment shown in (a). Symbols: ●, microsomes; ○, extract.

Fig. 5. Competition curves for the specific binding of ${}^{125}\text{I}$ -labelled human somatotropin to the solubilized receptors

${}^{125}\text{I}$ -labelled human somatotropin (150000 c.p.m.) was incubated with $15\mu\text{g}$ of solubilized proteins in the presence of increasing amounts of bovine somatotropin (Δ), equine somatotropin (∇), human prolactin ($\blacklozenge$), rat prolactin ($\blacktriangle$), bovine prolactin ($\diamond$), human choriomammotropin ($\square$), ovine prolactin ($\blacksquare$) and human somatotropin ($\circ$). A parallel incubation was also done with ${}^{125}\text{I}$ -labelled human somatotropin, liver microsomes ($66\mu\text{g}$ of protein) and increasing amounts of human somatotropin ($\bullet$). The procedure was as described in the Materials and methods section. Results are expressed as the ratio of the specific binding at each concentration of unlabelled hormone (B) to the specific binding in the absence of additions (B_0).

tropin to the receptors is challenged by different hormones.

The inhibition by human somatotropin is quite similar for both solubilized and microsomal receptors, although the curve for the microsomes is slightly displaced to the left. Various lactogenic hormones, like human, rat, bovine and ovine prolactins, as well as human choriomammotropin, inhibit the binding of ${}^{125}\text{I}$ -labelled human somatotropin to the solubilized receptors. All these curves are markedly displaced to the right when compared with that given by human somatotropin. Though it is possible that the presence of Triton X-100 may affect the activity of these hormones as competitors, their diminished potency cannot be totally attributed to this phenomenon since they are also less effective than human somatotropin in competing for the binding to the membrane receptors (J. S. Bonifacino, S. H. Sánchez & A. C. Paladini, unpublished work).

Non-lactogenic somatotropins, like those of bovine and equine origins and other unrelated hormones (follitropin, lutropin, thyrotropin and insulin), do not inhibit the specific binding.

Analysis of the competition data

Dissociation equilibrium constants and binding-capacity values were obtained from Scatchard analysis of competition or saturation curves for the solubilized or microsomal receptors (Table 2). The two methods gave similar results for each receptor preparation, but the comparison of the values for each parameter corresponding to solubilized or microsomal receptors disclosed significant differences.

The dissociation equilibrium constant calculated

Table 2. K_D and binding-capacity values for the microsomes or the solubilized receptors obtained by different methods. These parameters of the interaction of ^{125}I -labelled human somatotropin and the receptors were calculated: (a) by the ratio of rate constants (Figs. 2 and 3); (b) from saturation experiments (Fig. 4); (c) from competition experiments using human somatotropin (Fig. 5); (d) from competition experiments using ^{127}I -modified human somatotropin. Means $\pm$ s.d. are given for the numbers of independent experiments shown in parentheses. The statistical significance (Student's t test) of the differences in the homologous parameters for the microsomes and the extract, are indicated as follows: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

Method	Microsomes		Extract	
	$10^{10} \times$ Dissociation equilibrium constant (M)	Binding capacity (fmol/mg)	$10^{10} \times$ Dissociation equilibrium constant (M)	Binding capacity (fmol/mg)
k_{-1}/k_1	—	—	1.6	—
Saturation	1.3 ± 0.5 (5)*	440 ± 190 (5)***	2.2 ± 0.8 (7)*	2900 ± 580 (7)***
Competition with human somatotropin	2.2 ± 0.6 (6)**	450 ± 260 (6)***	3.6 ± 0.8 (4)**	3850 ± 870 (4)***
Competition with ^{127}I -modified human somatotropin	2.3 ± 0.0 (3)	790 ± 70 (3)	2.7 ± 1.3 (3)	3440 ± 1990 (3)

from kinetic data obtained with the extract was similar to the other values. Also, ^{127}I -labelled human somatotropin, prepared under identical conditions with those used for ^{125}I -labelling, was a competitor to ^{125}I -labelled human somatotropin, indistinguishable from human somatotropin as judged by the similar K_d values obtained with both hormones (Table 2). These results validate the use of ^{125}I -labelled human somatotropin as a ligand with the same affinity for the receptor as unlabelled human somatotropin.

Receptor and ^{125}I -labelled human somatotropin degradation during the incubation

To eliminate these complicating factors in our working conditions, control experiments were done.

The microsomal extract was incubated for 15 h at 25°C before measurement of the specific binding, as described in the Materials and methods section. No differences were found in comparison with a sample of extracts that was not pre-incubated.

The possible degradation of ^{125}I -labelled human somatotropin on incubation for 15 h at 25°C with the microsomal extract was investigated by analysing the integrity of the remaining free labelled hormone by three methods: precipitation with 10% (w/v) trichloroacetic acid, chromatography on Sephadex G-100 and ability to rebind to fresh microsomal extract. No indications of degradation were found by the first two methods. The rebinding experiment was done as follows. After the incubation the unbound labelled hormone was obtained by chromatography on Sephadex G-100 as indicated by Bonifacino *et al.* (1978), and its ability to interact with fresh receptors was measured in comparison with that of a non-treated ^{125}I -labelled human somatotropin sample. The results obtained, expressed as a percentage of specific binding, were 12.5 ± 0.7 and 12.3 ± 2.2 for non-treated and unbound ^{125}I -labelled human somatotropin samples respectively. The conclusion

Table 3. Effect of different enzymic digestions on binding of ^{125}I -labelled human somatotropin to the solubilized receptors

The solubilized receptors (0.3 mg of protein) were incubated with the enzymes for 1 h at 25°C in 25 mM-Tris/HCl buffer. The neuraminidase treatment was done at pH 6 in the presence of 0.025% (w/v) bovine serum albumin. All other incubations were at pH 7.5. With phospholipases C and A_2 the medium contained 2.5 mM- CaCl_2 . The enzymes were not removed at the end of the treatment. To stop trypsin action the inhibitor from ovomucoid was used at a concentration of $50 \mu\text{g}/\text{ml}$. In each case a control without enzyme was carried out in parallel. The specific binding was measured as indicated in the Materials and methods section. Results are means $\pm$ s.d. for three independent experiments.

Enzyme	Enzyme/solubilized proteins ratio (w/v)	Specific binding of ^{125}I -labelled human somatotropin (% of control)
None (control)	—	100 ± 2
Trypsin	1:300	20 ± 1
Neuraminidase	1:60	88 ± 6
Phospholipase C	1:60	97 ± 18
Phospholipase A_2	1:250	121 ± 0

was drawn that on incubation with the soluble receptors the labelled hormone is neither degraded nor inactivated.

Effect of different enzymic treatments on the binding capacity of the solubilized receptors

To explore the chemical nature of the receptors the extract was treated with different enzymes (Table 3). Trypsin, at an enzyme/substrate ratio of 1:300 (w/v), caused an 80% decrease in the specific

Fig. 6. Effect of different treatments on particulate and solubilized receptors

(a) Microsomal suspension (●) or extract (○) (both 0.3 mg of protein) was treated with increasing concentrations of trypsin. Conditions and measurement were as indicated in the legend to Table 3. (b) Microsomal suspension (●) or extract (○) (both 0.3 mg of protein in 25 mM-Tris/HCl buffer, pH 7.5) was kept for 15 min at the indicated temperatures. Specific binding was measured as described in the text and results are expressed as percentages of that of homologous preparations kept at 0°C (control). (c) To the microsomal suspension (●) or extract (○) (both 0.3 mg of protein), 1 M-HCl or 1 M-NaOH was added to adjust the pH to the values indicated. The samples were incubated for 15 min at 25°C and then the pH was adjusted immediately to 7.5 with the addition of 5 mM-Tris/HCl buffer, pH 7.5. Specific binding was measured as described in the text and results are expressed as percentages of that of homologous preparations kept at pH 7.5 (control).

binding, which suggests the importance of a protein component in the receptor molecule.

Treatment with neuraminidase slightly decreased the specific binding, whereas phospholipase A₂ caused an increase and phospholipase C had no effect.

Comparative study of the sensitivity of the receptors in the microsomes or in solubilized form to trypsin, heat and pH

As shown in Fig. 6(a), at trypsin concentrations higher than 2.5 mg/ml, the solubilized receptors are more sensitive to hydrolysis than those in the particulate form. At 10 mg/ml the activity practically disappears from the extract when 20% is still present in the microsomes. Heat treatment (Fig. 6b) also shows the higher resistance of the particulate receptors: 50% are still active after 15 min at 43°C. The solubilized receptors reach this extent of inactivation at 37°C.

The curves in Fig. 6(c) indicate that, although very similar, the sensitivity of the solubilized receptors to extreme pH variations is slightly higher than that of the particulate form.

These results suggest a greater exposure to enzymic or physicochemical attack of regions in the soluble receptors important for their hormone-binding function.

Discussion

Until now it has not been possible to isolate any receptor for polypeptide or protein hormones in

sufficient quality and quantity for complete chemical characterization. The principal difficulties encountered derive from the membrane localization of these receptors, of which they are integral constituents, and from the small amounts in which they are usually present.

In the present work we have been able to surmount the first of these stumbling blocks for the lactogenic receptors present in the liver of female rats. Triton X-100 treatment of liver microsomes has succeeded in solubilizing the receptors with retention of their specific binding properties. Furthermore, a substantial increase in receptor activity was found in the extracts.

It was found that concentrations of detergent higher than 0.02% (w/v) interfere both with poly(ethylene glycol) precipitation of the hormone-receptor complex and with the specific binding. This effect, though, is less marked than that reported for other receptors, which completely lose the ability to bind their specific ligands (Arostam *et al.*, 1978; Aguilar *et al.*, 1979).

The reaction:

has been studied in detail, since it is fundamental to a recognition and eventual purification of the receptors.

The kinetics of the association process show that the reaction is second order with a rate constant of

similar magnitude to that of other hormone-solubilized receptor interactions (Cuatrecasas, 1972; Klett *et al.*, 1973; Dufau *et al.*, 1973). Dissociation of the complex is complete within 55 h at 25°C. This is not so when the receptor is on the membrane (Herington *et al.*, 1976b). Analysis of the dissociation kinetics indicated that this process is first order; the rate constant has a value close to that obtained for other solubilized receptors (Cuatrecasas, 1972; Klett *et al.*, 1973; Dufau *et al.*, 1973).

These observations strongly suggest that the interaction of ^{125}I -labelled human somatotropin with the solubilized receptors takes place according to a simple mechanism. Hence, the concentrations of all reactants at equilibrium must comply with the requirements of a bimolecular reaction. This prediction is confirmed by the linear nature of the Scatchard plots resulting whether the receptor is in the microsomes or in solubilized form and using saturation data or competition data obtained with either native human somatotropin or ^{127}I -modified human somatotropin as competitors.

We consider that this evidence indicates the presence of only one group of binding sites in the membrane and in the solubilized preparation. This is relevant in the clarification of a controversial point in the study of liver lactogenic receptors. Our results confirm the observations of Posner *et al.* (1974), Posner (1976), Herington *et al.* (1976a) and Baxter & Turtle (1978), using ^{125}I -labelled human somatotropin, and of Sherman *et al.* (1977) and Silverstein & Richards (1979), using ^{125}I -labelled prolactin. All these authors found one group of lactogenic receptors in liver membranes from female rats.

Our results are at variance with those of Herington *et al.* (1976b), who used liver microsomes from female rats, and those of Dang Vu & Bénard (1978), who used liver microsomes from oestrogen-treated male rats. These two groups of workers obtain curvilinear Scatchard plots, suggesting the existence of more than one group of binding sites.

The solubilized receptors, like those in the microsomes, are strictly specific for lactogenic hormones. No evidence of somatogenic receptors have been found in the extracts.

The fundamental parameters of the binding, dissociation equilibrium constant and binding capacity, were calculated by various methods, both for the solubilized receptor and the microsomal one, with comparable results. It was also clear that solubilization of the receptors causes a slight diminution of their affinity for ^{125}I -labelled human somatotropin.

Similar results have been found for somatotropin receptors in rabbit liver (McIntosh *et al.*, 1976) and for the lutropin receptors in rat testis (Dufau *et al.*, 1973). In both cases the affinity decreases after solubilization. The reverse effect is also known, i.e.

prolactin receptors in rabbit mammary gland (Shiu & Friesen, 1974) and in rabbit liver (Waters & Friesen, 1979) increase by four to five times their affinity on solubilization. These observations suggest that the physical milieu in which the receptors are immersed is important for the modulation of their affinity for the specific ligand.

Another interesting feature of the solubilization is the great increase in total binding activity that accompanies the procedure. Since an increase in affinity can be discarded, the phenomenon is attributed to the exposure of new receptors by detergent action. This conclusion is supported by similar observations made when liver microsomes (Du Caju *et al.*, 1978; Sánchez *et al.*, 1979) or isolated hepatocytes (Postel-Vinay & Desbuquois, 1977) are treated with phospholipase A_2 .

To explain this phenomenon two different models can be suggested. The first one hypothesizes that the receptors, located in the lipid bilayer of the membrane, are inhibited or masked by the phospholipids, as was shown by Cuatrecasas (1971) for the insulin receptor in liver. The second model predicts the presence of membrane vesicles bearing the receptor on the internal face, where they cannot be reached by the labelled hormone. This last possibility seems to be more probable, since it is known that the majority of the lactogenic receptors present in 100000 g pellet from a liver homogenate are located in fragments of the Golgi apparatus (Bergeron *et al.*, 1978), which is a highly vesiculated membranous system (Morré, 1977). Furthermore, the hormone binding increases by disruption of these vesicles through successive freeze-thawing cycles (Bergeron *et al.*, 1978).

The liver microsomal extract obtained with Triton X-100 has a binding capacity approx. 7 times greater than that of the starting microsomes with a total capacity of about 3000 fmol of ^{125}I -labelled human somatotropin/mg of protein. Hence, it is the richest source of receptors for polypeptide and protein hormones described so far. Other solubilized preparations, like those for lutropin/human choriogonadotropin (Dufau *et al.*, 1973), follitropin (Abou-Issa & Reichert, 1977), insulin (Cuatrecasas, 1972), somatotropin (McIntosh *et al.*, 1976; Herington & Veith, 1977; Waters & Friesen, 1979) and prolactin (Shiu & Friesen, 1974), have ligand-binding capacities not higher than 700 fmol/mg of protein. The only exception, to the best of our knowledge, is a preparation for angiotensin from rat aorta (Devynck *et al.*, 1974), which has a capacity of 2000 fmol/mg.

In summary, the solubilized preparation in the present paper is obtained from a rich source of microsomes like the rat liver and has a relatively high binding capacity, high affinity and great stability. These properties make it a material of

choice for the attempted purification of the lactogenic receptor.

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