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Presence of Specific Growth Hormone Binding Sites in Rainbow Trout (*Oncorhynchus mykiss*) Tissues: Characterization of the Hepatic Receptor¹

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The present work outlines the presence of specific binding for chinook salmon growth hormone (sGH) in different tissue preparations of rainbow trout. Optimal incubation conditions (pH, Tris, MgCl₂) were determined. Specific binding was very sensitive to salt concentration during incubation. The specific binding reached a plateau after 15 and 25 hr of incubation at 12 and 4°. At 20°, specific and nonspecific binding were not stable. Specific binding dissociation was slower than association and was only partial. The binding was saturable ($B_{\max} = 187 \pm 167$ pmol), of high affinity ($K_d = 2.4 \pm 0.8 \cdot 10^9 M^{-1}$), and very specific for GH, properties which are in agreement with the characteristics of hormonal receptors. Sea bream and mammalian GH appeared 2- and 30-fold, respectively, less potent than cold sGH₂ for displacing ¹²⁵I-sGH₂. Tissue preparations from ovary, testis, fat, skin, cartilage, gill, blood pellet, brain, spleen, kidney, and muscle showed significant saturable binding. © 1991 Academic Press, Inc.

In fish, interest in pituitary hormone receptors is increasing. In salmonids, using a homologous system, GtH receptors have been characterized in ovary (Salmon *et al.*, 1984; Breton *et al.*, 1986; Kanamori *et al.*, 1987; Kanamori and Nagahama, 1988; Breton and Sambroni, 1989) and in testis (Le Gac *et al.*, 1988). Only one study on prolactin (PRL) receptors was done with homologous hormone in tilapia kidney (Fryer, 1979a). Its presence in testis, ovary, intestine, gills, liver, and kidney was detected using ovine PRL (Edery *et al.*, 1984; Dauder *et al.*, 1990a,b).

Using bovine growth hormone (bGH), Tarpey and Nicoll (1985) demonstrated the presence of a specific receptor in the liver of mudsucker (*Gillichthys mirabilis*) and surgeon (*Acipenser transmontanus*). Char-

acterization of tilapia (*Sarotherodon mossambicus*) liver GH receptor was done by Fryer (1979b) using homologous hormone and specific binding was noted in gill and kidney microsomal membrane fraction. Using this last hormone, specific binding was also found in the liver of mudsucker, rainbow trout (*Oncorhynchus mykiss*), and chinook salmon (*O. tshawytscha*) (Fryer, 1979b), and one study indicated that stunted coho salmon (*O. kisutch*) have a decreased ability to bind GH by liver (Fryer and Bern, 1979). More recently, using chinook salmon GH, we demonstrated the presence of saturable binding in rainbow trout liver (Le Bail *et al.*, 1989; Niu *et al.*, submitted for publication).

The aim of this study was to characterize, *in vitro*, the specific binding of salmonid growth hormone (sGH) to rainbow trout liver. This paper reports on the incubation conditions, association and dissociation kinetics, hormonal specificity, determination of affinity constant and number of liver

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binding sites, and the presence of saturable binding in putative target tissues.

MATERIALS AND METHODS

Experimental Animals

One-year-old rainbow trout (*O. mykiss*) reared at Cornec (Finistère) fish farm were used. They were acclimated for more than 2 weeks in a recycling water unit, at 15°, under a natural photoperiod and were fed *ad libitum*.

Hormones

The purification of chinook salmon (*O. tshawytscha*) GH (sGH₁ and sGH₂) has been described previously (Le Bail *et al.*, 1989). Sea bream (*Sparus aurata*) GH (dGH) (Pagelson, Zohar, and Le Bail, unpublished data) and chinook GtH (sGtH) (Breton *et al.*, 1978), and PRL (sPRL) (Prunet and Houdebine, 1984) were purified in our laboratory. Ovine GH (oGH) and FSH (oFSH) and bovine GH (bGH) and TSH (bTSH) were obtained from the National Institute of Arthritis and Metabolic Diseases, National Institute of Health (Bethesda, MD). Recombinant human (rhGH) and bovine (rbGH) GH were a gift from Sanofi and Monsanto, respectively.

Preparation of ¹²⁵I-sGH₂

Five micrograms of chinook salmon GH₂ was iodinated by the chloramine-T method (Greenwood *et al.*, 1963) with the modification introduced by Martal (1972). Separation of radiolabeled sGH₂ from free ¹²⁵I was carried out by chromatography on a PD10 column (Pharmacia). The specific radioactivity (SA = radioactivity content/protein content) varied between 60 and 90 μCi/μg. The specific radioactivity was corrected with time, taking into account deiodination of the hormone and decrease of iodine radioactivity.

Preparation of Receptor Material

The entire procedure was carried out at 4° using chilled buffers. Tissues were minced and then homogenized in 20 mM Tris, 5 mM MgCl₂ (pH 7.5) containing 1 mM PMSF (5 ml/g initial tissue weight). A Polytron tissue grinder, with two successive 15-sec bursts at high speed, was used. The homogenate was passed through cheesecloth and centrifuged (3000g × 30 min). The pellet was resuspended in the same buffer and centrifuged again. The resulting pellet was resuspended in the assay buffer (20 mM Tris, 5 mM MgCl₂, 0.25 mg/ml soya bean trypsin inhibitor, 0.5 mM ascorbic acid, 1% BSA, 0.1% sodium azide, pH 7.5) generally at 2 g wet initial tissue weight/ml (×2). This suspension was used directly in the binding assay.

Nose cartilage was powdered in liquid nitrogen us-

ing a mortar, and then incubation was done as described above.

Binding Assay Procedure

Assays were performed in 5-ml polystyrene tubes (LES C¹⁶) containing 100 μl of ¹²⁵I-sGH₂ (generally 10,000 cpm), 100 μl of assay buffer containing, if required, unlabeled hormone as described under Results. After vortexing, the tubes were incubated (generally at 12° for 20 hr). At the end of incubation, 3 ml of chilled assay buffer was added and the tubes were centrifuged at 3000g for 10 min. The supernatant was discarded and the pellet-associated radioactivity was counted in a Packard (Multi-Prias 2) gamma counter (75% efficiency).

The nonspecific binding (NSB) was determined in the presence of an excess of unlabeled sGH (1000 ng/tube). The specifically bound (SB) radioactivity was calculated by subtracting the nonspecifically bound (mean of duplicate determination) from the total bound (TB) radioactivity (mean of duplicate determination).

Calculation

Affinity constants (K_d) and binding capacities (B_{max}) were estimated from the Scatchard (1949) transformation of saturation curves for specific binding of ¹²⁵I-sGH₂ to trout liver preparation. The maximum binding experiment showed that only a fraction of the hormone had the capacity to bind to the receptor which varied from 30 to 50%, according to the labeling and the age of the iodinated hormone. Therefore, the amount of hormone added to each tube was corrected by this factor before doing the Scatchard transformation.

RESULTS

¹²⁵I-sGH₂ Quality

When the labeling reaction medium was chromatographed in an ACA 54 (IBF) column, ¹²⁵I-sGH₂ binding activity was mainly observed in the first peak, in which the *B/T* ratio measured appeared relatively constant (Fig. 1). Therefore, the following separation of ¹²⁵I-sGH₂ from free ¹²⁵I was performed in a column of lesser resolution but of more convenience (PD10).

Incubation Conditions

Figure 2 shows the effect of incubation buffer pH on binding of labeled sGH to

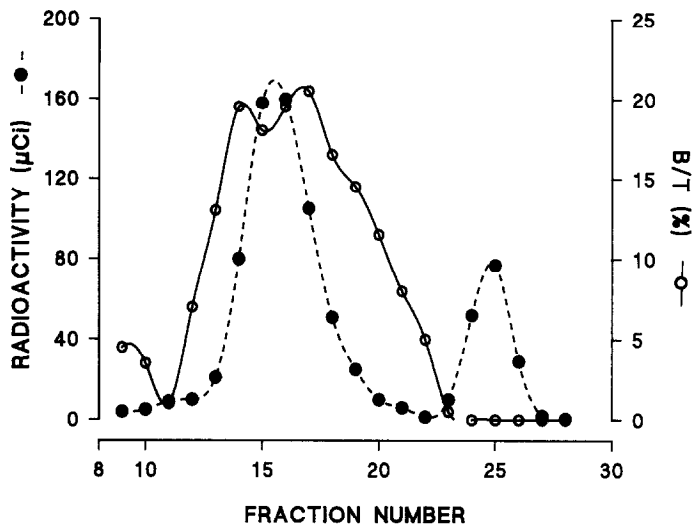


FIG. 1. Specific binding activity of ^{125}I -sGH₂ to liver preparation. After iodination, labeled sGH₂ was chromatographed on an ACA 54 (22 × 0.6 cm) column, and 20,000 cpm/100 µl was incubated (20 hr, 4°) with 100 µl of liver preparation (0.5 g/ml wet tissue) and 100 µl of buffer, with cold sGH (1 µg) added or omitted. Specific binding was expressed as a percentage of total radioactivity added (B/T).

trout liver preparations. Maximum specific binding occurred between pH 6.5 and pH 8. The nonspecific binding plot started at high values at low pH and then decreased dramatically up until pH 7 and more slowly thereafter. The best ratio between specific and nonspecific binding was obtained at pH 7.5.

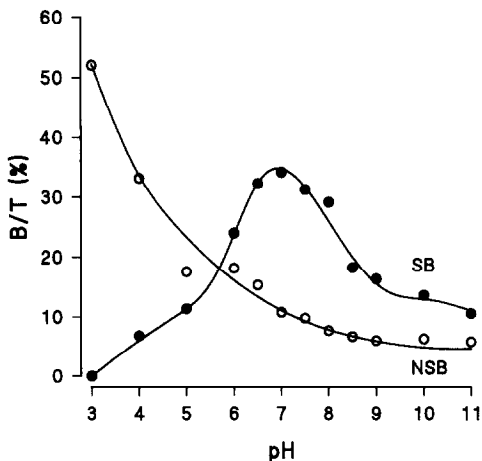


FIG. 2. Effect of pH on the specific (SB) and non-specific (NSB) binding of ^{125}I -sGH to liver preparation (100 µl of 1 g/ml wet tissue). The incubation conditions are described under Materials and Methods.

During incubation MgCl_2 concentrations greater than 10 mM decreased the specific binding (Fig. 3). A similar effect was obtained with Tris concentrations greater than 20 mM. Nonspecific binding was not sensitive to salt concentration at pH 7.5. The most favorable conditions appeared to be 20 mM Tris (for a high buffering capacity) and 5 mM MgCl_2 and thus were used for the following studies.

Association and Dissociation Studies

The effect of incubation duration on the receptor hormone complex formation was studied at 20°, 12°, and 4°. Under these conditions, apparent equilibrium of the specific binding was reached after 5, 15, and 25 hr of incubation, respectively (Fig. 4), after which specific binding was stable up until 60 hr of incubation at 12 and 4°. At 20°, specific binding decreased after 20 hr. Nonspecific binding increased quickly during the first 5 hr of incubation, then appeared relatively stable at 4°, increased slowly at 12°, and increased dramatically at 20°.

Dissociation of sGH from its binding

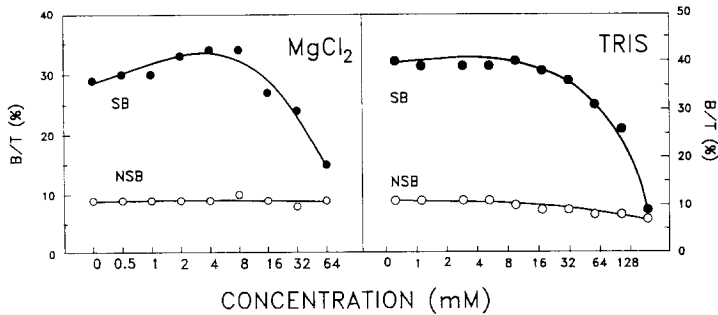


FIG. 3. Effect of MgCl_2 and Tris concentration on the specific (SB) and nonspecific (NSB) binding of ^{125}I -sGH₂ to liver preparation (100 μl of 1 g/ml wet tissue). MgCl_2 and Tris concentrations were expressed as final concentrations in millimoles.

sites was investigated after preincubation (12°, 20 hr) of the tissue preparation with labeled sGH. Because control specific binding showed some minor variation during this long incubation period (Fig. 5A), results from the dissociation kinetics were expressed as percentages of the control values for each point of the study (Fig. 5B). Under these conditions, dissociation appeared relatively rapid during the first 48 hr (about 45%), after which specific binding was nearly stable for at least 13 days of incubation.

Saturation and Competition Experiments

Figure 6 shows the percentage of labeled sGH binding to increasing amounts of liver preparation. Specific binding was essentially linear with up to 1 g wet liver/ml (final concentration of incubate) when it reached a plateau, demonstrating that up to 40% of the ^{125}I -sGH₂ may be bound to the receptors.

When a constant amount of liver preparation was incubated with an increasing concentration of ^{125}I -sGH₂, nonspecific binding behaved linearly and was not saturable, while specific binding was saturable (Fig. 7). The Scatchard derived curve revealed a single class of binding sites with a binding capacity of 187 ± 167 pmol ($n = 4$; 1.6 pmol/g tissue) and a high binding affinity ($K_a = 2.4 \pm 0.8 \cdot 10^9 \text{ M}^{-1}$, $N = 4$).

When incubation was carried out with a

fixed concentration of labeled hormone, specific binding could be competitively inhibited by increasing the concentration of unlabeled sGH₂ (Fig. 8). The concentration of cold sGH₂ required to obtain 50% displacement of ^{125}I -sGH₂ was about 40 ng/ml.

Gonadotropins, thyrotropins, and prolactin from mammals or chinook salmon had no significant effect on binding (Fig. 8). Natural or recombinant mammalian GH inhibited ^{125}I -sGH₂ binding, but 50% inhibition required a hormone concentration about 30-fold higher than that required when using sGH₂. Chinook salmon GH₁ and sea bream GH appeared to be 2-fold less effective than cold sGH₂.

^{125}I -sGH Binding to Different Tissue Preparations

The liver preparation bound ^{125}I -sGH₂ to a much greater extent than the other tissue preparations, especially if we took into account the tissue concentrations of each tissue (Fig. 9). However, preparations from ovary, testis, fat, skin, cartilage, gill, blood pellet, brain, spleen, kidney, and muscle revealed significant specific binding. Only intestine and stomach preparations were ineffective, but showed high nonspecific binding.

DISCUSSION

In the present study, we used a chinook

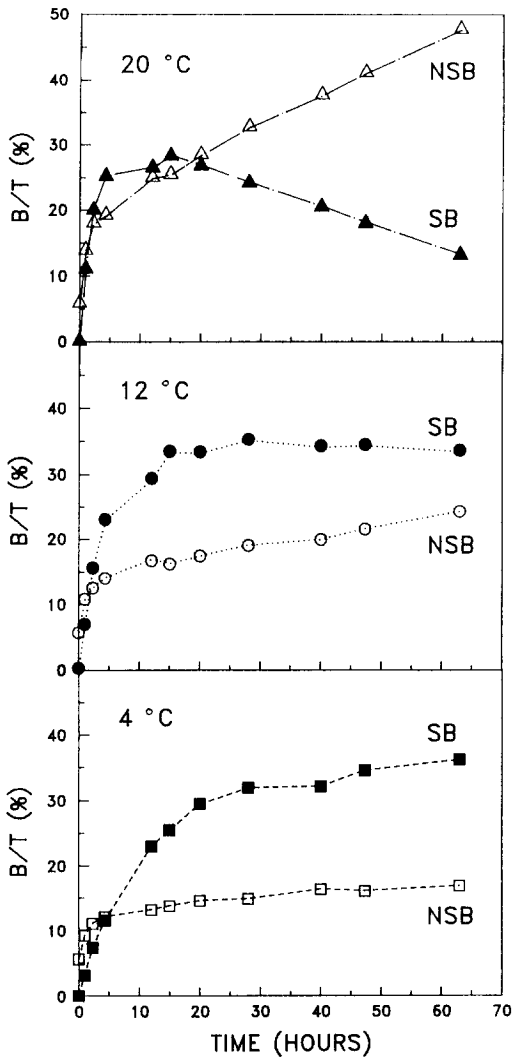


FIG. 4. Effect of time and temperature on specific (SB) and nonspecific (NSB) binding of ^{125}I -sGH₂ to liver preparation (100 μl of 2 g/ml wet tissue).

salmon GH to characterize GH receptors in rainbow trout liver. There is only one amino acid difference between the GHs of these two species (Hew *et al.*, 1989; Agellon and Chen, 1986), whereas the two forms of rainbow trout GHs differ with respect to 11 amino acids (Rentier-Delrue *et al.*, 1989). Taking these conditions into account, we consider that our study was done in a homologous system. Labeled sGH₂ shows a high and homogeneous ability to bind.

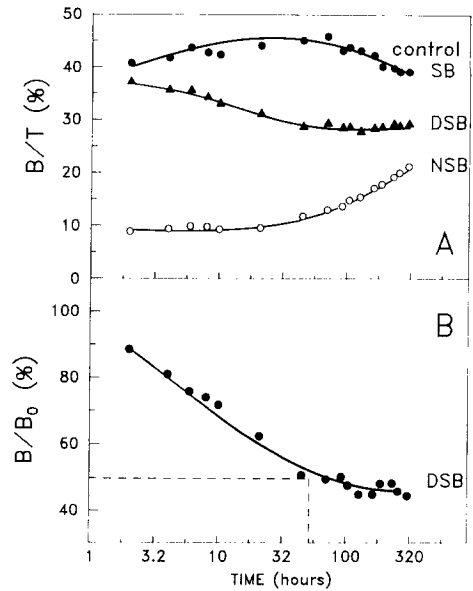


FIG. 5. Dissociation kinetics of ^{125}I -sGH₂ from hepatic preparation at 12°. After 20 hr of liver preparation (100 μl of 1 g/ml wet tissue) preincubation with ^{125}I -sGH₂ (20,000 cpm/100 μl) and 50 μl of buffer with (NSB) or without (SB) cold sGH (10 μg), cold sGH (10 μg /50 μl) (DSB = dissociated specific binding) or buffer (50 μl) (control SB and NSB) was added. Results were expressed as percentages of ^{125}I -sGH₂ added (A) or as percentages of control specific binding (B).

Before characterizing the receptors, we looked for the optimal incubation conditions. A crude liver preparation was used because the liver membrane preparation obtained by serial centrifugation in sucrose solution (Tsushima and Friesen, 1973) lost 30% of its binding capacity, nonspecific binding staying unchanged. Optimal incubation pHs obtained in this study corresponded to those found by Tarpey and Nicoll (1985) in mudsucker and sturgeon using bovine GH and that found by Breton *et al.* (1986) in rainbow trout using chinook GtH. An important sensitivity of the specific binding to salt concentration (Tris or MgCl_2) during incubation was observed. The incubation conditions (5 mM MgCl_2 , 20 mM Tris) we retained are different from the physiological situations, in which salt concentration is more than 10-fold higher. Un-

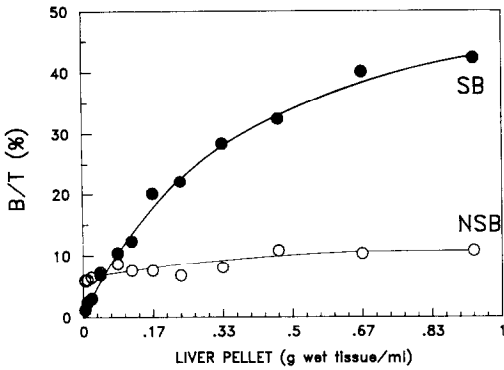


FIG. 6. Effect of the amount of liver preparation on specific (SB) and nonspecific (NSB) binding of ^{125}I -sGH₂. The incubation conditions are described under Materials and Methods.

der these conditions, the apparent K_a and the number of sites found in our study, as well as in other work done on GH receptors, might not reflect the *in vivo* situation.

The binding of ^{125}I -sGH to liver membranes was time and temperature dependent. The decline in specific binding observed after 15 hr at 20° could be due to a

degradation of labeled hormone, as the increase in nonspecific binding suggests. A similar phenomenon was previously reported with high, nonphysiological temperatures in trout (Ikuta *et al.*, 1989), eel (Hirano and Kishida, 1989), and mudsucker (Tarpey and Nicoll, 1985). At 4°, specific binding equilibrium was reached after 28 hr, which is in agreement with Ikuta *et al.* (1989), but which differs with results obtained in eel (12 hr) (Hirano and Kishida, 1989) and rabbit (80 hr) (Gerasimo *et al.*, 1979). While 50% of the binding could be displaced after 48 hr in the presence of a large excess of sGH, more than 45% of the specific binding could not be dissociated even after 13 days. Similar phenomena have been noted for GH/receptor binding in rabbit (Gerasimo *et al.*, 1979). Such nonreversible binding of the hormone to its receptor might correspond to a first step of the internalization process as was found in human chorionic gonadotrophin-receptor interaction (Catt *et al.*, 1980).

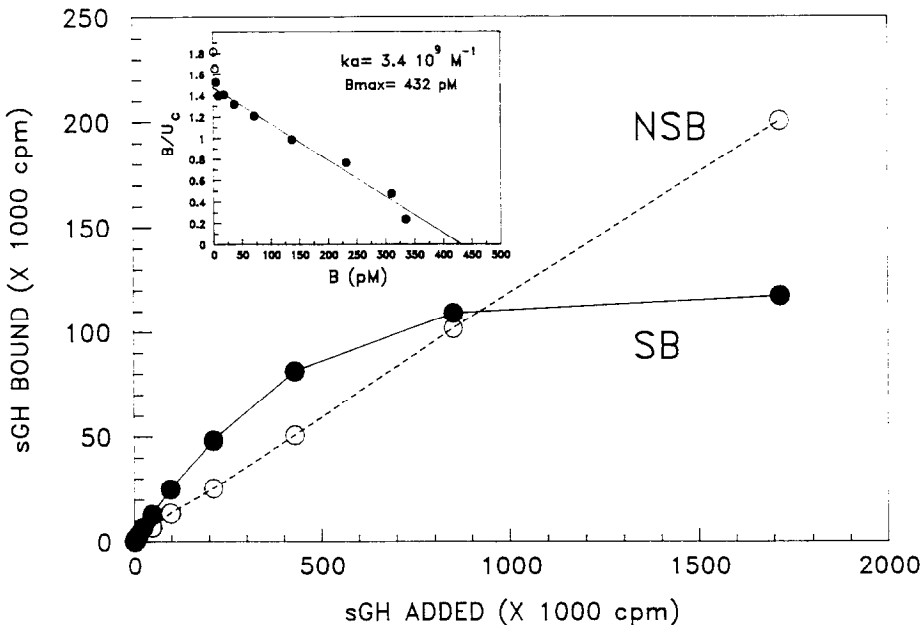


FIG. 7. Effect of increasing concentrations of ^{125}I -sGH₂ on specific (SB) and nonspecific (NSB) binding to liver preparation (100 μl of 1 g/ml wet tissue). The inset shows the derived Scatchard plot. The incubation was carried out at 12° (20 hr).

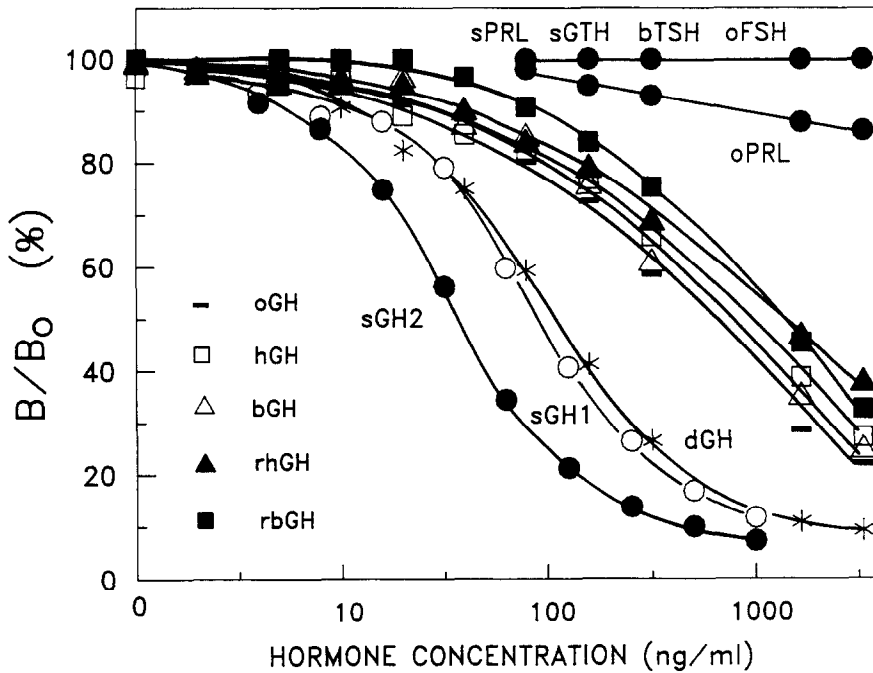


FIG. 8. Competitive inhibition curves for specific binding of ^{125}I -sGH₂ (20,000 cpm/100 μl) to liver preparation (100 μl of 0.5 g/ml wet tissue) by increasing various hormone preparations expressed in ng/ml (final concentration). Binding is expressed as a percentage of ^{125}I -sGH₂ specific binding in the absence of competition.

The Scatchard plot derived from the saturation curve gave only one class of binding site, showing an affinity constant ($K_a = 2.4 \pm 0.8 \cdot 10^9 \text{ M}^{-1}$, $N = 4$) similar to that found in trout by Ikuta *et al.* (1989) and of the same order of magnitude as those found in mammalian liver using homologous hormones as in rabbit (Hughes, 1979), cattle (Hung *et al.*, 1985), sheep (Glukman *et al.*, 1983), mice (Haro and Talamantes, 1985), and human (Carr and Friesen, 1976). The trout affinity constant appears to be one order of magnitude lower than that of tilapia (Fryer, 1979b) and two orders of magnitude lower than that of sturgeon (Tarpey and Nicoll, 1985) using bovine GH. We also found that mammalian GH could bind to the trout liver preparation but with a much lower affinity as shown by the high concentrations necessary to inhibit ^{125}I -sGH₂ binding. This result corroborates the lower biological activity of mammalian GH on

salmonid growth (see Donaldson *et al.*, 1979) as compared to the growth-promoting effect of the homologous hormone (Kawachi *et al.*, 1986). Conversely, salmon GH or salmonid pituitary extracts are able to bind mammalian receptors (Nicoll *et al.*, 1987; Le Bail *et al.*, 1989; Niu *et al.*, 1990) but also with a lower affinity than mammalian hormones. Sea bream GH was highly efficient in inhibiting the ^{125}I -sGH₂ binding, which suggests a similar structure between these two hormones. sGH₁ seemed to be less potent than sGH₂ in displacing labeled sGH₂. However, it is difficult to draw conclusions without knowing the "biochemical quality" of the sGH₁ used, which can vary from one purification to another.

The number of binding sites found (1.6 pmol/g tissue) was equivalent to the value reported by Ikuta *et al.* (1989) (2.46 pmol/g tissue) but appeared to be very variable. According to our observation (unpublished

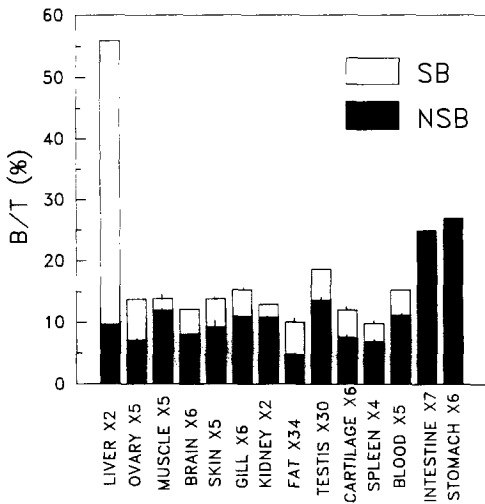


FIG. 9. Specific and nonspecific binding of ¹²⁵I-sGH₂ to different trout tissues. Tissue preparations (100 μl) at a given dilution (×2 = 2 g/ml of wet tissue) were incubated as described under Materials and Methods. The specific (SB) and the nonspecific (NSB) binding are expressed as percentages of the total added ¹²⁵I-sGH₂. Histograms are the means of duplicate determinations and the vertical bars represent the distance between the mean and the superior value. Absence of bar signifies that the duplicates coincide.

data), this variability can be explained in part by the different sexual stages and degrees of nutrition of the animal used in this study. Fryer and Bern (1979) and more recently Gray *et al.* (1989) demonstrated that specific binding of GH is lower in stunted than in normal coho salmon. In mammals, variations of GH binding were also observed during pregnancy (Posner, 1976; Gerasimo *et al.*, 1979) and starvation (see Postel-Vinay *et al.*, 1987). All the properties observed with the trout liver preparation, a finite number of binding sites, high affinity, and hormone specificity, are characteristic of hormonal receptors.

Binding sites were looked for in other tissues. Only intestine and stomach did not show binding sites. This could result from ¹²⁵I-sGH degradation by digestive enzymes as suggested by the high nonspecific binding observed. All other tissues tested demonstrated saturable binding but at a level

considerably lower than that of the liver preparation. The presence of saturable GH binding in kidney and gill was also noted by Ikuta *et al.* (1989) in rainbow trout and masu salmon (*O. masou*) and by Fryer (1979b) in tilapia. The presence of GH receptors in these osmoregulatory organs together with the facilitating effect of GH pretreatment for salmonid adaptation to seawater (Komourdijan *et al.*, 1976; Clarke *et al.*, 1977; Bolton *et al.*, 1987; Boeuf *et al.*, 1990) would support the hypothesis of a role for GH in osmoregulation. In mammals, direct effects of GH or the presence of GH receptors was found in tissues implicated in growth function, such as fat (Fagin *et al.*, 1980; Gavin *et al.*, 1982; Digirolamo *et al.*, 1986), skin (Murphy *et al.*, 1983), cartilage (Eden *et al.*, 1983), and muscle (Daughaday, 1977). Saturable GH binding was also found in muscle of rainbow trout, masu salmon, and char (*Salvelinus pluvius*) (Ikuta *et al.*, 1989). The presence of a putative GH receptor in brain suggests a possible GH autofeedback control via the hypothalamus. In blood cells, the saturable binding cannot be attributed to possible serum contamination since our previous work has shown that in trout, blood plasma proteins do not specifically bind salmonid GH (Niu *et al.*, 1990), but it can be explained by the presence of lymphocytes in which GH receptor was characterized in human (Lesniak *et al.*, 1974).

In rat gonads, a GH receptor has not yet been demonstrated, but *in vitro* studies have shown that GH was able to increase the IGF₁ secretion by ovary (Hsu and Hammond, 1980) and testes (Tres *et al.*, 1983). In fish, a strong relationship exists between growth and reproduction (Le Bail, 1988), and injection of sGH can increase the steroid production (Singh *et al.*, 1988; Van der Kraak *et al.*, 1989). *In vitro*, GH alone or in synergy with GtH increases the steroid production by gonads (Singh *et al.*, 1988; Van der Kraak *et al.*, 1989; Le Gac *et al.*, unpublished data). So, the GH saturable bind-

ing we found in trout testis and ovary are in agreement with these physiological data.

However, saturable binding alone cannot be equated with the presence of receptor and further investigation is necessary to establish the presence of GH receptor in tissues other than liver.

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