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Radioimmunoassay for GH-Release Inhibiting Hormone[1] (38631)

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Recently, Brazeau *et al.* (1) isolated growth hormone release-inhibiting hormone (GH-RIH) from ovine hypothalamic extracts and characterized the structure as H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH. The synthetic tetradecapeptide was as active as natural GH-RIH in both *in vitro* and *in vivo* assay systems (1–5). However, it is not clear whether this tetradecapeptide represents physiological GH-RIH which controls GH secretion in animals and humans. An approach to the study of this problem may be the production of an antibody against GH-RIH which would enable a sensitive and specific radioimmunoassay method to be established for determining GH-RIH in biological materials together with immunohistochemical studies on GH-RIH. This report deals with the development of such a system.

Materials and Methods. Immunogen preparation. Two and one-half mg of synthetic GH-RIH tetradecapeptide (Ayerst, No. 24910) and 12 mg human serum α-globulin (HSG) (Schwartz/Mann, Orangeburg, New York) were dissolved in 2 ml of ammonium acetate buffer 0.1 *M*, pH 7.0. To this solution was added dropwise 1.3 ml of 0.02 *M* glutaraldehyde solution. After stirring for 5 hr at room temperature, the reaction mixture was dialyzed against 2 l of water at 4° for 24 hr. The water was changed twice during the dialysis. The solution was then lyophilized, and the weight of final product was 16.1 mg, indicating that nearly all GH-RIH was conjugated with HSG. It could, therefore, be assumed that 1 mg of the conjugate contained approximately 155 μg GH-RIH.

Immunization. Four mg of GH-RIH-HSG conjugate was dissolved in 2 ml 0.9% NaCl. To this was added 10 mg of desiccated *Mycobacterium tuberculosis* (Difco Lab, Detroit) and 2 ml complete Freund's adjuvant (Difco Lab, Detroit), whereupon the mixture was emulsified by an Omni-mixer (Sorvall, Newton, CT). One and two-tenth ml of the emulsion, equivalent to 190 μg GH-RIH, was given to each of the three mixed-bred rabbits by multiple site injection. Each rabbit also received 0.5 ml of pertussis vaccine (Eli Lilly and Co., Indianapolis, IN) subcutaneously. Immunization was repeated every 2 wk until antibody with a sufficiently high titer was generated.

Preparation of labelled hormone. Since GH-RIH is lacking an amino acid which can be readily iodinated, Tyr[1]-GH-RIH was synthesized by a solid phase method (4), and 1 mg of this peptide was dissolved in 1 ml 0.1 *M* acetic acid to make a stock solution. Five μg of Tyr[1]-GH-RIH were radiolabelled with ^{125}I using lactoperoxidase and purified on a carboxymethylcellulose (CMC) column as described elsewhere (6). The CMC column was first eluted with 0.002 *M* ammonium acetate buffer, pH 4.6, until 16 ml of the effluent was collected. The buffer was then replaced by 0.2 *M* ammonium acetate buffer, pH 4.6. Two ml fractions were collected and the radioactivity of each fraction determined in a Packard Autogamma Counter.

Fractions containing the main peak were pooled, lyophilized, and stored at 4°. Lyophilized ^{125}I-Tyr[1]-GH-RIH was dissolved in a small volume of 0.002 *M* ammonium acetate buffer and repurified on the CMC column as described above.

Radioimmunoassay (RIA) for GH-RIH. The method of RIA for GH-RIH was similar to that used for LH-RH (6). The diluent for reagents was 0.1% gelatine made up in 0.01 *M* phosphate/0.14 *M* NaCl/0.25 *M* ethylenedinitrilotetraacetic acid disodium, pH 7.4 (gelatine-PBS). To each disposable reaction tube (0.9 × 7.5 cm) was added 0.4 ml gelatine-PBS, 0.1 ml antiserum of appropriate

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dilution, 0.1 ml standard solution in a dose range from 1 pg to 1000 pg or unknowns and 0.1 ml labelled Tyr^1-GH-RIH (approximately 10,000 cpm). The reaction mixture was incubated at 4° for 48 hr and the bound and free hormone were separated by dextran-coated charcoal as described elsewhere (6). The radioactivity of the bound labelled hormone was determined in the Autogamma Counter.

Extracts of the hypothalami of rats and pig. Rat hypothalami were removed from normal male rats, homogenized in 2 *N* acetic acid, and lyophilized. Pig hypothalamic extract was prepared as described previously (7). These preparations were dissolved in gelatine-PBS and assayed for GH-RIH in increasing dilutions.

Hypothalamic pituitary hormones, and synthetic peptides used for testing cross-reactivity. Thyrotropin-releasing hormone (TRH) (Abbott), LH-RH (Sankyo Co.), MSH-release inhibiting hormone (MRIH): Pro-Leu-Gly-NH_2 (8), and a decapeptide formerly proposed as a GH-releasing hormone (GH-RH); Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala (Merck) (9), were of synthetic origin. Other synthetic peptides included linear GH-RIH and the ring portion of GH-RIH; [Des-Ala^1, Des-Gly^2]-GH-RIH, prepared as described previously (4).

Pituitary hormones tested were arginine vasopressin (10), ACTH (Cortisyn, Organon), oxytocin (Syntocinon, Sandoz), highly purified ovine LH (gift from Dr. Harold Papkoff), rat FSH (NIAMD-rat-FSH-I-1), rat GH (NIAMD-rat-GH-I-1), human GH (HS 1216C), LH (LER 960), FSH (LER 1366), TSH (NIH), and bovine TSH (NIH-TSH-B5).

Removal and extraction of GH-RIH from plasma. Heparinized sheep plasma was treated with activated charcoal to remove GH-RIH as described previously for removal of LH-RH (6). Extraction of GH-RIH was carried out using acetone in a similar method to that used for the extraction of vasopressin (11).

Results. An iodination of 70–80% was usually achieved as judged by the chromatographic pattern on CMC. Figure 1 illustrates the elution pattern of radioactivity when the iodination mixture was chromatographed on a CMC column. Peak I contained free iodide (6). Peak II contained very little immunoreactive material. Peak III, which was the most prominent, was antibody-bindable.

The elution pattern of radioactivity when peak III from CMC column was rechromatographed on CMC after storage was similar to that for the first chromatography. The main peak was found in the same area corresponding to peak III on the first chromatography. A small peak for free ^{125}I (peak I) and damaged hormone (peak II) was also observed. The specific activity of CMC re-

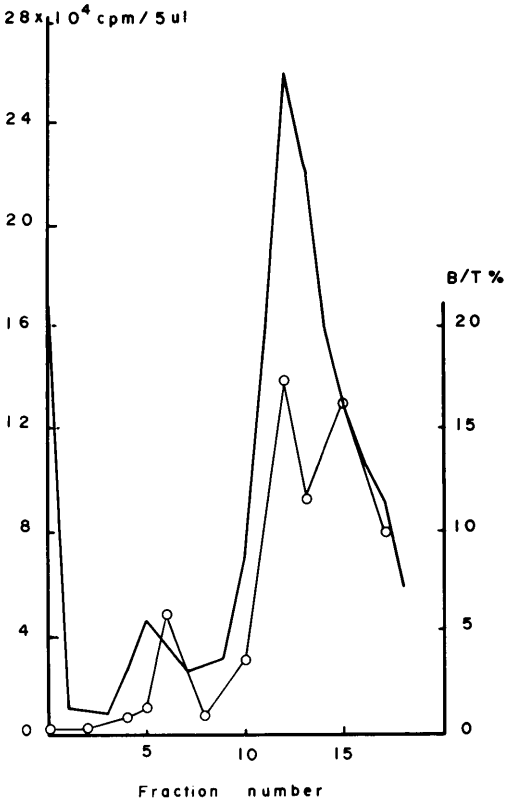

FIG. 1. Elution pattern of ^{125}I-Tyr^1-GIF on CMC column. ——radioactivity; O——O binding activity to the antiserum. B: radioactivity bound to GH-RIH antibody; T: total radioactivity. Peak I (fraction 0, eluted by 0.002 *M* ammonium acetate buffer, pH 4.6) corresponds to free iodide. After 16 ml of the effluent was collected, the buffer was changed to 0.2 *M* ammonium acetate, pH 4.6. Peak II (fractions 3–7) contained labelled products with little immunoreactivity. Peak III (fractions 11–15) contained labelled products with greatest immunoreactivity.

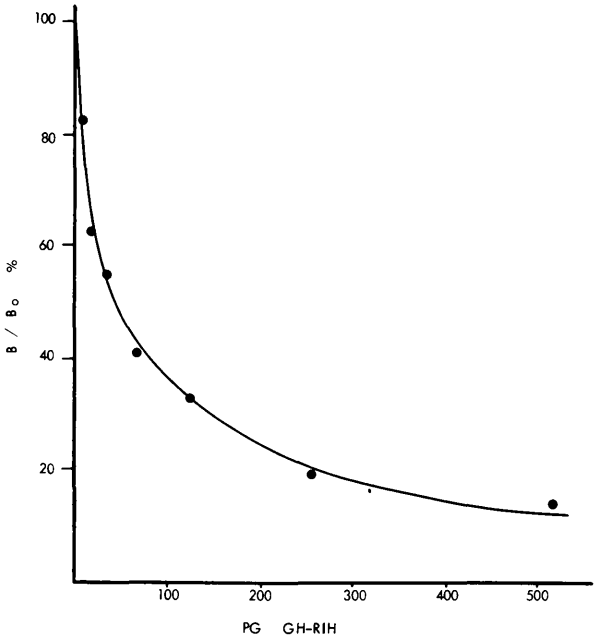

FIG. 2. Dose response curve of radioimmunoassay for GH-RIH. B and Bo: bound radioactivity with and without unlabelled hormone, respectively. Antiserum No. 103 was used at 1:7000 dilution. Nonspecific binding after treatment with dextran-coated charcoal was 1.3%. The ratio of bound radioactivity (B/T) without presence of unlabelled GH-RIH was 20%.

purified labelled hormone, which was calculated from the standard curve using various amounts of labelled hormone and fixed concentration of antiserum was 330 μCi/μg.

Sera from all three rabbits immunized with GH-RIH-HSG conjugate showed significant binding with ^{125}I labelled Tyr^{1}-GH-RIH after four immunizations. Antiserum No. 103 showed the greatest immunoreactivity and bound 70% of the labelled hormone in the presence of excess amount of antiserum. The binding was inhibited by the addition of unlabelled GH-RIH and the inhibition was dose-related, enabling us to establish the dose-response curve of the RIA for GH-RIH (Fig. 2). With this antiserum at dilution of 1:7000, the minimum detectable dose as determined by the amount for the lower limit of 95% confidence limits of the buffer control was 4 pg per tube. Within-assay coefficient of variation was 9.99% (assay of identical sample containing about 64 pg in eight replicates in one assay).

Measured concentrations of immunoreactive GH-RIH in rat and pig hypothalamic extracts fell linearly with dilutions (Fig. 3). This suggests that the material assayed was indistinguishable immunologically from the standard GH-RIH tetradecapeptide. Various hypothalamic and pituitary hormone preparations did not interfere with the RIA for GH-RIH, indicating that it is fairly specific (Fig. 4). However, linear GH-RIH and the ring portion of GH-RIH tetradecapeptide cross-reacted in the RIA system by 17 and 67%, respectively.

Washing plasma with charcoal removed 99% of ^{125}I-Tyr^{1}-GH-RIH added to the plasma. As shown in Fig. 5, both untreated sheep plasma and charcoal-washed, GH-RIH-free plasma exhibited a dose-related inhibition of ^{125}I-Tyr^{1}-GH-RIH antibody binding. Deproteinization of plasma with acetone eliminated this inhibitory effect. This procedure may not have removed endogenous GH-RIH in the plasma, since 90% of ^{125}I-Tyr^{1}-GH-RIH added to plasma was recovered in the acetone extract. On the other hand, recovery of 40 pg GH-RIH added to plasma containing Trasyrol (FBA Pharmaceuticals, New York) averaged 60% after

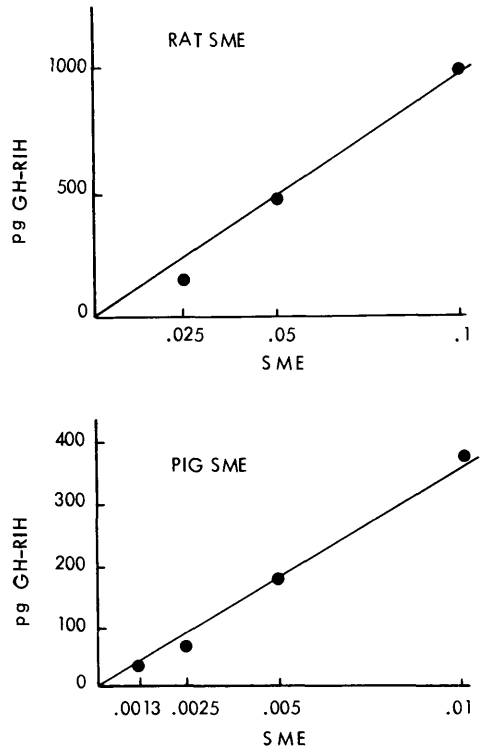

FIG. 3. Amounts of GH-RIH determined, as a function of the dilution of crude rat and pig hypothalamic extracts (SME) in the RIA for GH-RIH.

acetone extraction when determined by radioimmunoassay.

Discussion. Since the GH-RIH molecule is lacking tyrosine or histidine residues which can be readily iodinated, Tyr[1]-GH-RIH was synthesized in order that it could be labelled. Therefore, the antiserum to be generated had to bind GH-RIH and Tyr[1]-GH-RIH in a competitive manner. Since binding of an antibody with a hapten antigen does not seem to involve the portion of molecule through which the hapten is conjugated with a carrier protein (12, 13), conjugation of GH-RIH through alanine in position 1 was attempted by using glutaraldehyde which couples hapten and protein by forming Schiff's base with their free amino groups (14, 15). Since there are three primary amino groups in the molecule of GH-RIH (Ala[1], Lys[4], Lys[9]), the coupling with HSG could have taken place through either one, two, or all of these amino groups. In any case, the atigenic determinant of the antibody thus generated did not involve alanine in *N*-terminus as judged by the competitive binding of GH-RIH and Tyr[1]-GH-RIH with the antibody.

Since only two peptides related to GH-

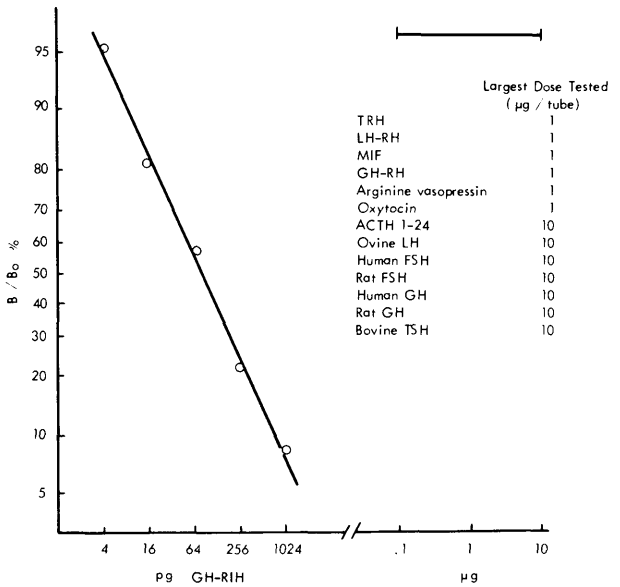

FIG. 4. Lack of cross-reaction with various hypothalamic and pituitary hormones in the RIA system for GH-RIH. B and Bo: bound radioactivity with and without presence of unlabelled hormone. The single line at the upper right of the figures applies to all of the substances tested for cross-reactivity.

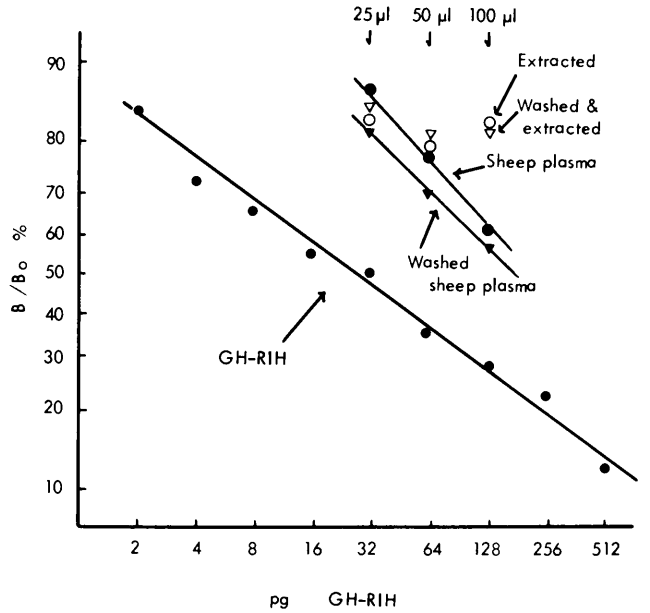

FIG. 5. Dose-response curve of untreated and charcoal-washed sheep plasma before and after acetone extraction in the RIA system for GH-RIH. B and Bo: bound radioactivity with and without presence of unlabelled hormone.

RIH, linear GH-RIH and [Des-Ala^1, Des-Gly^2]-GH-RIH were tested for cross-reaction, exact information on the antigenic determinant is not available at the moment. Considerable cross-reaction of these peptides in the RIA system suggests that the antigenic determinant for the antiserum No. 103 involves the amino acid sequence from position 3 to 14, or part of it, and that the ring structure is not necessary for immunological binding.

In using an immunohistochemical technique with the antiserum used in the present study, we have observed that GH-RIH is contained in the secretory granules of many nerve endings, mainly located in the external zone of the median eminence of the rat (16).

Our recent studies indicate that the median eminence of the rat contains the greatest concentration of radioimmunoassayable GH-RIH, but that it is not confined only to the hypothalamus. The samples of septum and preoptic area, midbrain, brainstem, thalamus and cortex all have significant amounts of GH-RIH (unpublished observation). The distribution of GH-RIH in the rat brain as measured by radioimmunoassay has been in good agreement with that measured by bioassay by Vale *et al.* (17). Further work is necessary before it can be concluded that the GH-RIH tetradecapeptide acts as a neurotransmitter in addition to a hypophysiotropic hormone.

Dose-related inhibition of ^{125}I-Tyr-GH-RIH antibody binding by GH-RIH free plasma and the elimination of inhibition by deproteinization suggest: (a) presence of substance(s) immunologically indistinguishable from GH-RIH in plasma protein, or (b) nonspecific interference of this RIA system with plasma protein.

Lower recovery after acetone extraction of added GH-RIH, as measured by RIA, than that of added ^{125}I-Tyr^1-GH-RIH, as determined by radioactivity, might be explained by a greater loss due to adsorption on the glass during the extraction procedure of unlabelled GH-RIH, or some other reason. Although the nature of the acetone-unextractable GH-RIH-like substance in plasma is still unknown, it is recommended to deproteinize plasma before assaying for GH-RIH.

Summary. The synthetic growth hormone release inhibiting hormone (GH-RIH) was conjugated with human serum globulin using glutaraldehyde and administered to rabbits.

An antiserum thus generated bound 70% of ^{125}I-Tyr^{1}-GH-RIH. The binding was inhibited by unlabelled GH-RIH and the inhibition was dose-related, enabling us to establish a radioimmunoassay method for GH-RIH. The minimum detectable dose was 4 pg. A linearity was demonstrated for immunoreactive GH-RIH of extracts of rat and pig hypothalami, indicating that they contained substance(s) indistinguishable from GH-RIH. Various hypothalamic and pituitary hormones did not interfere with the radioimmunoassay, but considerable cross-reaction was observed for linear GH-RIH and the ring portion of GH-RIH, suggesting that the antigenic determinant involved the amino acid sequence from position 3–14 of GH-RIH or part of it. Plasma protein appears to contain substance(s) immunologically indistinguishable from GH-RIH or to interfere, in a nonspecific manner, with the radioimmunoassay system for GH-RIH.

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1. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R., Science **179,** 77, (1973).
2. Vale, W., Brazeau, P., Grant, G., Nassey, A., Burgus, R., Rivier, J., Ling, N., and Guillemin, R., C. R. Acad. Sci. Paris Series D **275,** 2913 (1972).
3. Rivier, R., Brazeau, P., Vale, W., Ling, N., Burgus, R., Gilirs, C., Hardley, J., and Guillemin, R., C. R. Acad. Sci. Paris Series D **276,** 2737 (1973).
4. Coy, D. H., Coy, E. J., Arimura, A., and Schally, A. V., Biochem. Biophys. Res. Commun. **54,** 1267 (1973).
5. Martin, J. B., Endocrinology **94,** 497 (1974).
6. Arimura, A., Sato, H., Kumasaka, T., Worobec, R. B., Debeljuk, L., Dunn, J., and Schally, A. V., Endocrinology **93,** 1092 (1973).
7. Schally, A. V., Arimura, A., Bowers, C. Y., Kastin, A. J., Sawano, S., and Redding, T., Recent Progr. Hormone Res. **24,** 497 (1968).
8. Nair, R. M. G., Kastin, A. J., and Schally, A. V., Biochem. Biophys. Res. Commun. **43,** 1376 (1971).
9. Veber, D., Bennett, C., Milkowski, J., Gal, G., Denkwalter, R., and Hirschmann, R., Biochem. Biophys. Res. Commun. **45,** 235 (1971).
10. Schally, A. V., Bowers, C. Y., Carter, W. H., and Hearn, A. C., Arch. Biochem. Biophys. **107,** 332 (1964).
11. Husain, M. K., Fernand, N., Shapiro, M., Kagan, A., and Glick, S. M., J. Clin. Endocrinol. Metabol. **37,** 616 (1973).
12. Niswender, G. D., and Midgley, A. R., *in* "Immunological Method in Steroid Determination" (F. G. Peron and B. L. Caldwell, eds.) p. 149. Appleton–Century–Crofts, New York (1970).
13. Arimura, A., Sato, H., Coy, D. H., Worobec, R. B., Schally, A. V., Yanaihara, N., Hashimoto, T., Yanaihara, C., and Sukura, N., Acta Endocrinologica, **78,** 222 (1975).
14. Halbeeb, A. F. S. A., and Hiramoto, R., Arch Biochem. Biophys. **126,** 16 (1968).
15. Avrameas, S., Ternynck, T., Immunochemistry **6,** 53 (1969).
16. Pelletier, G., Labrie, F., Arimura, A., and Schally, A. V., J. Anatomy, **140,** 445 (1974).
17. Vale, W., Rivier, C., Palkovitis, M., Saavedna, J. M., and Brownstein, M., Program of the 56th Annual Meeting, The Endocrine Society, Abstract No. 146 (1974).

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