PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 140-145 (1976).

A Rapid Radioimmunoassay Method of Growth Hormone in Dog Plasma[1] (39161)

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The dog is a very useful animal model in experimental endocrinology; it is relatively easy to handle, it has readily available superficial vessels for repetitive blood withdrawal or prolonged infusions and responds similarly to the human to many of the stimuli which are effective to activate or inhibit hormonal secretions (1, 2).

With regard to growth hormone (GH), although methods for the isolation and purification of canine GH (cGH) have been described previously (3, 4), so far, only two reports of specific radioimmunoassay for cGH (5, 6) and two short notes (7, 8) on this subject have been published.

The present study (1) describes a homologous radioimmunoassay method for cGH in the plasma which requires only 40 hr, with no loss of sensitivity; (2) reports on the cross-reactivity of cGH with GH preparations from several other species; and (3) shows that the dog is a valuable species for further work on the regulation of GH secretion.

Materials and Methods. 1. *Radioimmunoassay. Hormone preparations.* Highly purified cGH (D887A, 2.0 IU/mg) for iodination and standards was kindly supplied by Dr. A. E. Wilhelmi (3), Emory University, Atlanta, Ga. Human growth hormone (HGH) and bovine GH (BGH) were prepared in our laboratories by the method of Li (9) and ovine prolactin (OPRL) was prepared by the method of Cole and Li (10). Ovine GH (OGH) was a gift from Dr. F. Hertelendy, Washington University, St. Louis, Mo., and purified pig GH (PGH, NIH P482, 1.1 USPU/mg) was supplied by the Endocrine Study Section of NIH, Bethesda, Md.

Radioiodinated canine growth hormone. Five μg cGH were iodinated by the method of Greenwood *et al.* (11), using 2.3 mCi of [^{125}I]NaI (Sorin, Saluggia, Vercelli, Italy) and 100 μg of chloramine T. Unreacted iodine-125 was separated from that bound to the hormone on a strongly basic anion exchange resin (Zerolit FFCl$^-$, U.W. Softeners Ltd., London) column (8 × 1 cm) eluted with 0.05 *M* phosphate buffer, pH 7.5. The protein peak was collected in 2-ml fractions, and the tube containing the highest radioactivity was selected for the assay because of its maximum binding affinity for the antibody prepared to cGH. The elution pattern showed the labeled hormone between the third and sixth tube. When submitted to a chromatoelectrophoresis (16 V/cm, 2 hr) using Whatman 3 MM paper strips (25 × 5 cm) with 0.06 *M* Veronal buffer, pH 8.6, labeled cGH thus prepared exhibited a single radioactive peak at the origin of the chromatogram. The specific activity obtained was in the order of 300 μCi/μg. The tracer was stored frozen until use; good standard curves have been obtained for at least 4 weeks after iodination.

Antisera. Antisera to cGH were raised in monkeys, using a total immunizing dose of 3.1 mg over about a 3-month period. The immunization schedule was as follows: two male rhesus monkeys received 1.0 mg cGH in complete Freund's adjuvant, sc, every 3 weeks for three doses, followed by one booster injection of 0.1 mg. The final bleeding was performed 2 weeks after the booster injection. The antisera was vialed and freeze-dried (1 ml antiserum diluted 1 to 15,000 in 0.04 *M* phosphate buffer, pH 7.4, 0.1% BSA). The antisera was stored at 4°C until use.

Anti-monkey gamma globulin (AMGG) sera were prepared in goats. Animals were immunized every 3 weeks with 1.0–2.0 mg

[1] Supported in part by Contract No. CT 74.00232.04 of the Consiglio Nazionale delle Ricerche, Rome, Italy.

of monkey gamma globulin emulsified in complete Freund's adjuvant and were bled 2 weeks after the fourth immunization.

Assay procedure. All dilutions were made in 0.04 *M* phosphate–saline buffer, pH 7.4, containing 5% bovine serum albumin. Plastic test tubes (Luckham LP3) were used for incubation. One hundred μl of plasma sample or 100 μl of standard cGH (1.0–100 ng/ml) was added to 0.5 ml of diluent in the incubation tube, followed by 0.1 ml of diluted anti-cGH and 0.1 ml of [^{125}I]cGH (10,000 cpm, sp act 300 μCi/μg).

The appropriate dilution of anti-cGH (usually 1:150,000) is that which binds 25–40% of labeled cGH added when no competing cGH is present in the system. After shaking, the tubes were placed at 4°C for 20 hr. Then 0.1 ml of diluted precipitating AMGG was added. The tubes were shaken and incubated another 20 hr at 4°C. The appropriate dilution of AMGG is one which precipitates monkey gamma globulin in the system maximally. The precipitates in the tubes were then collected by microfiltration through "Oxoid" membrane filter discs (2.5 cm) mounted on a Millipore microanalysis filter holder held in a vacuum filtration flask. This method does not require the addition of MGG as a carrier to obtain insoluble complexes. The precipitates were counted in a well-type gamma counter and the percentage of labeled cGH precipitated was calculated.

Specificity of the assay. Fresh-frozen dog and rat pituitaries were homogenized at 4°C in 1 ml of 0.01 *M* phosphate buffer, pH 7.5, using a motor-driven Potter blender. After centrifugation at 18,000 rpm for 10 min at 4°C, the obtained supernatants (dilutions 1/10,000 to 1/80,000) were studied for their ability to compete with the labeled cGH in the anti-cGH-labeled cGH system. Dilution responses of dog plasma samples were also studied. Similarly, dilutions of purified GH of other species were substituted for cGH in the anti-cGH-labeled cGH system. The hormones were weighed and after being dissolved in 0.1 *M* ammonium bicarbonate, their protein titer was assayed spectrophotometrically by the ultraviolet method according to Warboury and Christian (12). They were then diluted in 0.04 *M* phosphate buffer pH 7.4, 5% bovine serum albumin, to an initial concentration of 1000 ng/ml.

2. *Animal experiments. Animals.* All experiments were performed on unanesthetized trained male and female beagles (4- to 5-yr-old) following an overnight fast and starting at 8:00–9:00 AM. Animals were kept in the resting state for about 1 hr before the start of the experiment. Blood was collected from the cephalic vein into heparinized chilled tubes, centrifuged immediately and the separated plasma was stored at −20°C until plasma concentrations of cGH and glucose were analyzed. Plasma glucose levels were determined by Glucosio Test, Sclavo, Siena.

Effect of insulin hypoglycemia. Regular insulin (Iletin, Lilly, 0.4 U/kg body wt) in 10 ml of saline was injected intravenously in 10 normal adult dogs (six males and four females). Blood samples were drawn 30 min and immediately before the injection and at 30-min intervals up to 150 min. An identical study was conducted using 10 ml of saline alone instead of insulin in four normal male dogs.

Effect of glucose ingestion. Glucose (1.0 g/kg) was administered orally to four normal male dogs and blood samples were drawn at the usual time intervals.

Results. Figure 1 shows a typical standard curve obtained with this assay method.

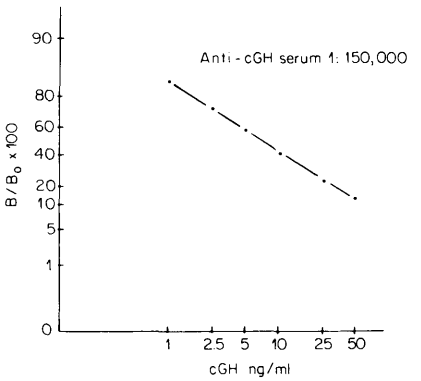

FIG. 1. Standard curve for the radioimmunoassay of cGH. The concentrations of the standard cGH solution are reported on the abscissa, and each point is the mean value of duplicate determinations. The ordinate is a logit scale. This standard curve was obtained using 1:150,000 diluted monkey anti-cGH serum.

cGH concentrations were in the range 1 to 50 ng/ml and the antiserum used was diluted 1:150,000. Since 100 μl of the standard solution was used in the incubation, the assay could detect displacement by 0.1 ng of cGH using 100 μl of plasma. Serial dilutions of canine plasma samples and canine pituitary extracts were parallel to those of the standard curve (no significant difference was present between the angular coefficients of the three lines calculated by an original program for the IBM model 360 computer, which compares the slope using the statistical tables reported by Snedecor and Cochran (13)), indicating indistinguishable immunologic reactions for both plasma and pituitary GH (Fig. 2). Intraassay variation was 3% while a reference plasma showed an interassay variation of 16%.

Figure 3 shows the cross-reactivity between cGH and human, ovine, bovine, and porcine GH and ovine prolactin. Concentrations used for the various hormones ranged between 1 to 1000 ng/ml. PGH and cGH not only cross-reacted, but completely overlapped in the same concentration range. A partial degree of overlapping between antigenic sites of BGH, OGH, and cGH was exhibited, but the antibody affinity was different from cGH. HGH and OPRL did not cross-react with anti-cGH to any extent. Though not presented in Fig. 3, a complete parallelism was present between cGH and serial dilutions of rat pituitary extracts.

Plasma cGH concentrations after an overnight fast were 2.0 ± 0.4 ng/ml (mean ± SE) in 10 normal adult unanesthetized dogs (six males and four females). The intravenous injection of 10 ml saline did not produce any consistent change in plasma glucose or cGH levels throughout the test period. Following insulin injection (0.4 U/kg iv) in 10 animals, plasma glucose fell promptly from 80.8 ± 4.2 at 0 time to 28.6 ± 2.3 mg/100 ml at 30 min, and hypoglycemia continued for 2 hr. The average of peak cGH values, usually obtained at 60 or 90 min, was 17.5 ± 4.1 ng/ml. The mean cGH values at 30, 60, 90, and 120 min were significantly higher than base line values. Glucose ingestion (1.0 g/kg) in four male dogs caused a rise in blood glucose levels reaching a peak at 30 min (160 ± 41 mg/100 ml) with a return to base line at 90–120 min. Hyperglycemia re-

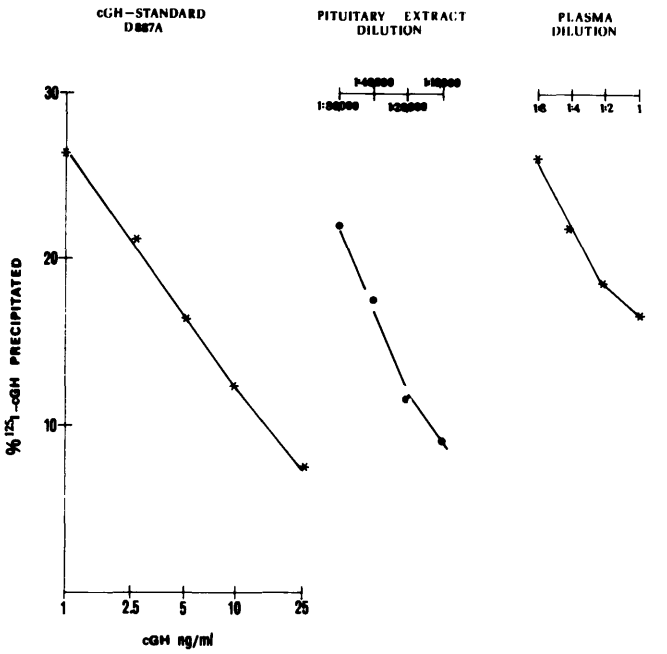

FIG. 2. Dilution response curves for both dog pituitary extracts and plasma samples compared with the standard curve obtained with purified cGH. The standard curve was obtained by plotting the percentage of ^{125}I-labeled cGH precipitated vs log cGH concentrations.

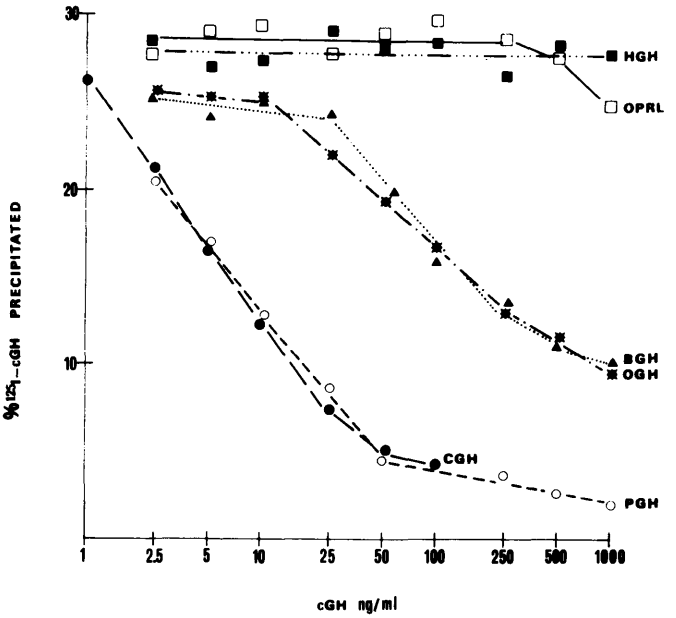

FIG. 3. Effect of varying concentrations (expressed logarithmically) of purified human, bovine, ovine, and porcine GH, and ovine prolactin in the radioimmunoassay for canine GH.

sulting from the glucose ingestion did not significantly depress the initial plasma cGH level, but cGH levels found throughout this period never exceeded 2.0 ng/ml. Figure 4 is representative of the pattern of cGH and blood glucose responses in two individual dogs following the above stimuli.

Discussion. The method described allows a rapid determination of cGH in the plasma using a sensitive and specific [^{125}I]double antibody system. The parallelism found between the pituitary and plasma hormone and cGH standard indicates that the latter was not damaged during the extraction and underlines the similar immunoreactivity of pituitary and plasma GH.

The method, although having a sensitivity similar to that reported by Tsushima *et al.* (5) and Lovinger *et al.* (6), has the advantage of rapidity since it can be completed in 40 hr instead of 96 (Tsushima *et al.*) or 144 hr (Lovinger *et al.*) avoiding the preincubation of the cold hormone with the specific antibody before adding the tracer. This is surely due to the higher affinity constant of the monkey antiserum used.

A partial cross-reactivity was reported by Tsushima *et al.* (5) between PGH and cGH; in this study, not only parallelism but a perfect correspondence was found between the two hormones. It can be concluded that the antigenic site(s) of PGH and cGH being recognized with our antiserum, at the dilution employed, are identical and have the same affinity. As reported by the Japanese workers (5), PGH and cGH gave two perfectly parallel curves and only showed a shift in the higher dose range. This fact might be accounted for by the greater impurity present in the PGH they used, i.e, by lack of correlation between the calculated amount and the hormone actually present in their preparation.

The complete overlapping found between PGH and cGH in our study suggests that the former, when our antiserum is used, can substitute for the latter hormone in a heterologous radioimmunoassay for cGH, a fact of noticeable practical advantage on considering the greater availability of PGH than cGH. In addition, since rat pituitary extracts showed complete parallelism with cGH, it appears that the system anti-cGH serum and PGH as standard and tracer can be used for rat GH radioimmunoassay.

The present study demonstrated also the existence of cross-reaction between cGH and BGH and OGH, the latter hormones

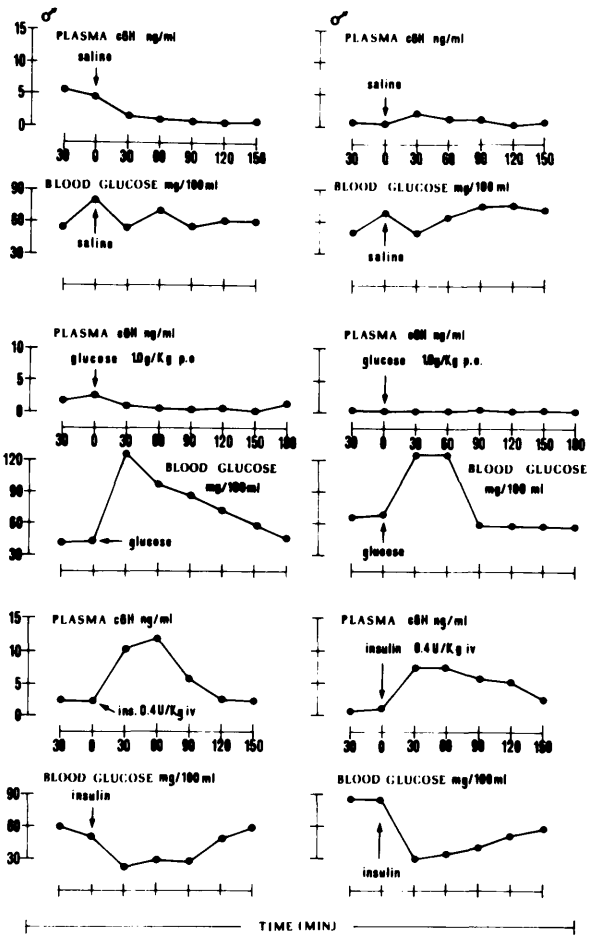

FIG. 4. Plasma GH and glucose levels in two individual male dogs given saline, glucose, or insulin (for further details, see text).

behaving identically in the system. HGH did not cross-react at any level, while OPRL induced a slight displacement of iodinated cGH at a concentration of 1000 ng/ml; this fact might be attributed to contamination of the hormone by ovine GH.

Validation of the assay was obtained by studying plasma GH levels in dogs under conditions known to affect GH secretion in other animal species. Fasting levels of plasma GH were comparable to those reported by Tsushima *et al.* (5) and Lovinger *et al.* (6) in mogrel adult dogs; in newborn puppies, higher base line GH levels have been reported (5). Insulin injection was followed by a clear-cut elevation of plasma cGH, a fact which by no means can be attributed to the stress of the injection or blood sampling, because no GH rise was observed following administration of saline in control experiments. Hyperglycemia did not significantly alter preinfusion GH levels, although cGH values in single plasma determinations did not exceed 2.0 ng/ml. Thus, the dog resembles the primate (14) more than the rat or the rabbit, in which hypoglycemia induces a marked fall (15) or no significant rise (16) of plasma GH, respectively, and appears more responsive than the pig, whose GH response to hypoglycemia is quite sluggish (17). In addition, Lovinger *et al.* (6) have shown that L-dopa is a potent stimulus for secretion of GH in the dog, as it is in man (18). Therefore, this animal species appears to be valuable for further work on the regulation of growth hormone secretion.

Summary. A rapid and sensitive homolo-

gous radioimmunoassay (RIA) system is described for the measurement of growth hormone (GH) in dog plasma. The method requires only 40 hr and is able to detect concentrations of GH as low as 1.0 ng/ml of dog plasma. Antiserum to canine growth hormone (cGH) prepared in monkeys, exhibited a complete cross-reactivity with porcine GH, suggesting that the latter can substitute cGH in a heterologous radioimmunoassay for cGH. Studies on GH regulation performed with this RIA in the unanesthetized dog showed that this species resembles the primate more than the rat or the rabbit.

1. Altszuler, N., Steele, R., Rathgeb, I., and De Bodo, R. C., *in* "Growth Hormone" (A. Pecile and E. E. Müller, Eds.), p. 309, Excerpta Med. Int. Congr. Ser. No. 158 (1968).
2. Van Loon, G. R., Hilger, L., King, A., Boryczka, A. T., and Ganong, W. F., Endocrinology **88,** 1404 (1971).
3. Wilhelmi, A. E., Yale J. Biol. Med. **41,** 199 (1968).
4. Hashimoto, C., Irie, M., Matsuzaki, F., Tsushima, T., and Shizumi, K., Biochem. Biophys. Acta **175,** 231 (1969).
5. Tsushima, T., Irie, M., and Sakuma, M., Endocrinology **89,** 685 (1971).
6. Lovinger, R. D., Connors, M. H., Kaplan, S. L., Ganong, W. F., and Grumbach, M. M., Endocrinology **95,** 1317 (1974).
7. Hampshire, J., Altszuler, N., Steele, R., and Greene, L. S., Fed. Proc. **266,** (1973), abstract.
8. Lovinger, R. D., Connors, M. H., Boryczka, A. T., Kaplan, S. L., and Grumbach, M. M., Endocrinology **92,** A-82 (1973), abstract.
9. Li, C. H., Liu, W. K., and Dixon, J. S., Arch. Biochem. Biophys. Suppl. **1,** 327 (1962).
10. Cole, R. D., and Li, C. H., J. Biol. Chem. **213,** 197 (1955).
11. Greenwood, F. C., Hunter, W. M., and Glover, S. S., Biochem. J. **89,** 114 (1963).
12. Warboury, D., and Christian, S., Biochem. Z. **310,** 384 (1941).
13. Snedecor, G. W., and Cochran, W. G., "Statistical Methods," Iowa State Univ. Press, Ames (1967).
14. Glick, S. M., Roth, J., Yalow, R., and Berson, S. A., Recent Progr. Horm. Res. **21,** 241 (1965).
15. Schalch, D. S., and Reichlin, S., *in* "Growth Hormone" (A. Pecile, E. E. Müller, Eds.), p. 211, Excerpta Med. Int. Congr. Ser. No. 158 (1968).
16. Garcia, J. F., and Geschwind, I. I., *in* "Growth Hormone" (A. Pecile, E. E. Müller, Eds.), p. 267, Excerpta Med. Int. Congr. Ser. No. 158 (1968).
17. Machlin, L. J., Horino, M., Hertelendy, F., and Kipnis, D. M., Endocrinology **82,** 369 (1968).
18. Boyd, A. E., Lebovitz, H. E., and Pfeiffer, J. B., New England J. Med. **238,** 1425 (1970).

Received July 25, 1975. P.S.E.B.M. 1976, Vol. 151.