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Immunochemical and Biological Properties of Horse Parathyroid Hormone (41211)

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Abstract. Intact, biologically active parathyroid hormone (1–84) extracted from horse parathyroid glands was detected by heterologous amino terminal radioimmunoassays and heterologous carboxyl terminal radioimmunoassay using antibovine parathyroid hormone antibodies. The immunochemical behavior of horse parathyroid hormone suggests that this hormone possesses a 1–34 amino terminal portion very similar to that of the human one and structural differences in the 53–84 portion from those of the three known parathyroid hormones.

The structures of Parathyroid hormone (PTH) from three different species are established (1–3); their sequences differ predominantly in the midregion of the molecule. The establishment of the structure of horse parathyroid hormone presents a phylogenetic interest and could contribute to knowledge of structure–activity relationship of this group of such hormones. Due to the low content of the parathyroid hormone in the gland of this species, a sensitive assay capable of detecting PTH at the picomole level, at each step of purification is necessary. Radioimmunoassay (RIA) was chosen for its sensitivity, specificity and easy methodology. Furthermore cross reaction studies afford a method for structural comparison of horse PTH with the hormone of other species, in particular those for which the amino acid sequence is known; similar immunochemical behavior can provide an useful prediction of similar secondary structure between a new and a known peptide. On the basis of immunochemical comparisons, of rat and human calcitonin (4) we predicted that rat calcitonin would have a secondary structure quite similar to human calcitonin and the sequence of the rat hormone would be very similar to that of the human molecule. This was confirmed when we established the sequence of the rat calcitonin and showed that it differed by only two amino acids from human calcitonin (5). We have therefore studied the cross reaction of extracted partially purified horse PTH with antibodies specific for bovine and porcine PTH (C-terminal region) and for human PTH (two proposed N-terminal regions). Raising specific antibodies to horse parathyroid hormone would have entailed the use of large quantities of extracts.

Material and Methods. *Extraction of horse PTH.* Glands were collected during the removal of fat from thyroid glands. Parathyroids, approximately 0.2–0.3 cm in size, were found either in the fat surrounding the thyroid or on the capsule of the thyroid itself. 74g of tissue were frozen and immediately lyophilized. After defatting in the cold the tissue was extracted according to the procedure described by Aurbach (6), except that at the last step excess of trichloroacetic acid was removed by washing the hydrochloric acid solution of TCA precipitate with peroxide free ether (100 ml × 3) and filtering through a Biogel P 10 column equilibrated with acetic acid 0.2 *N*. We obtained 12 mg of partially purified horse parathyroid extract which was eluted in the same volume as active bovine PTH. Detection was carried out by a C-terminal RIA using antibodies antibovine PTH.

Peptide hormones. Synthetic 1–34 N-terminal fragment of human PTH according to the sequence proposed by Niall (7): (1–34 h PTH Niall) was synthetized by the Merrifield method (8–9).[1]

[1] Synthesis made in our laboratory by Dr. P. Rivaille.

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Synthetic 1–34 N-terminal fragment of human PTH according to the sequence proposed by Brewer (10)[2] (1–34 h PTH Brewer) was a generous gift of Dr Arnaud (11).

Synthetic 1–34 N-terminal fragment of bovine PTH (12) (1–34 b PTH) was purchased from Beckman.

Human PTH (h PTH) was extracted from parathyroid adenoma in our laboratory (13).

Bovine PTH (b PTH) was generously given by Dr. Parsons (Aurbach batch).[3]

Synthetic 28–48, 44–68, and 53–84 fragments of human PTH were synthetized by the Merrifield method.[1]

Bioassay. Bioassay of horse parathyroid hormone was performed by measuring in vitro its half maximal stimulation of rat kidney cortex adenylate cyclase (14). The cortex was dissected from medullary tissue and minced, and the tissue was homogenized in 5 × 10-2 *M* Tris–HCl, pH 7.4; 0.25 *M* sucrose, and 10-3 *M* EDTA in an Omni mixer. Membranes were prepared according to the procedure described by Heidrich (15), omitting the free-flow electrophoresis step. Total adenosine triphosphatase activity was enriched by a factor of 5–7. Membrane extracts were stored in a 5 × 10-2 *M* Tris–HCl–DMSO buffer and kept in small aliquots under liquid nitrogen (16). The stability of the enzyme activity was tested regularly by measuring the response to submaximal stimulation by PTH and fluoride (10-2 *M*). Adenylate cyclase assay was performed using standard procedure with an ATP regenerating system but with 2.5 × 10-3 *M* ATP and 4.5 × 10-3 *M* magnesium. Assay of basal and newly formed cAMP was performed by a competitive protein binding assay (17) using a commercial kit Amersham TRK 432. The response of three concentrations of standard 1–34 b PTH and three concentrations of horse extract were used within the linear part of the log–dose response curves for calculation of biological activity, according to parallel-line assay methods (18).

Antibodies. Two types of N- and C-terminal RIA were run using different antibodies.

Three goat antisera against the 1–34 h PTH Niall (13) which show complete cross-reaction with human PTH, partial cross reaction with synthetic 1–34 h PTH Brewer and no cross-reaction with b PTH.

Three goat antisera against synthetic 1–34 h PTH Brewer (20)[2] (generously given by Dr. Arnaud) which give partial cross-reaction with h PTH and 1–34 h PTH Niall and no cross-reaction with b PTH.

A guinea pig antiserum against natural bovine PTH (19) (As 1D153) which gives complete cross-reaction with h PTH and no cross reaction with synthetic 1–34 h PTH Niall, 1–34 h PTH Brewer, and 53–84 h PTH.

A guinea pig antiserum against natural porcine PTH (As A150)[2] generously given by Dr. Arnaud which shows complete cross reaction with h PTH, b PTH, synthetic 53–84 h PTH, and no cross-reaction with 1–34 h PTH Niall and 1–34 h PTH Brewer.

Immunoassays. The nonequilibrium assays were performed by incubation of the antiserum in barbital buffer 0.05 *M*, pH 8.6, during 3 days in the presence of different concentrations of unlabeled hormone or parathyroid extract, then 4 days in the presence of 2000 cpm (= 10 pg) of the corresponding ^{125}I-labeled hormone. Free hormone was separated from bound hormone by adsorption on Dextran–charcoal (21). Results are expressed in nanograms per milliliter equivalent of standard hormone.

Mild acid cleavage was achieved by incubating horse parathyroid extract in acetic acid solution (0.1 M) during 2 months at 4°. Separation of fragments was obtained by gel filtration on an Ultrogel Ac 202 column equilibrated with 0.05 *M* barbital buffer, pH 8.6, in the presence of 6% of human plasma. The column was previously calibrated with ^{125}I-labeled bovine PTH and ^{125}I-labeled 1–34 h PTH Niall. Fractions were assayed by N- and C-terminal RIA.

Results. The extract of horse parathyroid was bioassayed first *in vitro* against the active synthetic 1–34 b PTH. As parallel log–dose response curves were obtained (Fig. 1) the relative specific activity of the

[2] Generously given by Dr. Arnaud (University of California, San Francisco).

[3] Generous gift from Dr. Parsons (MRC London).

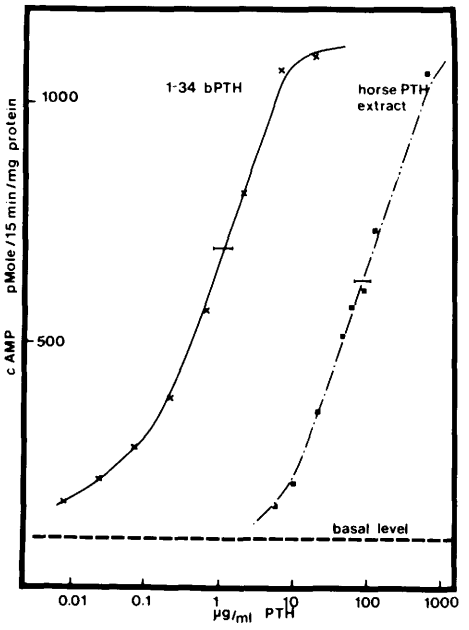

FIG. 1. Dose response curves of (1–34) b PTH x and horse parathyroid extract ■ in the rat renal cortical adenylate cyclase *in vitro* assay. Adenylate cyclase is assayed with an ATP regenerating system; cAMP is measured by a competitive protein binding assay.

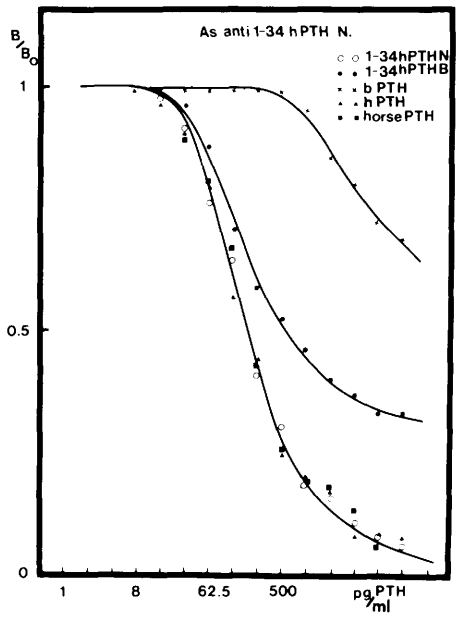

FIG. 2. Competitive inhibition of binding of ^{125}I-labeled (1–34) h PTH Niall by horse parathyroid extract ■; h PTH ▲; b PTH x; (1–34) h PTH Niall ○; (1–34) h PTH Brewer * in a nonequilibrium radioimmunoassay with anti-(1–34) h PTH Niall 1/250,000. Initial B/F ≈ 0.48.

extract could be calculated: 100 μg was equivalent to 1 μg of 1–34 b PTH, i.e., 280 pmole or 60 MRC units/mg since the 1–34 b PTH was found in this system to possess a specific activity around 6000 MRC units/mg.

Similar results were found when horse parathyroid extract was studied with the N-terminal immunochemical systems using antisera anti-1–34 h PTH Niall diluted 1/250,000 with a sensitivity up to 70 pg/ml. In this system horse extract gave displacement curve parallel to those of the human PTH and synthetic 1–34 h PTH Niall fragment (Fig. 2) and 100 μg of horse parathyroid extract corresponded to 1.2 μg of 1–34 h PTH Niall fragment. In the immunochemical system using antisera raised against synthetic 1–34 h PTH Brewer fragment horse extract gave only partial cross reaction (Fig. 3) and the displacement curve was very similar to those shown by 1–34 h PTH Niall fragment and human PTH.

The antibodies against the 1–84 b PTH (As 1D153) and anti 1–84 p PTH (As A150) bind the complete molecule of human, bovine, and porcine PTH but not synthetic 1–34 N-terminal fragments of bovine or human PTH. They are directed toward the C-terminal part of bovine, porcine, and human PTH (35–84).

Figure 4 shows the results obtained with horse parathyroid extract in the presence of antibovine PTH (As 1D153). Human PTH, bovine PTH and horse parathyroid extract give parallel curves and the immunoassays give for the horse parathyroid extract equal value versus those found in the N-terminal immunochemical system Niall. But in the presence of antibodies anti p PTH (As A150) the horse extract does not give any cross reaction and is differentiated by this RIA from PTH of other species (Fig. 5).

Discussion. The similar behavior of horse parathyroid extract and human PTH in C-terminal and N-terminal RIA indicates that RIA may be used to detect and mea-

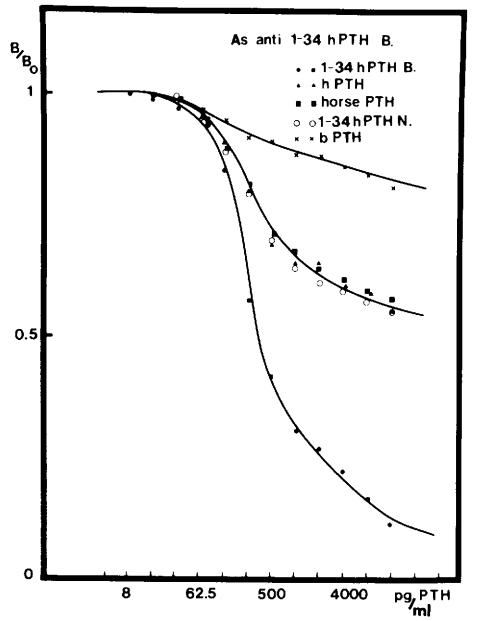

FIG. 3. Competitive inhibition of binding of ^{125}I-labeled (1–34) h PTH B by horse parathyroid extract ■; h PTH ▲; b PTH x; (1–34) h PTH Niall ○; (1–34) h PTH Brewer * in a nonequilibrium radioimmunoassay with goat anti-(1–34) h PTH Brewer 1/12,000. Initial B/F ≃ 0.51.

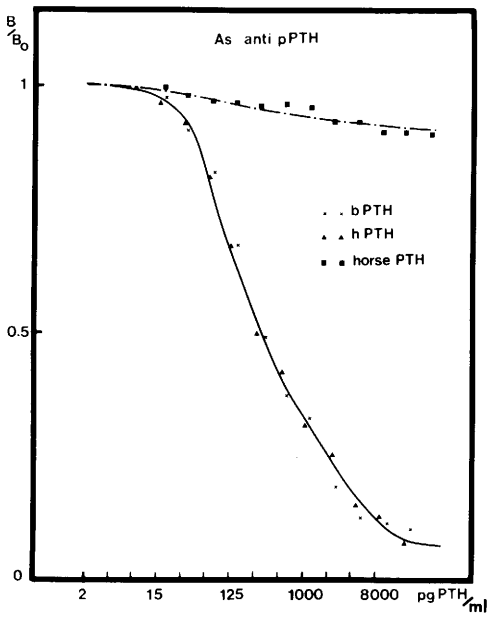

FIG. 5. Competitive inhibition of binding of ^{125}I-labeled bovine PTH by horse parathyroid extract ■; h PTH ▲; b PTH x; in C-terminal radioimmunoassays with guinea pig anti-porcine PTH (As A150) 1/15,000. Initial B/F 0.51.

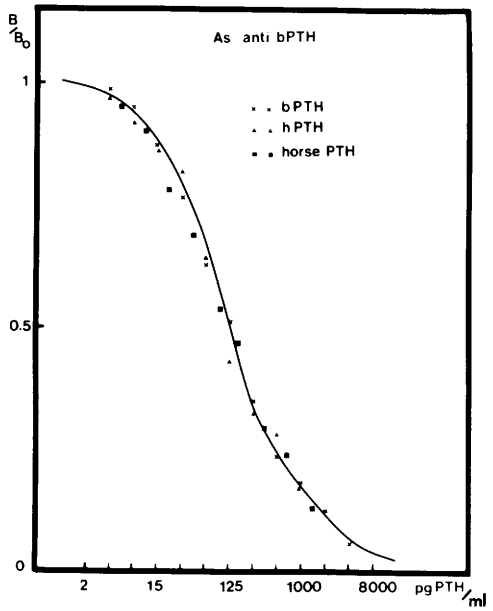

FIG. 4. Competitive inhibition of binding of ^{125}I-labelled bovine PTH by horse parathyroid extract ■; h PTH ▲; b PTH x; in C-terminal radioimmunoassays with guinea pig anti-bovine PTH (As 1D153) 1/40,000. Initial B/F 0.50.

sure the content of active PTH in the extract. The agreement between results of *in vitro* bioassay and RIA indicates a content of 2 to 3 ng of horse PTH in each lobe with a specific activity close to that of bovine PTH (6000 MRC units/mg). The extract apparently contains the complete molecule (1–84) in as much as elution volumes of horse PTH and b PTH were similar on the Biogel P 10 column.

Our immunochemical studies on PTH from different species show that N-terminal RIAs specific for both reported structures of human PTH are sensitive to differences in amino acid sequence of 1–34 fragments. As in both systems horse PTH gives cross reactivity almost identical to human PTH and synthetic 1–34 h PTH Niall, it suggests that structure of the N-terminal region of horse PTH is closely related to the human one, a consequence of a high similarity in the amino acid sequence.

Furthermore mild acid treatment of horse PTH cleaves it in several fragments only one of which is recognized by N-terminal

RIA. Results of gel filtration suggest an apparent molecular weight of approximately 3000 daltons similar to the 1–29 biologically active fragment produced by cleavage at Asp in position 30 for bovine PTH (22).

In the C-terminal systems using anti porcine antibodies (As A150) the horse PTH differs in cross reactivity from PTH of other species. Our immunochemical studies support the supposition that horse PTH differs from porcine, bovine, and human PTH in the C-terminal region (53–84) for the following reason. Anti-porcine PTH antibodies (As A150) which show total cross-reaction with synthetic 53–84 fragment of human PTH cross-react with all known PTH with the exception of horse PTH.

As anti-bovine PTH antibodies (As 1D153) which cross-react with horse PTH show no cross-reaction with fragment 53–84 of human PTH. The data do suggest that in the region of the site for these antibodies the structure of horse molecule is similar to bovine and human PTH.

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