A RADIOIMMUNOASSAY FOR PARATHYROID HORMONE IN MAN

1. Development of a radioimmunoassay for bovine PTH

By

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ABSTRACT

Bovine parathyroid hormone (PTH) was purified up to the Sephadex-stage. The biological activity of the preparations was about 2000 U/mg solid material. Rabbits and guinea-pigs were immunized with crude and Sephadex-purified material. Antisera elicited in two guinea-pigs proved to be useful for further studies. Purification of 125I-labelled bovine PTH was carried out by adsorption to and desorption from antibodies or by means of microfine silicate. The purified labelled hormone showed excellent binding to the antibodies. Maximum sensitivity of the assay system of 10 pg per incubation medium could be achieved by a pre-incubation method. The selectivity of the system was studied.

For the development of a radioimmunoassay for parathyroid hormone which can be used for human plasma, the plasma human hormone is preferred. Up to the present, however, it has been impossible to obtain the purified human hormone in sufficient quantity to produce antibodies.

The second best choice is to develop a system involving the use of an animal hormone, which elicits antibodies cross reacting with the human hormone. Radioimmunoassays for bovine parathyroid hormone have been set up by several investigators. Virtually complete or partial cross reactivity of the

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human hormone with the anti-bovine PTH antibodies has been reported. Successful immunization of rabbits with parathyroid hormone (PTH) preparations has been described by Tashjian et al. (1962) and Williams et al. (1964).

Berson et al. (1963) immunized rabbits and guinea-pigs with crude PTH preparations. The antibodies elicited in a guinea-pig were useful for a sensitive radioimmunoassay. This species proved to be the most suitable for the development of bovine PTH (b-PTH) antisera. Thus Sherwood et al. (1967) used guinea-pig b-PTH antibodies in a radioimmunoassay with which they could measure picogram (pg) amounts of hormone in bovine, ovine and caprine plasma.

This study reports on the successful production of antibodies against b-PTH preparations in guinea-pigs. Details of the iodination procedure and subsequent purification of $^{125}$I-b-PTH are described as well as the conditions for a sensitive radioimmunoassay for bovine parathyroid hormone.

**M A T E R I A L**

Defatted and acetone-dried bovine parathyroid glands were extracted with aqueous phenol 70 % w/v (Aurbach 1959). The extract was fractionated with acetone and acetic acid, ether and sodium chloride and the active principle precipitated with trichloroacetic acid (TCA-PTH) (Rasmussen et al. 1964). Cysteine (0.01 m) was added at two steps during the isolation in order to minimize loss of biological activity. The crude product (TCA-PTH) was further purified by two successive gel filtration experiments on Sephadex G-100 (2.0–2.9 × 95 cm), according to Aurbach & Potts (1964). On amino acid analysis the Sephadex-purified PTH preparation was found to resemble closely the amino acid composition of carboxy-methylcellulose purified bovine PTH preparations (Potts & Aurbach 1965; Hawker et al. 1966). However, by means of polyacrylamide gel electrophoresis at pH 4.3 and 2.8, our Sephadex-purified PTH preparations proved to be heterogeneous (2–3 bands).

In spite of these observations, they were found to have near-maximum biological activity, viz. 2000 U/mg solid material. This value is about equal to that (2000–3000 U/mg found by Potts et al. 1966).

The biological activity of the preparations was tested in parathyroidectomized rats according to Munson's method (Munson 1961) with some modification. The modifications consisted of a substitute for the low-calcium diet used by Munson (1961). A mixture of proteins, fats, minerals and vitamins (Calcinon®; Nutricia, Zoetermeer) was mixed with an equal amount of sucrose. With distilled water a paste was made, which was readily eaten by the rats. The paste contained 30 mg Ca/100 g. The animals were placed on this diet for three days before parathyroidectomy (PTX); 2. PTX was performed by electric cautery 24 h before decapitation. Eighteen hours after PTX blood was drawn by orbital puncture and analysed for calcium. Animals which had not responded adequately to PTX (serum calcium > 4.0 meq/l) were discarded. For detail of the purification, the PTH-preparations and the bioassay cf. Lequim (1969).

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* Kindly performed by Ir. B. Goverde, N. V. Organon, Oss.
Production of antibodies

Eleven guinea-pigs and five rabbits were used for immunization. Of the guinea-pigs only two (310 and 311) gave antibodies with high affinity for the antigen suitable for a sensitive radioimmunoassay. Of these two antisera one (311) was of particular interest because it also reacted with human PTH (cf. Lequin et al. 1970). The quality of the antibodies produced in rabbits was less satisfactory. The parathyroid hormone preparations used for immunization were crude TCA-PTH 111 (biological activity 240 U/mg) and the highly purified Sephadex-PTH 135-3 (2000 U/mg).

The guinea-pigs 310 and 311 (males) were injected intramuscularly (im) with 2 mg of TCA-PTH 111 dissolved in 0.5 ml of acidified (pH 2) phys. saline; the solution was homogenized with an equal volume of complete Freund’s adjuvant. Thereafter they received 1 or 2 mg of the crude preparation im without adjuvant, at four-weekly intervals. A total of 11 mg was given over a period of 6 months. The animals were then injected im with the highly purified PTH preparation (0.1 mg) at monthly or bimonthly intervals. A total of 0.7 mg was given per animal in 10 months. During the whole course of immunization 4040 U of crude and purified PTH preparations were administered to each animal.

Iodination of bovine PTH

Sephadex-purified PTH 135-3 was used for iodination. The chloramine-T method of Hunter & Greenwood (1962) was used for labelling the hormone with $^{125}$I (Radiochemical Centre, Amersham). The hormone was dissolved in a small amount of 0.005 M HCl and diluted with 0.05 M phosphate buffer to a concentration of 5 µg/10 µl.

Chloramine-T (British Drug House) was dissolved in 0.05 M phosphate buffer so that 10 µl contained 100 µg.

During the last months the micromodification for the iodination procedure proposed by Brown & Reith (1967) has been used. Microlitre quantities of the reagents and air – for mixing purposes – are sucked in a polyethylene tube in the sequence of 40 µl air, 10 µl Chloramine-T solutions, 5 µl air, 5 µl 0.5 M phosphate buffer, 5 µl air, 10 µl hormone solution, 5 µl air, and 5 µl 0.5 M phosphate buffer. Subsequently they are expelled into the reaction tube into which 2 mCi $^{125}$I has previously been pipetted (10–30 µl – the volume depending on the specific activity).

The iodination reaction is stopped by addition of sodium metabisulphite (240 µg/0.1 ml) and thereafter carrier iodide is added (2 mg KI/0.2 ml).

The iodination time never exceeded 1 min and was usually 30 seconds.

The iodination mixture – acidified with a few drops of 1 N HCl – is transferred to a Sephadex G-15 column (110 × 6.5 mm), in order to separate the labelled hormone from unreacted $^{125}$I. The column is slowly eluted with pH 3 buffer (for buffer composition see below).

Specific activities of 100–250 µCi/µg were usually obtained.

Conditions for the prevention of adsorption of PTH to glassware

Because it was found in earlier experiments that parathyroid hormone is absorbed to glassware (see also Rosselin et al. 1966), precautions were taken to minimize this loss. Tubes* made of neutral glass proved to be particularly useful for this purpose.

They have been used for the iodinations, for collection of eluates from Sephadex columns and also for the incubation mixtures of the radioimmunoassay.

There is less glass-adsorption at acid pH. Therefore the procedure of purification of the iodination mixture as well as the dilution of standard and labelled hormone for the immunoassay were done with a buffer consisting of 0.14 M sodium chloride and 0.25% bovine serum albumin (BSA: N. V. Poviet, Amsterdam) adjusted to pH 3.2 with 1 N HCl and to which 10^-4 Merthiolate (Eli Lilly) was added as preservative.

This buffer will now be called pH 3 buffer.

**Purification of ^125^I-PTH**

Labelled PTH was purified by means of antibodies as described for the purification of ^125^I-glucagon (Schopman et al. 1967). Antiserum is added to the fraction(s) of the Sephadex G-15 column, so that 50% of the hormone is bound to the antibodies when incubated overnight at 4°C. The amount of antiserum is calculated on the basis of the specific activity obtained for the labelled hormone and the binding capacity of the antiserum used – e.g. undiluted antiserum 310 binds 1.6 µg/ml. The antibody-bound ^125^I-hormone complex is isolated by gelfiltration on Sephadex G-75 (0.1 M phosphate buffer pH 7.5). The complex is dissociated by acidification of the mixture to pH 2 and incubation overnight at 4°C. The purified labelled hormone is recovered by gelfiltration on Sephadex G-75 (pH 3 buffer). For details of the procedure cf. Schopman et al. (1967).

Another method of purification was introduced by Yalow & Berson (1966). They took advantage of the adsorption of parathyroid hormone to glass. After iodination, the labelled PTH is adsorbed into microfine silicate (Quso G-32; Philadelphia Quartz Co.) in the presence of a large amount of normal human plasma. The labelled PTH is then desorbed from the silica by a mixture of 20% acetone in 1% acetic acid. It was found, however, that desorption was optimal with 40% acetone in 1% acetic acid, as also mentioned by Yalow (1968).

Acetone and acid are removed by evaporation under vacuum. The residue is taken up in 1 ml pH 3 buffer. This mixture still contains small amounts of human plasma proteins; these are removed by passage over a Sephadex G-75 column (pH 3 buffer as eluent).

^125^I-PTH prepared by either method was relatively stable and could be used for several weeks when stored at -15°C.

**Preparation of incubation mixtures**

The incubation mixtures are made up to a total volume of 0.5 ml with 0.1 M phosphate buffer pH 7.5 to which 1% BSA and 10^-4 Merthiolate are added. The volume is found by subtracting the following additions from the final volume: 1. 50 µl hypoparathyroid plasma; 2. 50 µl Trasylol (Bayer; 500 KIU); 3. 0, 10, 25 or 50 µl of standard solutions containing 1 pg/µl or 10, 20, 30, 40, 50 or 75 µl of a standard solution of 10 pg/µl or an appropriate amount of the test samples.

The standard (PTH 135-3) and the test samples are dissolved in and diluted with pH 3 buffer to a concentration of 1 ng/µl. The solutions of 1 pg/µl and 10 pg/µl are always freshly prepared. The stock-solution of the standard does not deteriorate when stored at -15°C for months; 4. 25 µl of labelled hormone solution containing 6000-10 000 cpm and pH 3 buffer as diluent is used; 5. 50 µl of antiserum at appropriate dilution, the diluent for the antiserum being 0.14 M sodium chloride containing 0.01% BSA and 10^-4 Merthiolate.
After addition of the reagents the tubes are stoppered and mixed on a Vortex-mixer. Incubation takes 3–4 days at 4°C.

**Separation of bound and free hormone**

Different methods for separation of antibody-bound (B) and free (F) hormone were used during the experiments. Hydrodynamic flow on Whatman 3 MM paper (Berson et al. 1957) with 0.1 M barbitone buffer pH 8.6 gave rather variable results.

In this method the free hormone is adsorbed onto paper at the spot at which the samples are applied. The antibody-bound hormone is displaced by the buffer flow.

Adsorption of free hormone (F) to microfine silicate (Quso G-32; 2.5 mg in a volume of 1.0 ml of 1.0 M phosphate buffer pH 7.5 was added per tube) gave better results (Rosselin et al. 1966), but a modification of the dextran-coated charcoal method described by Herbert et al. (1965) for insulin, proved to be the most reliable. 1 ml of a dextran-coated charcoal suspension is added to the tubes. The suspension consists of 2 g charcoal in 200 ml 0.1 M phosphate buffer pH 7.5 containing 0.3 g Dextran-80 (Pharmacia). After mixing, the tubes are centrifuged at 3000 r.p.m. for 5 min. B and F are separated by means of a Pasteur pipette. Both fractions are counted in a gamma-spectrometer (Baird Atomic 707).

The separation of B and F by paper, Quso or dextran-coated charcoal depends on the amount of serum proteins present in the incubation mixtures.

For Quso separation a total amount of 200 µl of serum/plasma was found to be optimal, whereas for separation on paper or by charcoal a total amount of 100–150 µl was required.

**Statistical analysis**

In the experiment shown in Fig. 5 the inhibition curves were transformed to a plot of % bound vs. log pg added.

This transformation yields a slightly S-shaped curve; by rejecting the extreme points of the upper and lower part of the curve, a linear part is obtained. The regression coefficients were calculated for this linear part. A significance test was done for differences between the regression coefficient of each test preparation and that of the standard (PTH 135-3). The differences in slope between the two lines compared is considered significant for P < 0.05.

**RESULTS**

Antibodies against bovine PTH preparations have been successfully elicited in guinea-pigs. Fig. 1 gives the results obtained with antiserum 311 in the course of a year and a half. The amounts of 125I-PTH used to test these antisera, were different but these differences are relatively small in comparison with the differences in antibody titre of the sera. All three curves could be used to establish the useful range of antiserum dilution for a sensitive radioimmunoassay. The antisera of guinea-pigs 310 and 311 could be used in a final dilution of 1:10 000 to 1:24 000.

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* Kindly performed by Mr. A. Delver of the Statistical Dept. of N.V. Organon, Oss.
The antibodies showed high affinity for the antigen. The value for the equilibrium constant $K$ for the antigen-antibody reaction was $4.9 \times 10^{10}$ L/M for antiserum 310 and $1.7 \times 10^{10}$ L/M for antiserum 311.

The purification of $^{125}$I-PTH proved to be difficult. In earlier experiments the labelled hormone was purified by gelfiltration on a Sephadex G-75 column (0.1 M phosphate buffer pH 7.5 containing 0.25% BSA and $10^{-4}$ Merthiolate) as is usual in this laboratory for the purification of labelled insulin and human growth hormone. The purified $^{125}$I-PTH obtained by this procedure though only slightly damaged as measured on Whatman 3 MM paper, showed what is in our experience poor binding to a relatively large amount of antibodies (Fig. 2).

This poor binding was not due to the fact that little antibody had been produced, since large amounts of unlabelled hormone were required to inhibit the binding of the labelled hormone. It seemed more likely that the $^{125}$I-PTH still contained labelled artifacts which did not react with the antibodies. An attempt was therefore made to purify labelled PTH by binding to antibodies as described by Schopman et al. (1967). As shown in Fig. 2 this purification resulted in an increased binding of $^{125}$I-PTH to a fixed amount of antibodies.

Because of its longer half-life, we used $^{125}$I for all labelling experiments rather than $^{131}$I. As 1 mCi of $^{125}$I represents about 8 times the number of iodide atoms of $^{131}$I, and as parathyroid hormone contains only one tyrosine residue per molecule, the poor binding of $^{125}$I-PTH in the earlier experiments could
Purification of $^{125}$I-PTH and $^{131}$I-PTH.

Ordinate: percentage bound to a fixed amount of antibodies of gel filtration experiments on Sephadex G-75 columns (300 X 13.5 mm).

Abscissa: Left: fractions of purification experiments of the tracer hormones by single gel filtration at pH 7.5.

Right: fractions of the final gel filtration at pH 3.2 after adsorption of the tracer hormones to and dissociation from the antibodies (see text).

25 µl $^{125}$I-PTH contained 140 pg of hormone; 25 µl of $^{131}$I-PTH contained 50 pg.

have been due to over-iodination. Therefore a labelling experiment with $^{131}$I was performed for comparison. Fig. 2 shows that $^{131}$I-PTH purified by gel filtration on G-75 (pH 7.5) did not bind better than $^{125}$I-PTH.

It was found that labelled hormone preparations purified via either antiserum 310 or 311, gave virtually the same standard curves independent of which of the antisera was used in the incubation mixtures.

Fig. 3 shows the results for antiserum 311 in the incubation mixtures. Other antisera were not tested for their usefulness in this purification procedure.

Purification of labelled PTH by adsorption to microfine silicate (Quso) in the presence of human plasma, as suggested by Yalow & Berson (1966), gave good results. It was necessary, however, to remove the small amounts of human plasma proteins present in the final product, by gel filtration on Sephadex G-75. The $^{133}$I-PTH obtained by this procedure showed excellent binding to antibodies and gave the same highly sensitive standard curve as did the labelled hormone purified via antibodies.

Schopman et al. (1967) have shown that there is a definite pH-optimum for the antigen-antibody reaction of $^{125}$I-glucagon with rabbit antibeef-porc glucagon antibodies. This pH dependence was also studied for the guinea-pig-anti-b-PTH antibodies. In phosphate buffer – pH range 6.5–8.0 – optimal B/F ratios were obtained (Fig. 4) and hence 0.1 m phosphate buffer pH 7.5 was chosen as the medium for the incubation mixtures.

With the present system standard curves were produced with which very small amounts of hormone could be detected.
Progressive decrease of the Bound/Free (B/F) ratio with increasing amounts of unlabelled parathyroid hormone (picograms PTH 135-3). Antiserum 311 was used in the incubation mixtures (1:12 000). The tracer hormone was purified via antibodies (antiserum 310 ———; antiserum 311 ———).
Inhibition curves (B/F ratio vs. increasing amounts of unlabelled hormone) for various PTH preparations with different biological activity. The data are plotted as % Bound vs. log picogram unlabelled hormone; the lines have been calculated by the method of least squares (25 µl of ¹²⁵I-PTH contained 40 pg PTH; allowance was made for this amount in the calculation of the regression lines).

**Fig. 5.**

The slopes of the calculated regression lines of all the preparations, except PTH 135–2, were not significantly different from that of the standard \( P > 0.05 \). For PTH 135–2 a significance level \( 0.05 > P < 0.01 \) was found.

In the insert: The standard curve is shown for PTH 135–3 plotted as B/F ratio vs. pg unlabelled hormone added.

Immunoelectrophoresis of normal guinea-pig serum and immune anti-b-PTH guinea-pig serum was carried out. The grooves were filled with ¹²⁵I-PTH and thereafter with rabbit anti-normal guinea-pig serum or rabbit anti guinea-pig IgG.

 Autoradiography showed that the labelled hormone – purified via antibodies – reacted only with gammaglobulin of the guinea-pig anti-b-PTH sera. Aspecific binding was not observed since no radioactivity was adsorbed to normal guinea-pig serum proteins or other components of the immune guinea-pig serum. A variety of other polypeptide hormones such as human growth hormone, beef-porc glucagon, human-, bovine- and porcine insulin as well as porcine calcitonin sulphoxyde did not depress the binding of labelled hormone to antibodies.

PTH preparations with different biological activities were tested in the immunological system. The results shown in Fig. 5 indicate that preparations

651
with a biological activity of 2000 U/mg – viz. the Sephadex-purified preparations 121-2 and 121-3 as well as a carboxymethylcellulose purified PTH preparation (prepared by Dr. G. D. Aurbach) – gave inhibition curves parallel to that of the standard (PTH 135-3) ($P > 0.05$).

Crude TCA-PTH 111 (biological activity 240 U/mg also behaved like the standard ($P > 0.05$). However, with regard to the PTH preparation 135-1 and 135-2 which are minor peaks preceding PTH 135-3 on a gelfiltration experiment on Sephadex G-100 – the latter is immunologically different from the standard ($0.05 > P > 0.01$), while the former is not statistically different ($P > 0.05$). This indicates that by refiltration over G-100, immunological different polypeptides are separated from the main PTH polypeptide(s). The immunological activities of the preparations are given in Table 1. For the purpose of calculation, the biological and immunological activities of the standard 135-3 are assumed to be equal. It is interesting to note that except for the crude PTH preparation, the relative immunological activities of the different preparations agree closely with the biological activities. Such an agreement between the two activities of polypeptide hormone is not always found.

**DISCUSSION**

Bovine parathyroid hormone proved to be a rather weak antigen when injected into rabbits and guinea-pigs, as could have been expected from data in the literature. The most useful antisera with a high affinity for the antigen, were obtained from two guinea-pigs; they could be used in a radioimmunological

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Immunological* activity</th>
<th>Biological activity</th>
<th>I/B** index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex-PTH 135-3</td>
<td>2000</td>
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<tr>
<td>CMC-PTH***</td>
<td>2050</td>
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<tr>
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<td>620</td>
<td>240</td>
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* PTH 135-3 as standard with an assumed potency of 2000 immunological U/mg.
** Ratio of immunological/biological activities.
*** Kindly made available by Dr. G. D. Aurbach (N. I. H. Bethesda) for use as standard in the bioassay of PTH preparations. The biological activity of this material was stated to be 2000 U/mg.
system with a sensitivity of 10–100 pg of hormone, depending on the conditions. This sensitivity is equal to that reported by Sherwood et al. (1967).

It was interesting to find in the procedure of purification of labelled hormone via antibodies that the trace quantities of labelled hormone were not easily damaged by acidifying the antibody-125I-PTH complex to pH 2.

This means that the purification of bovine and perhaps also of human parathyroid hormone extracts can be performed by immunological methods. It is noteworthy that the immunological activities of several PTH preparations varied in accordance with the biological activities, with the exception of the crude TCA-PTH. Tashjian et al. (1964) also reported that the biological activities of different PTH preparations were in good agreement with their immunological activities (measured by complement-fixation technique). The close agreement could be explained if it were known that the same amino acid sequence is essential for biological and immunological activity.

Potts et al. (1968) showed that the biological and immunological centres are both situated in the same part of the molecule, viz. the first 35 N-terminal amino acids, and probably close to each other. It is not yet clear, however, to what extent they overlap. In view of this work, we are inclined to assume that the crude PTH preparation TCA-PTH, contains fragments of the PTH molecule which show some immunological reactivity in the system but which is biologically inactive.

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References


653
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