

PREPARATION OF BIOLOGICALLY ACTIVE TRITIUM-LABELLED 1-34 HUMAN PARATHYROID HORMONE

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1. Introduction

Tritiated bovine parathyroid hormone (1-84 bPTH), has already been prepared by Zull and Repke [1] with the technique of acetamidination of the free amino-groups of this peptide. A fully active, 80% labelled PTH was obtained [2]. We have applied to 1-34 human PTH (1-34 hPTH) labelling another substitution reaction previously used for luteinizing hormone [3]. This technique is based upon the reductive methylation of the free amino-groups with tritiated sodium borohydride. Using this technique we have labelled 1-34 human PTH with 25% efficiency (methylation of one free amino-group over four). The [³H]PTH-derivative obtained retains its full biological potency and specifically binds to its renal receptors.

2. Experimental

2.1. Materials

1-34 hPTH was a gift of Armour-Montagu (Paris, France). This synthetic peptide corresponds to the amino-acid sequence proposed by Niall and his colleagues [4]. It contains three lysine residues at positions 13, 26 and 27 and one free terminal amino-group belonging to a serine residue. ¹²⁵I-labelled 1-34 hPTH (120 µCi/µg) and anti 1-34 hPTH raised in the goat were donated by Dr Moukhtar (Paris, France). Tritiated sodium borohydride (5-15 Ci/mmol) was obtained from 'Centre d'Etudes Nucléaires' (Saclay, France). All chemicals and solvents were of analytical grade and obtained from

Sigma (St-Louis, Mo. USA) or Merck (Darmstadt, FRG).

2.2. Preparation of ³H-labelled 1-34 hPTH

The methylation reaction of 1-34 hPTH was performed as previously published for luteinizing hormone [3]. The final concentrations of tritiated sodium borohydride and 1-34 hPTH were 0.8 mg/ml and 1 mg/ml, respectively. The tritiated material was purified by filtration through a Sephadex G-50 (Pharmacia, Paris, France) column (1.5 × 30 cm). The column was eluted with 0.1 M ammonium acetate buffer, pH 4.7 and fractions of 1 ml collected. The following determinations were then performed in each tube:

- (1) 10 µl eluate without any treatment were kept for counting total radioactivity.
- (2) 10 µl were mixed with 300 µl human plasma; then 2 ml 10% trichloroacetic acid (TCA) were added. After centrifugation, 100 µl supernatant were counted. This corresponded to TCA non-precipitable radioactivity. Total radioactivity was obtained in the same conditions of protein concentration from a control tube in which water was substituted for the TCA solution. TCA precipitable radioactivity was the difference between total and TCA non-precipitable radioactivity. The fraction of TCA precipitable over total radioactivity obtained in these conditions was applied to total radioactivity measured without addition of protein to obtain the absolute value of TCA precipitable radioactivity.

(3) 10 μ l were incubated with an excess of 1–34 hPTH antibodies (1/250, final) in 500 μ l 0.05 M barbituric acid–sodium barbiturate buffer, pH 8.6, with 0.5% human serum albumin during 2 days at 4°C. Separation of bound and free radioactivity was performed using the charcoal–dextran technique and 100 μ l supernatant (bound radioactivity) counted. A control tube was similarly treated except for the addition of charcoal–dextran which was replaced by the same volume buffer, 100 μ l of this solution were counted and corresponds to total radioactivity. The ratio bound over total radioactivity obtained in these conditions was applied to total radioactivity directly measured, to obtain the absolute value of antibody bound radioactivity. Tritium was determined by scintillation in 8 ml Bray's solution [5] with a Packard Tri-carb liquid scintillation counter. Three elution patterns (total,

TCA precipitable and antibody bound radioactivity) were thus obtained (fig.1). The tubes with more than 90% antibody bound radioactivity were fractionated into 100 μ l aliquots and stored in liquid nitrogen.

2.3. Assay of ^3H -labelled 1–34 hPTH immunoreactivity

50 pg of ^{125}I -labelled 1–34 hPTH were incubated at 4°C for 2 days with specific anti-1–34 hPTH antibodies (1/160 000, final concentration) in 0.5 ml 0.05 M barbituric acid–sodium barbiturate buffer, pH 8.6, containing 0.5% human serum albumin and increasing amounts of either unlabelled (ng) or tritiated (μCi) 1–34 hPTH. Bound and free radioactivity were separated using the charcoal–dextran technique and ^{125}I was counted using a Nuclear Chicago spectrometer with 55% efficiency.

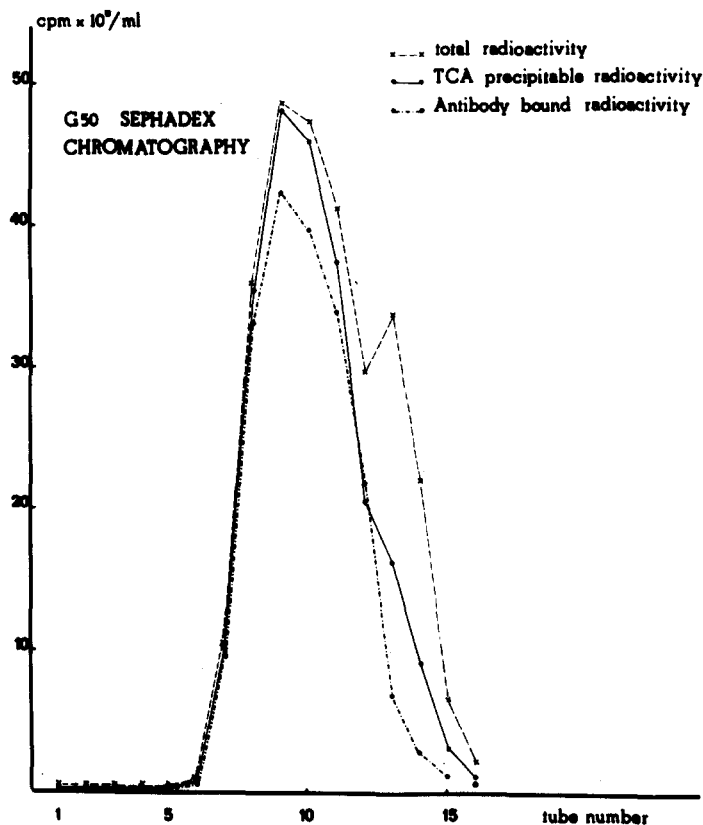


Fig.1. Gel-filtration of ^3H -labelled 1–34 hPTH on a Sephadex G-50 column. Elution patterns of total, TCA precipitable and antibody bound radioactivity are shown.

2.4. Biological assay of ^3H -labelled hPTH

Biological activity of ^3H -labelled hPTH was tested in vitro on a preparation of renal tubular membranes. These membranes were purified from the renal cortex of male Sprague-Dawley rats (Iffa-Credo, Fresnes, France) according to the technique of Fitzpatrick et al. [6]. Adenylate cyclase activity present in this preparation was measured in the presence of increasing doses of either unlabelled or ^3H -labelled 1–34 hPTH. 100 μg tubular membrane protein were incubated at 37°C for 20 min with hPTH in 100 μl 0.1 M Tris–HCl buffer, pH 7.5, containing 1 mM unlabelled ATP, 10 mM magnesium chloride and a regenerating system for ATP as previously described [7]. The enzymatic reaction was stopped by boiling the incubation milieu for 3 min after addition of 150 μl Tris–HCl buffer. The amount of 3′–5′-cyclic AMP formed was measured by radioimmunoassay using its iodinated [^{125}I]-succinyl derivative as a tracer (Institut Pasteur kit, Lyon, France). Adenylate cyclase activity was expressed as pmol cyclic AMP formed/20 min/mg tubular membrane protein.

2.5. Direct receptor studies with ^3H -labelled 1–34 hPTH

80 μg rat tubular membrane protein [6] and 2–5 pmol ^3H -labelled 1–34 hPTH were incubated at 10°C in 100 μl 50 mM Tris–HCl, pH 7.4, containing 5 mM dithiothreitol and 100 μM phenylmethylsulfonyl fluoride. At the end of the incubation period 3 ml of this same chilled Tris–HCl buffer were added. The total volume was filtered through a Millipore filter (HAWP, 0.45 μm). The filter was dried and ^3H -radioactivity counted. The blank value corresponding to radioactivity adsorbed on the filter in the absence of membranes was approximately 0.2% total radioactivity. Non specific binding was measured in the presence of 20 μM 1–34 hPTH and subtracted from total binding to obtain specific binding.

2.6. Indirect receptor studies with ^3H -labelled 1–34 hPTH

The labelled antibody membrane assay described by Mac Intosh and Hesck [8,9] with 1–84 bPTH and chicken renal membranes was used in the same conditions as those followed by these authors. Chicken membranes were initially preferred to rat membranes since they were considered as inactivating PTH at a

much slower rate [10]. Antiovine PTH antibody (Dr Bouillon, Rega Institute, Louvain, Belgium) cross-reacting with human PTH was purified according to Miles and Hales [11] and iodinated with ^{125}I by the technique of Hunter and Greenwood [12]. 1–84 Human parathyroid hormone (Standard 75/549, MRC Mill Hill, London) was incubated at increasing concentrations with renal cortical plasma membranes from chicken (100 μg protein/tube) in 200 μl 50 mM Tris–HCl buffer, pH 7.4, at 24°C for 2 h. Renal plasma membranes from chicken were prepared similarly to those obtained from rats [6]. After washing, membranes were incubated with labelled antibody for 1 h. After separation of the membranes from the incubation milieu and washing, ^{125}I bound was counted in a gamma counter (Berthold, Wildbad, FRG) with about 50% efficiency. Radioactivity due to non specific binding of antibody to membranes and to the tube was subtracted. The inhibitory effects of unlabelled and ^3H -labelled 1–34 hPTH on binding were compared. Increasing doses of these two preparations were incubated with 50 ng/ml 1–84 hPTH. Since the antibody used did not cross-react with 1–34 hPTH it was possible to estimate the inhibition of binding of 1–84 hPTH.

3. Results and discussion

3.1. Specific radioactivity and degree of alkylation of ^3H -labelled 1–34 hPTH

^3H -labelled 1–34 hPTH inhibited the binding of ^{125}I -labelled 1–34 hPTH to specific antibodies similarly to unlabelled 1–34 hPTH. The curves (B/T versus \log_2 dose) obtained with both preparations were parallel (fig.2). The slopes of the regression lines were -2.053 and -1.825 dose unit $^{-1}$ for unlabelled and tritiated 1–34 hPTH respectively ($F = 0.56$, non-significant). This allowed calculation of specific radioactivity of [^3H]hPTH which was 5.5 Ci/mmol. Since there are three lysine residues and a free terminal amino-group in the amino acid sequence of 1–34 human PTH, one can consider that four amino-groups are apt to be methylated. The specific radioactivity of sodium borohydride used as precursor was approximately 10 Ci/mmol. Admitting that the only tritium and the three hydrogens of sodium borohydride randomly participate to the exchange reaction, the

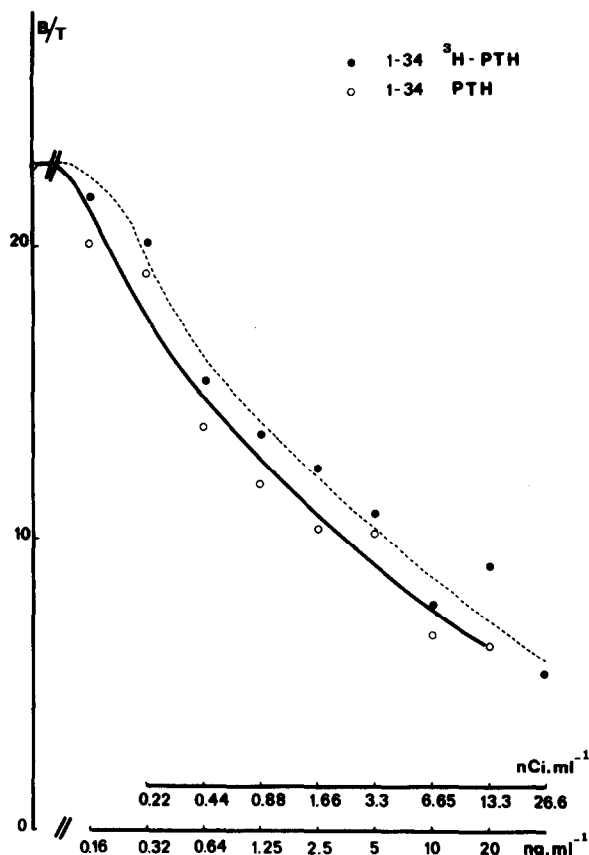


Fig. 2. Assay for ^3H -labelled 1–34 hPTH immunoreactivity. Bound over total ^{125}I -labelled 1–34 hPTH is plotted against increasing concentrations of either unlabelled (open circles) or ^3H -labelled (closed circles) 1–34 hPTH antibody. 1–34 hPTH antibodies were used at 1/160 000 final concentration.

theoretical maximum specific radioactivity would be 20 Ci/mmol if the four amino-groups had been methylated. The measured specific radioactivity corresponds to one-fourth of the theoretical maximum and indicates either dimethylation of one amino-group or monomethylation of two amino-groups over four. The present data does not allow to localize the site of

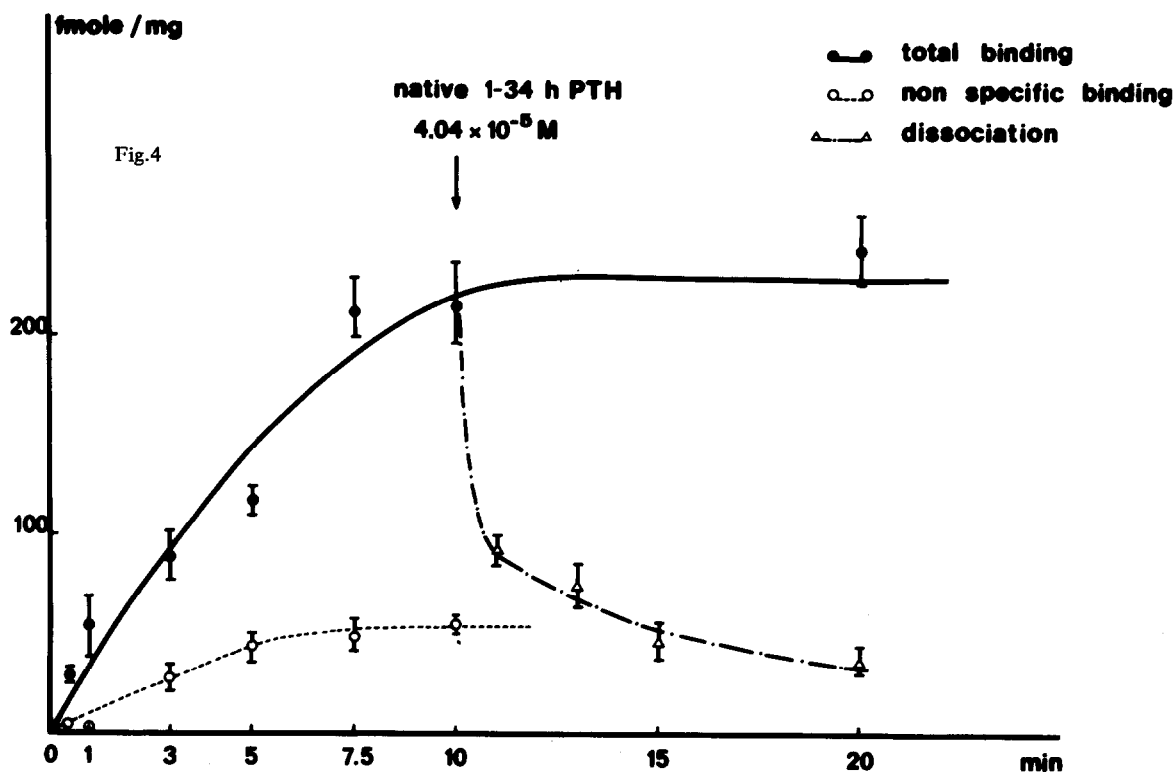
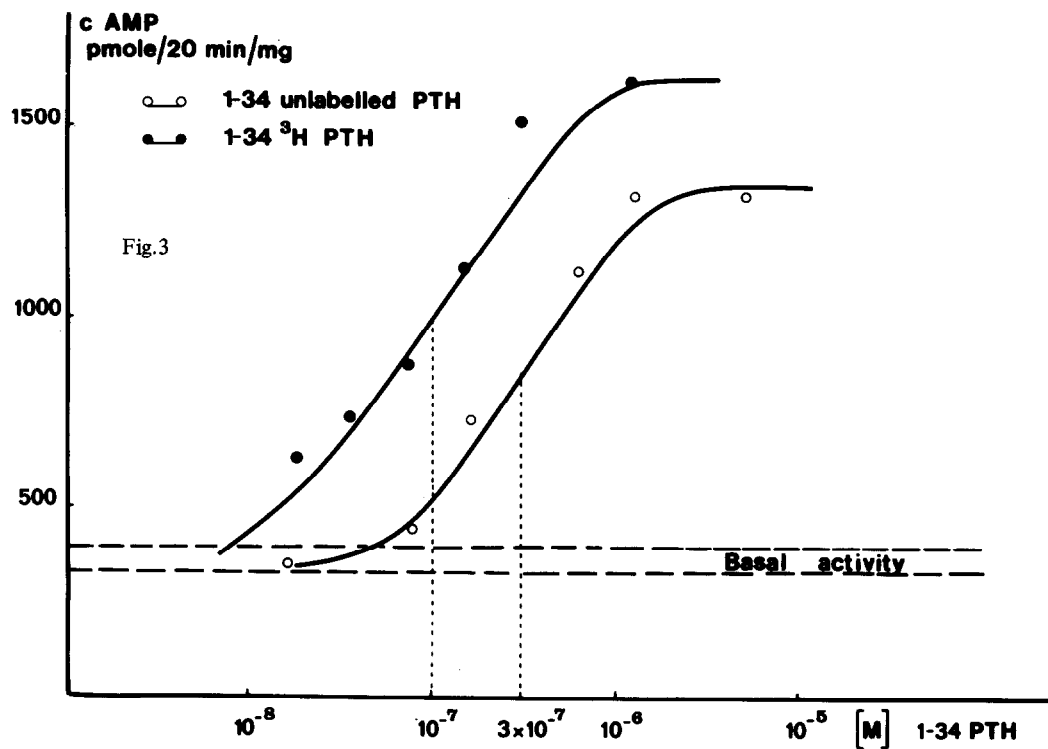
the substitution. Specific radioactivity of ^3H -labelled 1–34 hPTH is lower than that obtained by Zull and Chuang [2] with acetamidination of 1–84 bPTH. This is due to the ten free amino-groups present in the entire bovine peptide of which eight were reactive. However specific radioactivity of [^3H]acetonitrile used as precursor is four times smaller than that of sodium [^3H]borohydride.

3.2. Biological potency and binding to renal receptors of ^3H -labelled 1–34 hPTH

Adenylate cyclase activity present in tubular membranes was expressed on a weight basis using for calculation of ^3H -labelled 1–34 hPTH concentration its measured specific radioactivity. The curves obtained were parallel. Maximum stimulation was observed at 1 μM PTH and was approximately 20% greater with ^3H -labelled 1–34 hPTH. The apparent K_m were estimated from the concentrations corresponding to 50% maximum activity and were in the same range, 0.1 μM and 0.3 μM for ^3H -labelled and unlabelled 1–34 hPTH respectively (fig.3). This shows that the biological response to both PTH preparations was similar with a slightly greater potency for ^3H -labelled 1–34 hPTH. However the degree of accuracy obtained in measuring specific radioactivity or a difference in the rates of degradation of both preparations in the presence of renal membranes could easily explain this difference. Activation of adenylate cyclase by ^3H -labelled 1–34 hPTH suggested preliminary binding of the hormone to its renal receptors. Evidence for this binding was provided both by direct and indirect methods. Direct binding of ^3H -labelled 1–34 hPTH (20–50 nM, final concentration) was studied in time-course experiments (fig.4). The amount of ^3H -labelled 1–34 hPTH specifically bound increased with time and reached a plateau after 10 min incubation. Non specific binding was constant after 5 min and equal to 25% of the total binding observed at equilibrium. Addition of an excess of unlabelled 1–34 hPTH (40 μM) produced rapid dissociation of the hormone–

Fig. 3. Biological assay of ^3H -labelled 1–34 hPTH. Adenylate cyclase activity of renal tubular membranes is plotted against increasing concentrations of either unlabelled (open circles) or ^3H -labelled (closed circles) 1–34 hPTH.

Fig. 4. Time-course of ^3H -labelled 1–34 hPTH binding to rat renal tubular membranes. Total binding (closed circles) and non-specific binding (open circles) are shown. Dissociation of the hormone–receptor complex (open triangles) was obtained after addition of an excess of unlabelled 1–34 hPTH.



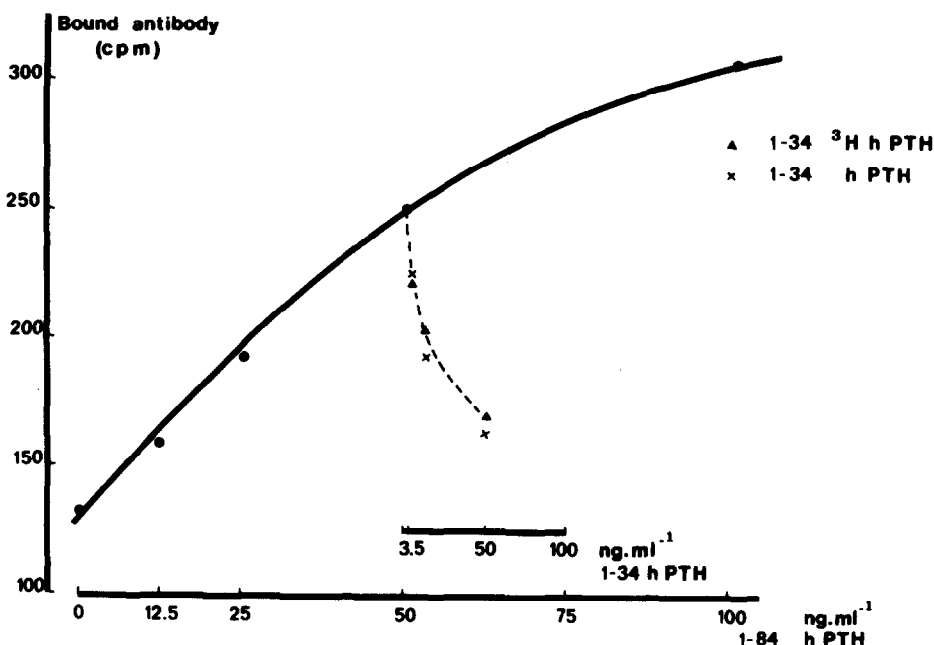


Fig.5. Binding of ^3H -labelled 1-34 hPTH to chicken renal membranes (labelled antibody membrane assay). [^{125}I]Antibody was measured as a function of 1-84 hPTH concentration (continuous line). Increasing concentrations of either unlabelled (crosses) or ^3H -labelled (closed triangles) 1-34 hPTH were added to a fixed amount (50 ng/ml) of 1-84 hPTH and inhibition of binding of the labelled antibody was studied (dotted line).

receptor complex since bound radioactivity reached the level of non-specific binding 5 min later. Indirect evidence for specific binding was given in the experiment shown on fig.5. ^3H -labelled 1-34 hPTH inhibited the binding of 1-84 hPTH to chicken renal membranes as well as unlabelled 1-34 hPTH. This demonstrates that the tritiated preparation has the same affinity for the renal receptor as the unlabelled peptide. Both direct and indirect methods show that ^3H -labelled 1-34 hPTH can be considered as an adequate tracer for binding studies.

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