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Evidence for a Precursor to Circulating Parathyroid Hormone

Louis M. Sherwood, John S. Rodman, Walter B. Lundberg

DEPARTMENT OF MEDICINE, BETH ISRAEL HOSPITAL, AND HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS 02215

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Abstract. Bovine parathyroid glands incubated in organ culture synthesize and secrete parathyroid hormone in response to physiologic alterations in cation concentration. Hormone synthesized in the glands is immunologically different and higher in molecular weight than that released into the culture medium or circulating in bovine blood, although it is identical to purified hormone extracted from the glands. On the basis of the physiologic responses of tissue and medium hormone to cation, the transfer of radioactive label from one moiety to another, and interconversion of the two forms by a gland homogenate, it is suggested that the parathyroid glands synthesize a precursor hormone (pro-parathormone) that is modified to the smaller molecule that circulates in blood.

Studies *in vivo*[1] and *in vitro*[2,3] have provided direct evidence that the secretion of parathyroid hormone (PTH) is regulated by the concentration of extracellular calcium. Using a radioimmunoassay to measure the hormone[4] and an *in vitro* test system in which physiologic function of the parathyroid glands can readily be controlled,[5] we now report investigations of the synthesis of PTH. These studies provide evidence that the hormone is initially synthesized in the glands as a polypeptide with a molecular weight similar to that of purified bovine PTH,[6] but that it is released as a smaller molecule which is immunologically different. Both the synthesis of hormone in the tissue and the secretion of the circulating molecule vary inversely with the extracellular calcium concentration.

Materials and Methods. The external parathyroid glands were removed from cows within minutes after slaughter, placed in sterile Hanks solution and kept at 4°C until the experiment was initiated (usually 1–2 hr). The glands were washed, stripped of surrounding fat, and cut into 1- to 2-mm^3 pieces for organ culture. Explants were placed in organ culture dishes as previously described[5] and incubated in BGJ[7] or F10 medium with 10% fetal calf serum under 5% CO_2 in air. Incubations were performed with leucine-free medium containing 3 μCi of [U-^{14}C]leucine per ml, and the concentrations of calcium and magnesium were varied according to the experimental protocol. Incubations were conducted for 1–72 hr, and immediately thereafter the tissue was homogenized in a solution of 8 M urea, 0.2 M HCl, and 0.14 M β-mercaptoethanol. Extraction was performed for 24 hr at 4°C and the suspension was centrifuged at 5000 $\times$ g for 15 min with the supernatant fraction being saved. Protein content of each tissue homogenate was determined by method of Lowry *et al.*[8]

Aliquots of tissue extract and supernatant medium were passed separately over columns of Sephadex G-100 (90 $\times$ 2.5 cm) and chromatographed at a rate of 20 ml/hr in 0.15 M ammonium acetate buffer (pH 5.0) containing 1 mg/ml egg white lysozyme to minimize

adsorption. Individual column fractions were analyzed for radioactivity and tested by radioimmunoassay as previously described.[5] Tissue and medium protein labeled with [^{14}C]leucine did not interfere with simultaneous radioimmunoassays of the samples, since the assays were performed with ^{131}I or ^{125}I and counted in a gamma well counter. Concentration of PTH in the culture medium was determined in the radioimmunoassay by comparison with pooled medium in which the concentration of PTH was determined by bioassay. Concentrations in the gland extracts were compared by radioimmunoassay with a standard curve of purified bovine PTH. Assays were run in duplicate at multiple dilutions. In several experiments, human parathyroid glands removed at the time of subtotal parathyroidectomy from patients with chronic renal failure and secondary hyperparathyroidism were also tested.

Results. The immunologic activities of the hormone in the culture medium (H_M) and of purified bovine were compared by radioimmunoassay at multiple dilutions (Fig. 1). When the concentrations of H_M were calculated from the standard curve against bovine PTH there was disagreement in the values obtained at different dilutions. The two curves could not be superimposed, regardless of the mathematical treatment applied. At high concentrations, H_M could completely displace the labeled hormone from antibody but it was only partially related immunologically to bovine PTH. A pool of H_M was therefore used as a radioimmunoassay standard after its content of hormone was determined by bioassay using the rat renal adenyl cyclase method.[9] Extracts of bovine parathyroid tissue (H_T), on the other hand, behaved exactly like purified

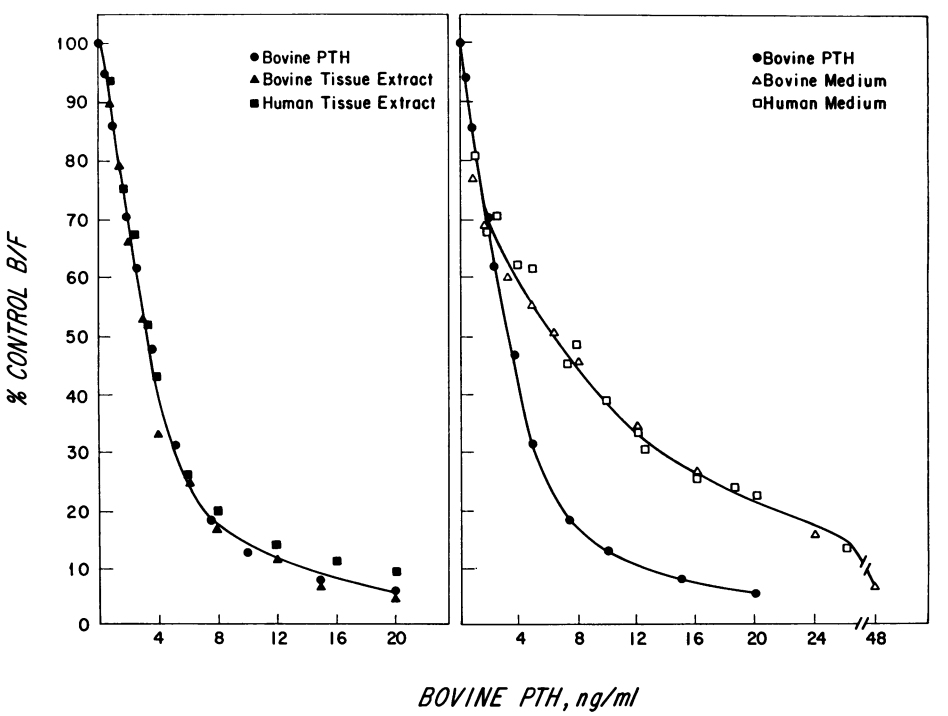

FIG. 1. Comparison of the immunologic activity of PTH in urea extracts of human and bovine glands and in medium from incubation of human and bovine glands. Similarities in the activity of pure hormone and of tissue extracts are indicated (*left*). Differences in the activity of pure hormone and medium hormone are indicated (*right*).

bovine PTH and caused parallel displacement of labeled hormone bound to antibody (see Fig. 1). No differences in immunologic behavior of H_T and purified hormone were noted in at least 12 different urea extraction procedures, suggesting that immunologic alteration of the hormone from tissue did not occur under the conditions used.

The possibility that artifacts or nonspecific alterations of hormone were responsible for the altered immunologic reactivity of H_M was ruled out by the following control experiments: (1) ^{131}I-labeled hormone incubated in organ culture with the explants for 12 hr showed no change in immunologic behavior or position of elution from Sephadex G-100. (2) Incubation of bovine PTH in medium from previously incubated glands or in control medium for 24 hr at either 4 or 37°C caused no alteration in immunologic activity. (3) Extracts of human hyperplastic parathyroid glands and medium from organ culture studies showed a similar dissociation of immunologic activity (Fig. 1).

When aliquots of medium from bovine glands incubated with [^{14}C]leucine were passed over Sephadex, a series of radioactive peaks was obtained. Labeled proteins appeared at the void volume, but there was a consistent late peak of radioactivity that preceded free leucine and varied in relation to the calcium concentration used in the incubation. When column fractions were tested for immunologic activity by radioimmunoassay, activity was noted only under this late peak (Fig. 2, *upper*). The height of the H_M peak varied inversely with the concentration of cation (calcium and magnesium) and paralleled the release of unlabeled immunoreactive hormone into the medium.[5]

When the tissue extracts were analyzed by Sephadex chromatography, several labeled proteins were noted, particularly after 24 hr. Usually a small peak or shoulder coinciding with the peak of radioactivity in the medium was observed (Fig. 2, *lower*). However, a larger peak of radioactivity usually appeared 8–10 fractions earlier. Radioimmunoassay of tissue extracts indicated that most of the H_T was eluted at the same point as the earlier peak of radioactivity and clearly earlier than H_M. In many instances a double peak for the tissue extract was observed, with the majority of counts and immunologic activity appearing in the first peak followed by a shoulder corresponding to the medium peak.

Better separation of H_T from other proteins was achieved with Bio Gel P10 (see Fig. 3, which also demonstrates the decrease in magnitude of the H_T peak, per mg of tissue protein, observed with increasing concentrations of calcium used in the incubation). Control experiments showed that bovine[^{131}I] PTH was eluted from the column at the same position as H_T, and that at very early time periods virtually all of the radioactive leucine was incorporated into the H_T peak.

Control experiments provided further evidence that the differential elution of H_T and H_M was a specific observation: (1) When the medium from glands incubated with radioactive leucine was mixed with an unlabeled 8 M-urea tissue extract, H_M still eluted at the same position. (2) The glands were extracted with 8 M urea and 0.2 N HCl. Under these conditions, it is most unlikely that early elution of H_T represented aggregation. Furthermore, H_T eluted at the same position as nonaggregated bovine PTH. (3) Incubation of H_T with a

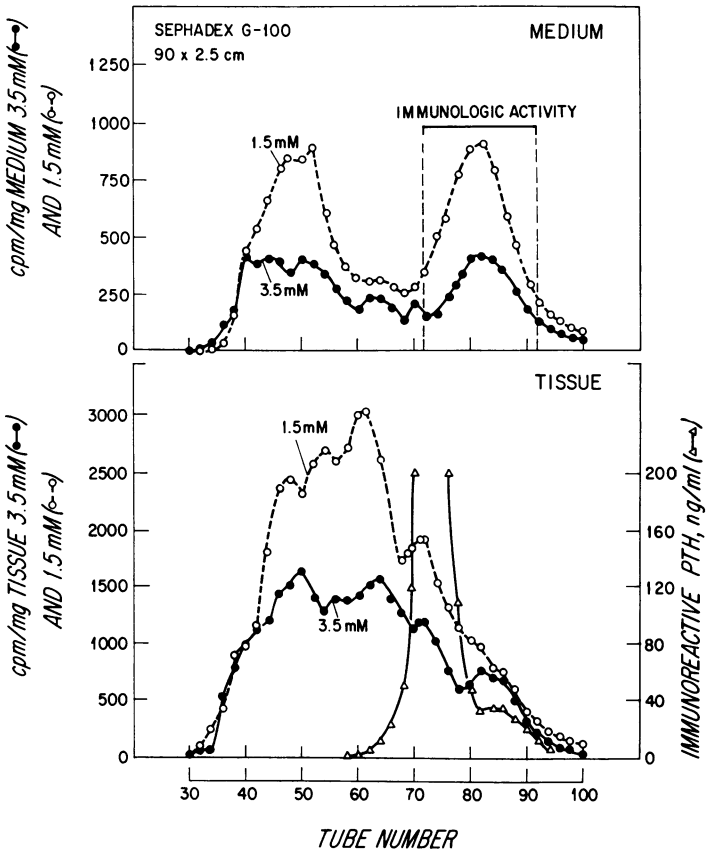

FIG. 2. Elution pattern on Sephadex G-100 in 0.15 M ammonium acetate buffer (pH 5.0) of medium (*upper*) and tissue extract (*lower*) of bovine glands incubated with [^{14}C]leucine for 72 hr. Patterns observed with 1.5 and 3.5 mM cation (calcium and magnesium) are indicated. Immunoreactive material from the medium (H_M) was consistently eluted later than that from tissue (H_T). Radioactivity and immunoreactivity of PTH are standardized per mg of tissue protein.

gland homogenate caused its conversion to H_M (see below). (4) The immunologic differences between H_T and H_M suggest that they may be chemically different.

An aliquot of bovine[^{131}I] PTH was incubated with a crude parathyroid gland homogenate made in 0.1 M Tris buffer, pH 7. After incubation at 37°C for 12 hr the hormone was passed over Bio Gel P10. PTH incubated with tissue homogenate was eluted at the position corresponding to H_M, which suggests its conversion to the lower molecular weight form (Fig. 4). Control experiments with other tissues failed to produce this type of conversion.

Immunologic studies of ^{14}C-labeled H_M indicated that it could be bound completely to antibody in the presence of excess anti-PTH but that it was readily displaced by incubation with an aliquot of unlabeled bovine PTH. Fig. 5 indicates the elution position of H_M before and after incubation with antibody and after displacement from antibody by bovine PTH.

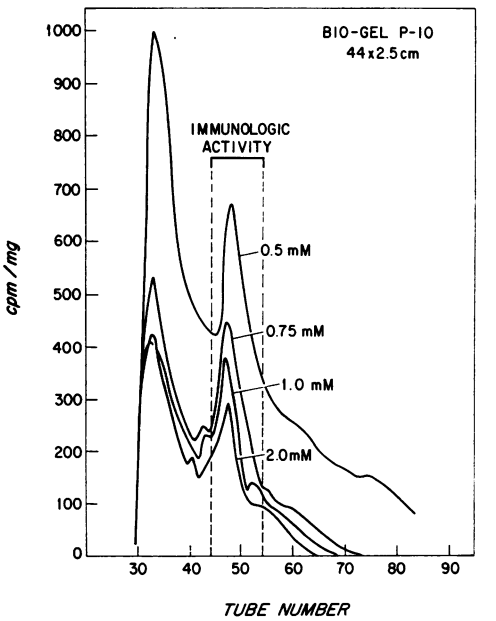

FIG. 3. Elution pattern on Bio Gel P10 in 0.15 M ammonium acetate buffer (pH 5.0) of tissue extracts of parathyroid glands incubated for 72 hr at four concentrations of calcium. Area of immunoreactive PTH and the progressive decreases in hormone synthesis with increasing calcium concentration are shown.

In order to prove that the hormone that circulates in bovine blood is similar to H_M, bovine plasma was extracted with microfine silica, as described by Yalow and Berson.[10] After the addition of 750 mg of QUSO32 (Philadelphia Quartz Co.) to 100 ml of fresh bovine plasma, the suspension was centrifuged at 5000 $\times$ g for 20 min. The protein was eluted from the silica with 50 ml of 20% acetone in 1% acetic acid, followed by 50 ml of water. The supernatant liquid was dried by rotary evaporation and lyophilization, and the powder was resuspended in 0.1 N acetic acid. In the radioimmunoassay, plasma extract behaved like H_M and was immunologically different from H_T (Fig. 6).

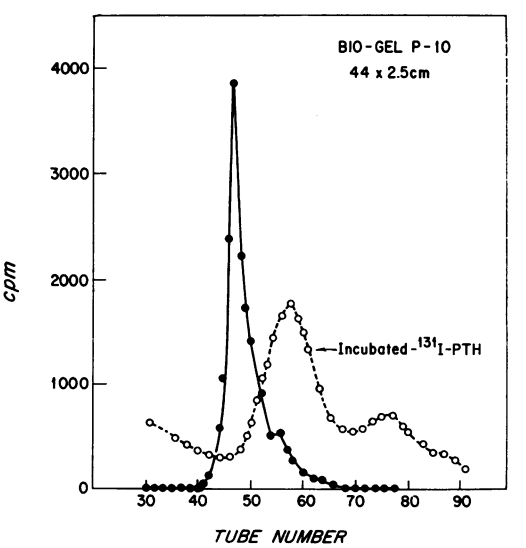

FIG. 4. Elution pattern of bovine [^{131}I] PTH on Bio Gel P10 before and after incubation with parathyroid-gland homogenate.

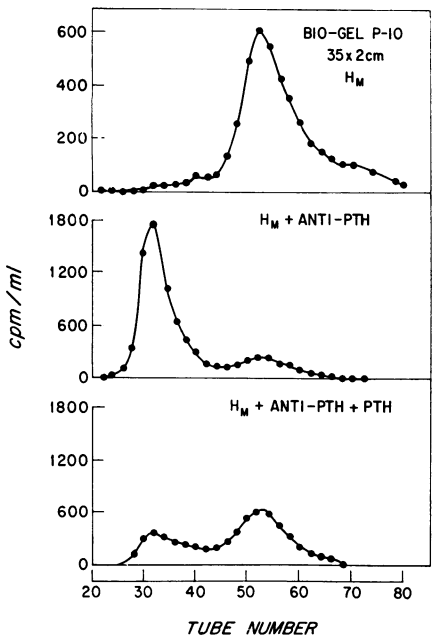

FIG. 5. Immunologic activity of H_M labeled with [^{14}C]leucine. *Upper panel,* elution pattern on Bio Gel P10 of H_M alone. *Middle panel,* elution pattern of H_M incubated with anti-bovine PTH for 24 hr. *Lower panel,* elution pattern of H_M incubated with anti-bovine PTH plus purified bovine PTH.

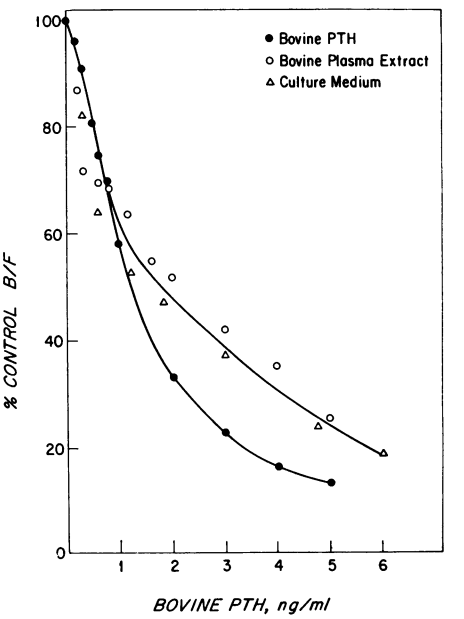

FIG. 6. Comparison of the immunologic activity of bovine PTH, medium from gland incubation, and an extract of bovine plasma made with microfine silica. Antibody dilution was 1/250,000.

Discussion. In an earlier study of PTH in human blood, Berson and Yalow[11] noted a dissociation between the immunologic activity of plasma PTH and the hormone in gland extracts with one antiserum and suggested that the circulating hormone might be immunochemically heterogeneous. Arnaud *et al.*[12] found no evidence for immunochemical heterogeneity of plasma PTH but detected a consistent difference in the immunologic activity of PTH in gland extracts and serum. These workers suggested that the hormone might be immunologically altered during extraction. We also noted consistent differences between the immunologic activity of pure bovine PTH and of tissue-extracted PTH, on the one hand, and of plasma PTH and of that released into culture medium on the other. Additional observations of the synthesis of hormone, however, provide an apparent explanation for what otherwise might seem confusing or discrepant observations. They also provide at least a partial understanding of the difficulties noted in many laboratories in developing a satisfactory radioimmunoassay for PTH in human blood.

Parathyroid hormone in normal bovine glands is synthesized as a higher molecular weight protein (H_T) that is immunologically identical to pure bovine PTH and has the same elution volume on gel filtration. Radioactive leucine is readily incorporated into H_T, but relatively little labeled protein appears in H_M for 2–3 hr. H_M is eluted from Sephadex G–100 8–10 tubes later than H_T and coincides with a peak of immunoreactive hormone. Control experiments indi-

cated that the differential elution of H_T and H_M was not related to aggregation, denaturation, or other artifacts, but represented a difference in exclusion volume explainable on the basis of differences in molecular weight or conformation. From their elution pattern on Bio Gel P10, molecular weights of approximately 9000 and 7000 would be estimated for H_T and H_M respectively. More convincing evidence for the existence of two species of hormone was provided by the conversion of H_T to H_M. Incubation of ^{131}I-labeled PTH with a gland homogenate converted H_T to a protein that was eluted from Bio Gel P10 in a position corresponding to H_M. H_M is immunologically related to H_T since it causes displacement of H_T from antibody (albeit with a different slope), and can be bound by anti-H_T.

These observations suggest, therefore, that H_T and H_M have a precursor–hormone relationship similar to that of proinsulin and insulin.[13] For consistency, the terms proparathormone and parathormone might be used to describe H_T and H_M respectively. Further support for this interpretation is suggested by the presence of a small shoulder of labeled protein and immunoreactive PTH on the downslope of the H_T peak, coincident with H_M (Fig. 2). Unlike the islet cell, which stores hormone primarily in the form of insulin,[13] the parathyroid may store its hormone as the prohormone, with conversion taking place when the gland is stimulated. In prolonged incubations, some labeled H_T is also released into the medium, a phenomenon similar to that noted for proinsulin both *in vitro* and *in vivo*.[14] Although H_T and H_M are related immunologically and differ in molecular weight, the chemical relation between them is still unclear. Control experiments indicate that H_M is not a nonspecific degradation product of H_T. The single tyrosine residue in iodinated PTH was still present after conversion to H_M, a finding that is consistent with the location of tyrosine near the amino-terminal or biologically-active end of the molecule.[15]

In addition to the above data, physiological studies documented the importance of the two species. Both H_T and H_M vary inversely with calcium concentration, indicating a physiologically relevant action of cation on the synthesis and secretion of PTH. In earlier studies in the rat, Raisz *et al.*[16] showed an inverse relation between calcium concentration and the release of calcium-mobilizing activity, the uptake and incorporation of amino acids, and the release of a labeled peak of low molecular weight protein. More recently, Hamilton and Cohn[17] noted an inverse relation between calcium and incorporation of amino acids into a band on polyacrylamide gel electrophoresis corresponding to PTH. The studies reported here examined for the first time, however, both the synthesized and the secreted hormone and suggest that they are different. Further analysis of the chemical and biological differences between the two moieties is currently in progress.

In reported radioimmunoassays for PTH, investigators have utilized partially purified hormone for immunizing guinea pigs, rabbits, or chickens. Although it has been possible to measure circulating PTH in both bovine[18] and human blood,[19–22] such studies, particularly of human blood, have been extremely difficult and the results have varied markedly. Since this report indicates that gland hormone may in fact be a precursor and that circulating hormone is only partially related immunologically, a logical explanation for some of the diffi-

culties is apparent. Antisera that distinguish poorly between H_T and H_M would tend to be more sensitive to plasma PTH,[11] and the use of plasma or culture medium PTH as standard would be indicated. Further investigation of the circulating hormone and of its chemical nature is essential before these and related problems can readily be resolved.

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Abbreviations: PTH, parathyroid hormone; H_M, hormone in the culture medium; H_T, hormone in parathyroid tissue extract.

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Correction. In the article "On a Third Kind of Characteristic Numbers of the Spheroidal Functions," by Donald R. Rhodes, which appeared in the September 1970 issue of *Proc. Nat. Acad. Sci. USA*, **67**, 351–355, all $\eta_{\alpha n}(c)$ and $\mu_{\alpha n}(c)$ should read $\nu_{\alpha n}(c)$.