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Subcellular Location of Human Parathyroid Hormone Immunoreactive Peptides and Preliminary Evidence for a Precursor to Human PTH[1] (38545)

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(Introduced by F. Grande)

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In 1963, Berson, Yalow, Aurbach and Potts reported the development of a radioimmunoassay for parathyroid hormone (PTH) (1). Even though this tool has been available for several years, the nature of the processes of synthesis, storage and secretion of PTH is incompletely understood. Evidence of a possible precursor in the biosynthesis of PTH has been shown by both Cohn *et al.* (2) and Kemper *et al.* in the cow (3), and by Habener *et al.* (4) and Chu *et al.* (5), in the human.

This communication will show evidence for the existence of multiple immunoreactive human PTH polypeptides in parathyroid glands, two of which appear to be larger than the previously described precursors to PTH. Furthermore, we will show the intracellular location of these polypeptides. These data provide a basis for formulation of a hypothesis that may explain the interrelationships of these polypeptides.

Methods. Preparation of subcellular fractions. The subcellular distribution of immunoreactive PTH (iPTH) was studied in human parathyroid tissue surgically obtained from a patient with severe secondary hyperparathyroidism due to chronic renal failure, and from another with a large parathyroid adenoma. A portion of the glands was immediately chilled, weighed and homogenized in 10 ml of 0.25 *M* sucrose at 0°. A one ml aliquot of the homogenate was removed; the remaining suspension was carried through differential centrifugation to obtain subcellular fractions. The speeds and times for centrifugation were identical to those we have used with parathyroid tissue and are described in detail elsewhere (6).

Radioimmunoassay for parathyroid hormone. PTH was assayed by a double antibody method similar to that originally described by Morgan and Lazarow (7) for separation of bound, from free, tracer. The chicken antibody to bovine PTH (bPTH), designated C-12, and another chicken anti-bPTH, designated C-14, were gifts from Dr. Claude Arnaud of the Mayo Clinic, Rochester, MN, and were used in our initial studies. Another chicken antibody to bPTH, designated C-6, was prepared in our laboratory and used for all other determinations of immunoreactive PTH (iPTH). The specificity of the three antibodies will be compared. Highly purified bovine PTH (Wilson Laboratories, Chicago, IL) was used for standards and for preparation of iodinated bovine PTH by the Hunter and Greenwood method (8). The specific activity of ^{125}I-bPTH was 100 mCi/mg. A human PTH material was prepared in our laboratory by urea HCl extraction by the method of Rasmussen *et al.* (9) and partially purified by column chromatography and was used as a working standard in the study shown in Fig. 1.

Gel filtration. Chromatography of the homogenate and subcellular fractions from the hyperplastic tissue was done after extraction with 0.2 *M* ammonium acetate, pH 4.6. The columns were 1.5 × 85 cm and contained Sephadex G-100 with the eluting buffer being 0.2 *M* ammonium acetate, pH 4.6.

To improve the resolution of iPTH peaks, gel filtration of subcellular fractions obtained from a human parathyroid adenoma was done by upward flow chromatography on Sephadex G-75 Superfine. Aliquots of the secretion granule fraction and microsomal fraction were prepared for column chromatography by extraction in 0.2 *M* acetic acid at 4° for 1 hr. Two-tenths molar Tris base

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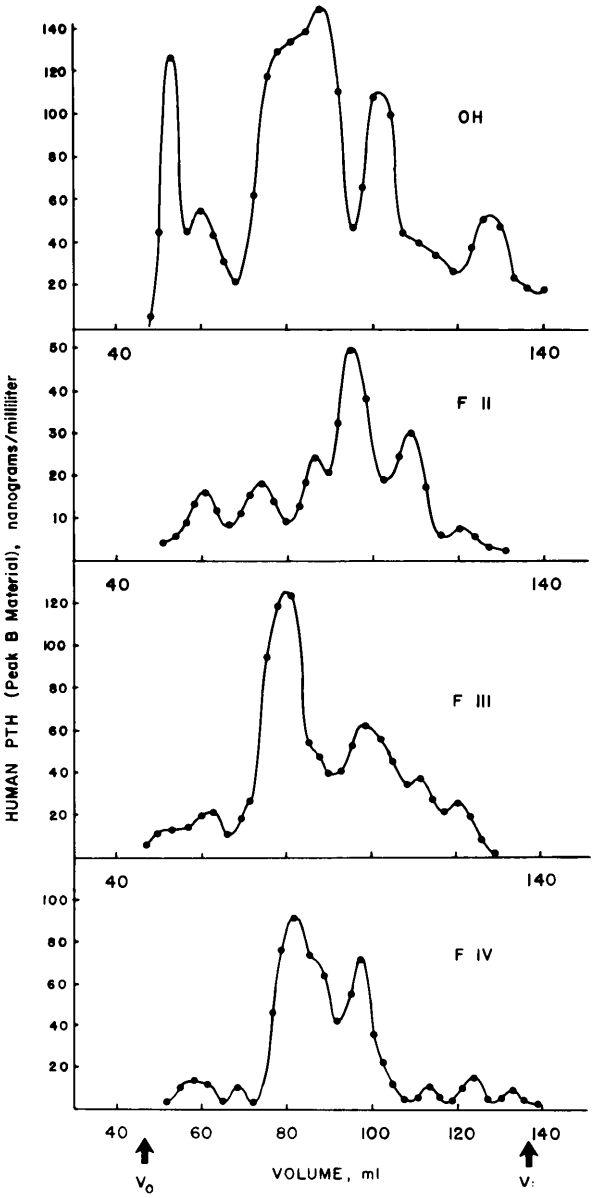

FIG. 1. Gel filtration of a homogenate and subcellular fractions of a hyperplastic human parathyroid gland. The homogenate and the subcellular fractions were suspended in 0.2 *M* ammonium acetate, pH 4.6, and then gel filtered on Sephadex G-100 in the same buffer. The effluent was analysed tube by tube for immunoreactive PTH using antiserum C-12. (OH = homogenate, F II = secretion granule and mitochondrial fraction, F III = microsomal fraction, F IV = final soluble fraction, V_0 = void volume, V_i = internal volume). PTH used for standards was a partially purified human PTH prepared in our laboratory and is referred to as Peak B material.

was added to bring the pH to 8.0 and undissolved material was removed by centrifugation. The clear supernatants were gel filtered on 1.5 × 60 cm columns of Sephadex G-75 Superfine in 0.13 *M* borate buffer, pH 8.4, with 1% bovine serum albumin added as a carrier. Blue Dextran and $Na^{125}I$ were included as internal markers to determine V_o and V_i for calculations of elution positions in terms of K_{av}.[2] The buffer was pumped at a

[2] $K_{av} = (V_e - V_o)/(V_i - V_o)$
V_e = elution volume observed for a given substance; V_o = void volume; V_i = total volume of the gel bed as determined by the elution position of $Na^{125}I$.

rate of 4.5 ml/hr; 1.5 ml fractions were collected. All column chromatography was done at 4°.

For purposes of estimating the molecular weights of the iPTH peptides, calibration of the column was done by gel filtering chymotrypsinogen (Pharmacia), myoglobin (Schwartz–Mann), highly purified bPTH (Wilson Laboratories), which was detected by radioimmunoassay, and ^{125}I-insulin (Abbott Laboratories). Their elution positions were expressed in terms of the K_{av} statistic. In determining the elution positions of these proteins of known molecular weight, Blue Dextran and Na^{125}I were included as internal markers. The eluting buffer for chymotrypsinogen and myoglobin was 0.13 *M* borate buffer, pH 8.4, without added bovine serum albumin, while for the bPTH and ^{125}I-insulin the same eluting buffer was used with 1% bovine serum albumin as carrier protein.

To validate the assumption that the preparation of highly purified bPTH from Wilson Laboratories had a molecular weight of 9500, this same preparation of bPTH, used in the gel filtration analysis described above, was further analysed by electrophoresis in sodium dodecyl sulfate (SDS)—urea acrylamide gels (3, 5). The polyacrylamide gels contained 10% acrylamide, 1.5% bisacrylamide, 8 *M* urea, 0.1% SDS and 0.1 *M* sodium phosphate buffer, pH 7.2. The running buffer contained 0.01 *M* sodium phosphate, pH 7.2, and 0.1% SDS. The proteins used for calibration were ovalbumin (Pharmacia), chymotrypsinogen (Pharmacia), cytochrome c (Schwartz–Mann), porcine proinsulin (a generous gift of Dr. Hosley, Eli Lilly) and the synthetic *N*-terminal tetratriacontapeptide of bPTH (Beckman). For detection of the highly purified bPTH (Wilson), the gels were sliced and extracted with 0.13 *M* borate buffer, pH 8.4 with 1% bovine serum albumin and aliquots of the extracts were assayed for bPTH by radioimmunoassay. Cytochrome c was included in all samples subjected to electrophoresis and served as an internal standard for each gel. The results were expressed as relative mobility which is defined as the ratio of the observed migration distance for the sample to the migration distance of cytochrome c in the same gel.

Results. Subcellular distribution and location of immunoreactive PTH. Studies of iPTH in subcellular fractions of human parathyroid material were done with fresh parathyroid tissue surgically obtained. Aliquots of the homogenate, secretion granule, and microsomal and soluble fractions from a hyperplastic gland were subjected to gel filtration and radioimmunoassay (antiserum C-12) on Sephadex G-100 (Fig. 1). In the homogenate (OH) some iPTH eluted just after the void volume, followed by three major peaks: the first peak eluting at about 80 ml, the second at 88 ml, and the third at 102 ml. The pattern of iPTH in the secretion granule fraction (F II) also consisted of multiple peaks; the predominant peak eluting at 95 ml, with a small peak ahead of it at 85 ml, and a component after it at 108 ml. With this antibody the microsome fraction (F III) contained the large iPTH at 80 ml with much less of the smaller forms of iPTH. The soluble fraction (F IV) contained three peaks of iPTH with the major peak at 80 ml.

The secretion granule fraction and microsome fraction from a parathyroid adenoma were then studied by chromatography on Sephadex G-75 Superfine and by radioimmunoassay using our antibody to bPTH, C-6 (Fig. 2). The microsome fraction was characterized by large quantities of a peptide of K_{av} 0.33, similar to the microsome fraction from a hyperplastic gland (Fig. 1), except that there seemed to be quantitatively more of the smaller peptides in this microsome fraction from an adenoma using our antibody, C-6. This difference can easily be accounted for by the insensitivity of C-12 antibody for the peptides eluting later than K_{av} 0.33. The secretion granule fraction from this adenoma (Fig. 2) showed predominantly a peptide at K_{av} 0.52, which is consistent with a molecular weight of 10,000. Other peptides at K_{av} 0.42 and 0.48 were also present. Relatively small amounts of the largest peptide at K_{av} 0.33 were present in the secretion granule fraction.

A direct comparison of our C-6 antibody to the C-12 and C-14 antibodies of Arnaud is shown in Fig. 3. The pattern of iPTH in this microsome fraction from a parathyroid adenoma as assayed by C-12 is similar to the pattern seen in the microsome fraction of hyperplastic parathyroid tissue shown in Fig. 1 with the exception that the resolution of the peaks of iPTH is vastly superior with

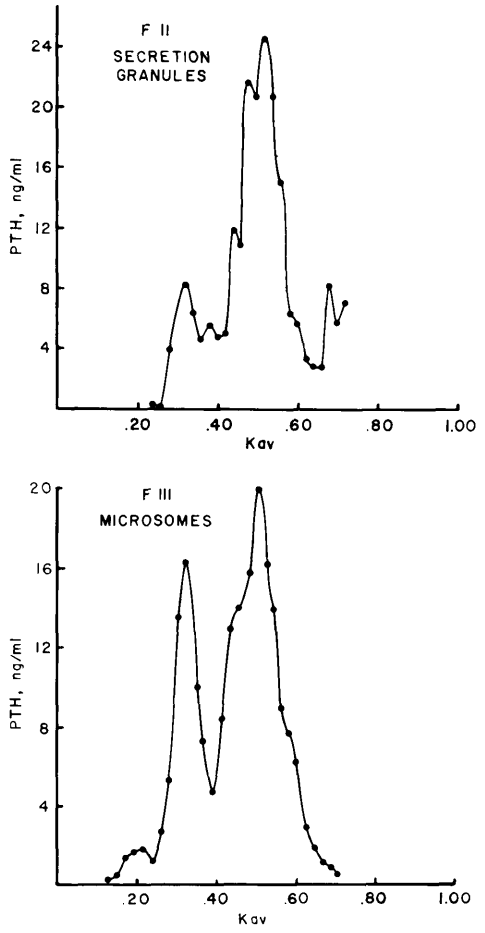

FIG. 2. Gel filtration of a secretion granule fraction (F III) and a microsomal fraction (F III) from an adenomatous human parathyroid gland. The fractions were suspended in 0.2 *M* acetic acid for one hour at 4° and then brought to pH 8.0 with 0.2 *M* Tris. The clear supernatants after centrifugation were gel filtered on Sephadex G-75 Superfine using 0.13 *M* borate buffer, pH 8.4, containing 1% bovine serum albumin and 0.01% merthiolate. The effluent was analysed for immunoreactive PTH using antibody C-6. Blue Dextran and ^{125}Na were cochromatographed with each sample and the relative elution volumes are expressed in terms of K_{av}.

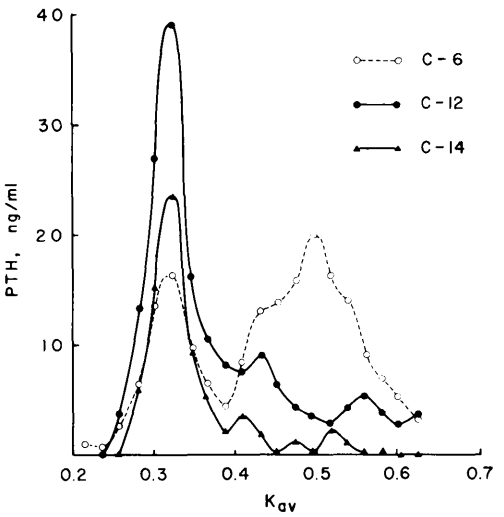

FIG. 3. Simultaneous assay of the chromatogram of a microsomal fraction (F III) shown in Fig. 5 for immunoreactive PTH using three antisera to bPTH: our C-6, and C-12 and C-14 of Dr. Arnaud.

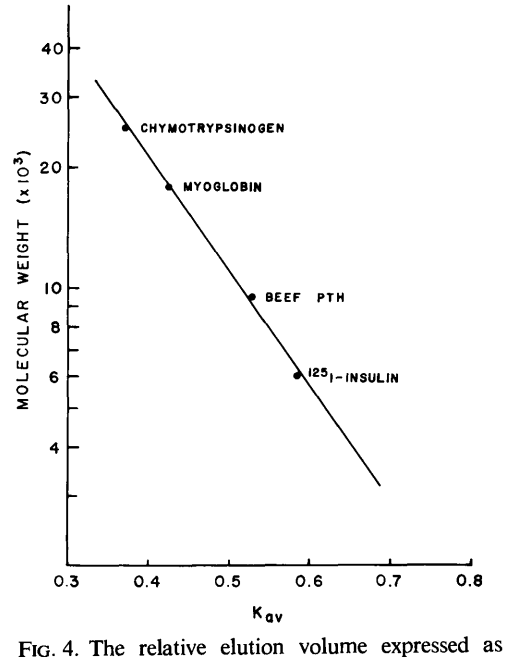

FIG. 4. The relative elution volume expressed as K_{av} for proteins of known molecular weight on a 1.5 × 60 cm column of Sephadex G-75 Superfine in 0.13 *M* borate buffer, pH 8.4. Each protein was chromatographed with Blue Dextran and Na^{125}I as internal markers.

the Sephadex G-75 Superfine. It can be seen that the antibodies designated C-12 and C-14 appeared to be relatively specific for the largest iPTH eluting at K_{av} 0.33 (estimated mol wt >25,000), and were relatively insensitive to all smaller iPTH peptides including the one eluting at K_{av} 0.52 which has an estimated mol wt of 10,000.

Estimation of molecular weight of the immunoreactive peptides. The elution profile of a series of proteins of known molecular weight was used to estimate the molecular weights of the iPTH peptides found in para-

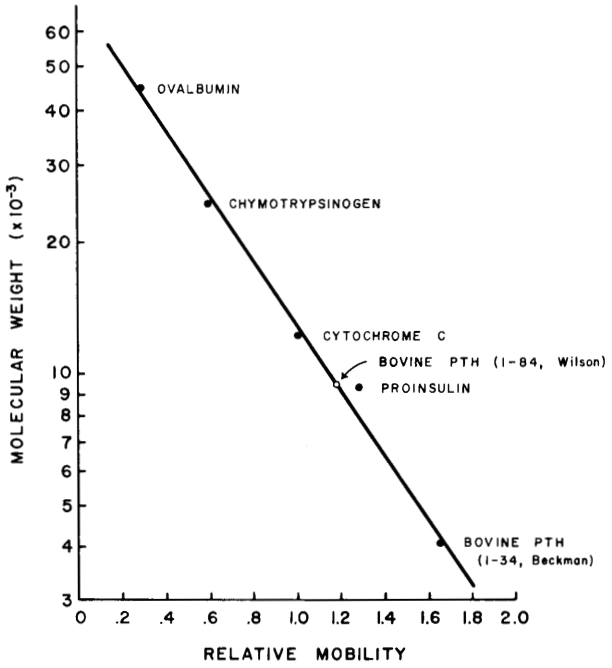

FIG. 5. Electrophoresis of a series of proteins of known molecular weight and highly purified bovine PTH (Wilson) on SDS–urea polyacrylamide gels. Each sample was electrophoresed with cytochrome c, which served as an internal marker. Bovine PTH (Wilson) was detected by radioimmunoassay. Relative mobility is ratio of observed migration distance of the sample to the migration distance of cytochrome c.

thyroid tissue (Fig. 4). Highly purified bPTH from Wilson Laboratories was found to elute at K_{av} 0.53–0.55. Immunoreactive PTH peptides eluting earlier have apparent mol wt of >25,000, 18,000 and 13,000 corresponding to the elution positions at K_{av} 0.33, 0.42 and 0.48 respectively.

That the highly purified bPTH from Wilson Laboratories used as a reference point in the molecular weight estimations was indeed intact bPTH and therefore had a molecular weight of 9500 was demonstrated by the results of the SDS—urea polyacrylamide electrophoresis shown in Fig. 5.

Discussion. Our studies of subcellular fractions of parathyroid tissue have shown the existence of a large peptide (mol wt >25,000) localized to the microsomal fraction; this suggests that it is the precursor polypeptide. A smaller peptide with a mol wt of 10,000 was localized to the secretion granule fraction. The iPTH peptides with estimated molecular weights of 18,000 and 13,000 may be intermediates formed in the process of conversion of the precursor by cleavage to PTH. We have previously alluded to the reports from other laboratories describing a precursor to bovine and human PTH with a molecular weight in the range of 11,500–12,500 (2–5). There were no indications in these reports of any forms of PTH larger than 12,500 daltons such as we have described. However, these other laboratories were studying acid–urea extracts of whole parathyroid tissue and did not examine subcellular fractions as we have done. The existence of this large iPTH peptide and its possible precursor role on biosynthesis of human PTH are supported by our studies of the incorporation of ^{3}H-leucine into this microsomal form of iPTH reported in detail separately (6). Their precursor to PTH of approximately 12,000 daltons is consistent with our formulation in which we postulate two intermediates of 18,000 and 13,000 daltons. The smaller intermediate may be the precursor reported by others.

The findings of a large iPTH peptide localized to the microsomes which contains elements of the protein biosynthetic apparatus suggests that it is the precursor to PTH. That this microsomal peptide is a form of

PTH is supported by the fact that it is recognized by three antibodies to bovine PTH which differ somewhat in their specificities and which are presumably reacting with separate antigenic determinants of the PTH molecule. A similar study on human hyperplastic parathyroid tissue also showed that this large form of iPTH is recognized by our C-6 antibody and the C-12 antibody of Arnaud (see Fig. 2 in Ref. (6)).

An alternate hypothesis to explain the finding of this iPTH with an apparent molecular weight of >25,000, is that it is a smaller iPTH peptide noncovalently bound to another molecule. Our studies to this point have not excluded this possibility. However, the findings of a large iPTH of >25,000 daltons in the serum of patients with hyperparathyroidism (10) tends to support the hypothesis that it is a discrete entity rather than some form of an aggregate of PTH.

We propose this microsomal iPTH is the precursor to PTH as a hypothesis and as a basis for further testing and study. Definitive proof that this microsomal iPTH is, in fact, the precursor to human PTH awaits isolation of sufficient quantities to permit determination of the amino acid sequence.

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