

## Proparathyroid Hormone: Identification of a Biosynthetic Precursor to Parathyroid Hormone

(tryptic peptides/molecular weight/protein synthesis/immunoassay)

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**ABSTRACT** Biosynthesis of a precursor to bovine parathyroid hormone has been demonstrated in slices of parathyroid tissue incubated *in vitro*. The proparathyroid hormone is 15-20 amino acids larger than the bovine hormone, and has a molecular weight of about 11,500 as determined by polyacrylamide gel electrophoresis. Upon incubation of parathyroid slices with [<sup>14</sup>C]amino acids, radioactivity is detected initially in the precursor. If incorporation of [<sup>14</sup>C]amino acids is inhibited after a short incubation either by replacement of radioactive amino acids with unlabeled amino acids or by addition of puromycin, the amount of radioactivity in the precursor decreases, while the radioactivity in the hormone continues to increase. The precursor is bound by an antibody that is specific for parathyroid hormone, and its binding can be inhibited by addition of the hormone. Analysis of tryptic digests indicates that the precursor and the hormone have common tryptic peptides, and that there are at least two additional peptides in the precursor.

Parathyroid hormone (PTH) regulates the concentration of plasma calcium in the circulation. The principal species of bovine PTH that is stored in, and extracted from, the parathyroid gland is a single polypeptide consisting of 84 amino acids (1, 2). It is known that insulin is synthesized as a precursor of higher molecular weight (3), and suggestive evidence has accumulated that several other peptide hormones have biosynthetic precursors (4-6). Thus, PTH may also be derived from a precursor. Cohn, *et al.* have recently suggested that bovine PTH may be synthesized as a molecule larger than 84 amino acids (7). However, the entire issue of a precursor to PTH has been confused by recent studies that suggest that PTH itself is an intracellular precursor of the circulating hormone, since the hormone secreted *in vitro* is smaller than 84 amino acids (8, 9). In this report, we present evidence that a precursor to PTH is synthesized in parathyroid slices during incubations *in vitro*. The precursor contains 100-105 amino acids, and its conversion to PTH is readily demonstrated in parathyroid slices.

### METHODS

Bovine parathyroid glands were obtained from a local abattoir and placed in cold Hank's balanced salt solution (Grand Island Biological Co.) immediately after removal from freshly killed animals. The glands were used in the experiments 2-3 hr later. The parathyroids were cut with a razor blade into

Abbreviations: PTH, bovine parathyroid hormone containing 84 amino acids; pro PTH, bovine proparathyroid hormone; SDS, sodium dodecyl sulfate.

slices about 0.5-mm thick, and slices from 1-2 glands were incubated for 15 min in 2-3 ml of Hank's solution supplemented with 5% fetal-calf serum. Then, 4  $\mu$ Ci/ml of a [<sup>14</sup>C]-amino acid mixture (New England Nuclear Corp.), with threonine and arginine omitted, and 10  $\mu$ Ci/ml of [<sup>3</sup>H]threonine were added to the incubation medium. Slices were then incubated for periods up to 150 min. In some experiments, after an incubation for 20 min with radioactive amino acids, the medium was decanted and the slices were rinsed with Hank's solution at 37°C, then incubated at 37°C in RPMI 1640 medium (Grand Island Biological Co.) containing unlabeled amino acids. In other experiments, 1 mM puromycin was added after parathyroid slices had been incubated for 20 min. At the end of an incubation, incorporation of radioactive amino acids was stopped by rapidly chilling the slices, washing them twice with cold Hank's solution, and then freezing the tissue in dry ice-ethanol.

The frozen slices were ground to a fine powder with a mortar and pestle, and 3-5 volumes of 8 M urea-0.2 N HCl were added. The resulting suspension was centrifuged at 10,000  $\times g$  for 10 min. The supernatant was adjusted to 10% trichloroacetic acid and three volumes of cold 10% Cl<sub>2</sub>CCOOH were added to precipitate protein. The precipitates were collected by centrifugation at 10,000  $\times g$  for 10 min and then redissolved in 1 ml of 0.1 NaOH. After 10 min at room temperature, protein was again precipitated with cold 10% Cl<sub>2</sub>CCOOH. After centrifugation for 10 min at 10,000  $\times g$ , the pellet was resuspended in 2 ml of distilled water and lyophilized to remove Cl<sub>2</sub>CCOOH. For gel electrophoresis, the lyophilized powder was extracted with 8 M urea-0.1 M acetic acid. This procedure recovered about 50% of the total radioactivity incorporated.

*Gel Electrophoresis.* The protein that had been extracted with 8 M urea-0.1 M acetic acid was analyzed (10) by electrophoresis on polyacrylamide gels at pH 4.4 that contained 10% acrylamide-0.25% bisacrylamide; all gel solutions contained 8 M urea.

The protein was also analyzed in sodium dodecyl sulfate (SDS)-urea acrylamide gels (11) that contained 10% acrylamide, 1.5% bisacrylamide, 0.1% SDS, 8 M urea, and 0.1 M sodium phosphate buffer, pH 7.2. The running buffer was 0.01 M sodium phosphate (pH 7.2)-0.1% SDS. Radioactivity in the gels was assayed by scintillation counting in 1 mm slices, which were incubated overnight with 1 ml of a solution containing NCS solubilizer (Amersham Searle)-water-scintil-

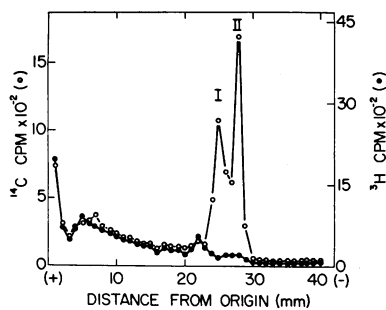


FIG. 1. Electrophoresis of protein extracted from parathyroid slices incubated for 60 min with radioactive amino acids. Slices of parathyroid glands were incubated with a mixture of [ $^{14}\text{C}$ ]-aminoacids and [ $^3\text{H}$ ]threonine, and protein was extracted from the parathyroid slices. Electrophoresis was performed in a urea-acrylamide gel at pH 4.4.

lation fluid 0.4:0.04:0.56 at room temperature. The distribution of immunoreactive PTH was compared with the radioactive profile of the gels by cutting each slice in half and assay of one half for radioactivity; the other half was shaken overnight at  $4^\circ\text{C}$  with 0.2 ml of 0.05 M Veronal buffer (pH 8.5)–10% human plasma. PTH in extracts from gel slices was assayed by a competitive-binding radioimmunoassay with  $^{125}\text{I}$ -labeled bovine PTH (12).

For isolation of radioactive protein in the peaks from the acrylamide gels, each slice was shaken with 0.2 ml of 0.1 N acetic acid overnight at  $4^\circ\text{C}$ . Aliquots of eluant from each gel slice were assayed for radioactivity by scintillation counting. Fractions from the peaks were combined and lyophilized to dryness. The lyophilized powder was dissolved in 0.1–0.4 ml of 0.15 N acetic acid, and passed through a BioGel P2 column equilibrated with 0.15 N acetic acid. Radioactive material eluting in the void volume of the column was pooled and lyophilized. The crosscontamination of protein in peaks I and II (see below) was less than 5%.

**Immunoprecipitation Studies.** The  $^{14}\text{C}$ -labeled protein isolated from pH 4.4 urea-acrylamide gels was incubated

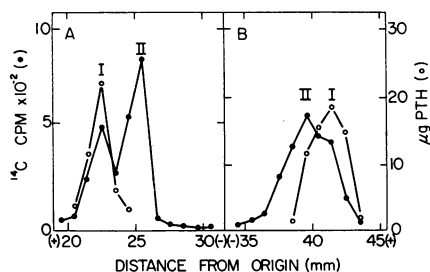


FIG. 2. Electrophoresis of protein extracted from parathyroid slices incubated for 60 min with radioactive amino acids. Electrophoresis was performed in both pH 4.4 urea and SDS-urea acrylamide gels. Half of each 1-mm gel slice was assayed for radioactivity, and the other half was assayed for immunoreactive PTH. PTH extracted and purified from glands was added as carrier to the sample before electrophoresis. Thus, the immunoreactive peak represents the 84-amino acid PTH. Only the regions of the gels containing peaks I and II are shown. (A) urea gel at pH 4.4. (B) SDS-urea gel.

at  $4^\circ\text{C}$  for 24 hr in a solution consisting of 0.05 M Veronal buffer (pH 8.5)–10% human plasma, and serum (diluted 1:400) from a guinea pig that had been immunized with a purified preparation of bovine PTH. Radioactivity bound to antiserum was separated from unbound radioactivity by precipitation of the antibody with rabbit antiserum to guinea pig globulin (diluted 1:6.5). Control incubations were done without the addition of anti-PTH serum, and also by addition of 2  $\mu\text{g}$  of bovine PTH with the antiserum.

**Trypsin Digest.**  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled proteins isolated from the peak fractions of gels were dissolved in 40  $\mu\text{l}$  of 0.2 M ammonium bicarbonate (pH 8.5) with 0.2 mg of PTH as carrier. 10  $\mu\text{g}$  of trypsin was added and, after 1 hr of incubation at  $37^\circ\text{C}$ , 10  $\mu\text{g}$  more of trypsin was added (13). After 2 hr of incubation, the reaction mix was spotted on Whatman no. 3 filter paper and electrophoresed for 2 hr at 4500 kV in pH 3.5 buffer containing 10% (v/v) acetic acid–1% (v/v) pyridine. Radioactivity in 1-cm strips on paper was assayed by scintillation counting. Radioactive peptide was eluted from the strips with 0.1 N acetic acid, lyophilized, redissolved in 0.1 N acetic acid, and chromatographed on Whatman no. 3 paper with a butanol/acetic acid/water 4:1:5 solvent.

## RESULTS

The major (84 amino acid) PTH contains no threonine (1, 2). Fig. 1 shows the profile of radioactivity of proteins extracted by urea-HCl from tissue slices incubated for 60 min with a mixture of [ $^{14}\text{C}$ ]aminoacids and [ $^3\text{H}$ ]threonine. Two major peaks of radioactivity are evident that contain 5–10% of the total  $^{14}\text{C}$ . Peak I has the same mobility as PTH run in a parallel gel, and peak II has a slightly greater mobility than peak I. There is no peak of [ $^3\text{H}$ ]threonine corresponding to peaks I and II. This result suggests that the proteins in peaks I and II are representative of the major isohormonal species of parathyroid hormone, which does not contain threonine (14).

To determine with certainty which of the two peaks corresponds to PTH, one half of a gel was assayed for radioactivity and the other half for the large amount of immunoreactive PTH (containing 84 amino acids) that is present in the tissue extract. The results of such an experiment are shown in Fig. 2A, which clearly demonstrates that the  $^{14}\text{C}$  present in peak I coelectrophoreses with PTH. If the protein is analyzed on a SDS-urea gel (Fig. 2B), the protein in peak II migrates

TABLE 1. Immunoprecipitation of peak II (proPTH)

	Radioactivity (cpm)		% of counts bound
	Bound*	Free	
Antiserum	258	17	94
Antiserum + PTH†	66	218	23
No antiserum	33	243	12

\* Peak II (proPTH) labeled with [ $^{14}\text{C}$ ]aminoacids was isolated by gel electrophoresis and incubated with guinea pig antiserum to PTH (dilution 1:400) for 24 hr. Antibody-bound radioactivity was precipitated by the addition of rabbit antiserum to guinea pig globulin.

† Unlabeled PTH (2  $\mu\text{g}$ ) was added at the beginning of incubation.

more slowly than the protein in peak I. In the urea gel at pH 4.4 mobility is a function of both charge and molecular weight, while in the SDS-urea gel mobility is a function of molecular weight only (11). Thus, the protein in peak II has a larger molecular weight than the protein in peak I, and its faster mobility on urea gels at pH 4.4 is the result of its higher content of positively-charged amino acids.

The protein in peak II was isolated and carrier PTH was added. The molecular weight of the protein in peak II was determined from its mobility on the SDS-urea acrylamide gel by calibration of the gel with cytochrome *c*, cyanogen bromide fragments of cytochrome *c*, PTH, and the large cyanogen bromide fragment of PTH (Fig. 3). From the slope of the curve, with immunoreactive PTH as an internal standard, the molecular weight of the peptide in peak II was estimated to be 11,500. Relative to PTH this is an increase of about 2000 daltons, which is equivalent to 15-20 additional amino acids.

Since the protein in peak II had a higher molecular weight than PTH, it seemed likely that it might be a precursor to PTH and should be synthesized before PTH. Fig. 4 summarizes the result of a kinetic study obtained by incubation of parathyroid slices with [<sup>14</sup>C]aminoacids for 10, 20, 45, or 150 min. Only the regions of the gels containing the two peaks of radioactivity are shown. The amount of radioactivity plotted has been corrected for the amount of protein extracted from each sample. At 10 min, all the radioactivity is present in peak II. At longer times, radioactivity appears in peak I in progressively larger amounts relative to peak II, although the total radioactivity in both peaks continues to increase up to an elapsed time of 150 min.

Two additional experiments were done to demonstrate that the radioactivity in peak I is actually derived from peak II. After 20 min of incubation with radioactive amino acids, they were removed and unlabeled amino acids were added to displace the isotope from rapidly turning-over molecules (see *Methods*). Additional incorporation of radioactivity into the peaks was effectively inhibited by this procedure. The amount of radioactivity in peak II began to decrease after an additional 10 min of incubation (Fig. 5, upper panel), and little radioactivity remained after 130 min. By contrast, the radio-

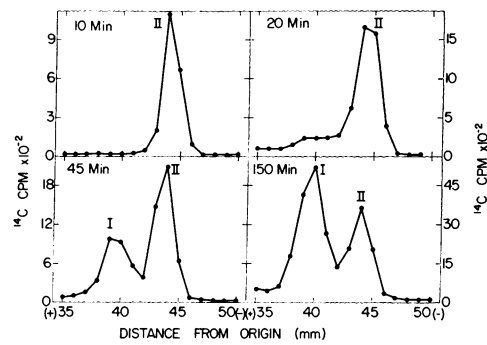


FIG. 4. Incorporation of [<sup>14</sup>C]aminoacids into proteins of peak I and II as a function of time. Parathyroid slices were incubated in four separate vessels for 10, 20, 45, and 150 min with [<sup>14</sup>C]-aminoacids. Incorporation was stopped and protein was extracted and analyzed on pH 4.4 urea gels. Only the region of the gels that contain peaks I and II is shown. Since different amounts of parathyroid slices were present in each incubation vessel, each point has been normalized by division of the amount of radioactivity by the amount of protein (mg) analyzed on each gel.

activity in peak I increased in the first 10 min; although the total radioactivity in this region of the gel decreased after 130 min, the radioactivity in peak I continued to increase. Identical results were obtained in a second experiment in which protein synthesis was stopped by the addition of puromycin after 20 min of incubation. Therefore, conversion of radioactivity from peak II to peak I occurs under conditions that allow protein synthesis to continue and under conditions that inhibit protein synthesis.

If the protein in peak II is a precursor of PTH, then about 80% of its sequence should be identical to PTH, and it should bind to antibodies specific for PTH. Contamination of the isolated, purified radioactive protein from peak II with radioactive PTH from peak I was less than 5% when it was re-electrophoresed on a urea gel at pH 4.4. Table 1 shows that over 90% of the radioactivity in peak II binds to an antibody that is specific for PTH. Added unlabeled PTH effectively

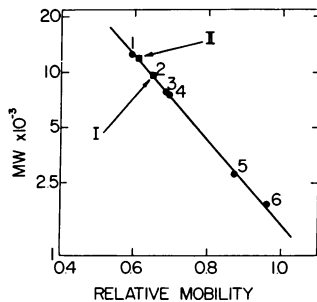


FIG. 3. A curve of the relative mobility of protein electrophoresed in SDS-urea acrylamide gels plotted against the logarithms of their molecular weight. The markers of known molecular weight are as follows: (1) cytochrome *c*, 12,300 (22); (2) PTH, 9600 (2); (4) cyanogen bromide fragment of PTH, 7600; (3, 5, 6) cyanogen bromide fragments of cytochrome *c*: (3) 7760; (5) 2780; (6) 1810. Solid squares indicate the mobilities of the proteins from peaks I and II.

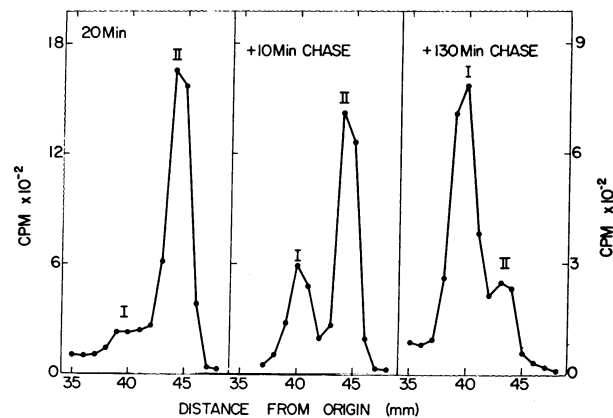


FIG. 5. Conversion of radioactivity in protein from peak II to peak I in the absence of additional incorporation of radioactive amino acids. The procedures are as described in Fig. 4. After a 20-min incubation with radioactive amino acids the medium was removed and fresh medium containing unlabeled amino acids was added. Results were analyzed at 20 min, 20 min + 10-min chase, and 20 min + 130-min chase.

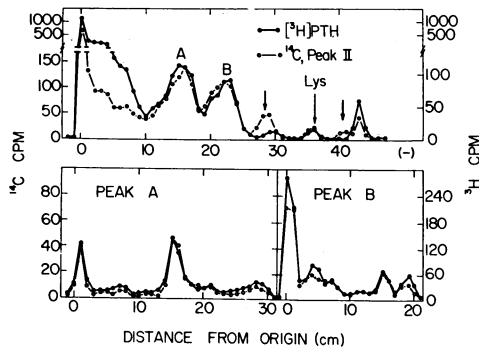


Fig. 6. Paper electrophoresis, followed by paper chromatography, of tryptic digests of PTH and protein from peak II. Slices were incubated for 120 min with either a [ $^{14}\text{C}$ ]amino acid mixture or a [ $^3\text{H}$ ]amino acid mixture. Radioactivity was isolated from peaks I (PTH) and II after urea gel electrophoresis at pH 4.4, with less than 5% crosscontamination. [ $^3\text{H}$ ]PTH was combined with [ $^{14}\text{C}$ ]protein from peak II, and then digested with trypsin. The products were analyzed by paper electrophoresis at pH 3.5 (*upper panel*). Protein from peaks A (fractions 12–18) and B (fractions 19–24) were eluted from the paper, and were analyzed by paper chromatography (*lower panels*). Similar results were obtained in an analysis of [ $^{14}\text{C}$ ]PTH combined with  $^3\text{H}$ -labeled protein from peak II. Unlabeled arrows point to new peptides from peak II protein.

competes with the radioactive labeled peptide in peak II for binding to the antibody. Thus, the protein in peak II contains common antigenic determinants with PTH.

To demonstrate further that the protein in peak II contains common amino-acid sequences with PTH, electrophoretic and chromatographic analyses were performed on tryptic peptides. Tissue slices were incubated with either [ $^3\text{H}$ ]amino acids or with [ $^{14}\text{C}$ ]amino acids. Protein from peaks I and II was isolated, and [ $^3\text{H}$ ]protein from peak I was combined with [ $^{14}\text{C}$ ]protein from peak II (and vice versa). These two samples were then digested with trypsin and electrophoresed on paper at pH 3.5. The results, shown in Fig. 6, (*upper panel*), indicate that most of the tryptic peptides are similar for the two proteins. Two major peaks of radioactivity that migrate from the origin are observed (13–18 cm, 20–25 cm), and these correspond to major spots that can be visualized with ninhydrin of purified PTH (15). Both lysine and dilysine (fastest-migrating peak) are liberated during the digestion (Fig 6, *upper panel*). There are two additional peaks (marked by *arrows*) in the material from peak II that migrate rapidly relative to the majority of the tryptic peptides; the fastest peak trails behind the dilysine peak. Since the protein in peak II is larger than PTH, but migrates faster on the urea-acrylamide gel, the additional 15–20 amino acids must contain a substantial number of basic amino acids. The extra, rapidly migrating, basic peptides observed after tryptic digestion are in agreement with this observation.

The peptides in each of the two major peaks of Fig. 6 (*upper panel*) were eluted and then chromatographed on paper (Fig. 6, *lower panel*). The first major peak (13–18 cm) separated into two major and one minor component. The second major peak (20–25 cm) separated into one major and four minor components. Each component contained both  $^3\text{H}$  and  $^{14}\text{C}$  radioactivity, thereby providing substantial evidence that the proteins in peaks I and II contain peptides with

identical sequences. An analysis of the  $^3\text{H}$ -to- $^{14}\text{C}$  ratio in the peak containing the C-terminal peptide suggests that the additional amino acids of the protein in peak II were added onto the C-terminal end.

## DISCUSSION

Our findings, in addition to the data presented by Cohn *et al.* (7), establish the existence of a precursor to parathyroid hormone. The peptide in peak II isolated from the electrophoretic gels is about 15–20 amino acids larger than PTH. Neither protein contains threonine. Radioactive amino acids are first detected in peak II, and, if further incorporation of radioactivity is blocked, the radioactivity in the protein of peak II is depleted with a corresponding increase in radioactivity of PTH in peak I. The protein in peak II contains peptides with sequences that are identical to those of PTH, since it can bind specifically to an antibody against PTH and has tryptic peptide peaks corresponding to PTH on electrophoresis and on paper chromatography. We conclude from these data that the protein in peak II is an intracellular precursor to PTH, and we suggest that it be designated parathyroid hormone (proPTH). However, it is clear that further work will be necessary to demonstrate that the proPTH described here, rather than an even larger polypeptide, is the direct product synthesized on the messenger RNA for PTH.

Little can be stated about the chemical nature or mechanism of the cleavage of proPTH. However, it is possible that the cleavage of the extra amino acids occurs in one step, since no intermediate molecules are observed on the acrylamide gel. The greater basicity of proPTH as compared to PTH, which is itself basic, suggests that a large percentage of the additional amino acids are lysine, arginine, or histidine. This hypothetical basic fragment would provide a good substrate for an enzyme with specificity similar to trypsin, comparable to the enzyme which cleaves proinsulin (16, 17). Cleavage by an enzyme with tryptic activity would require two steps if the extra amino acids are added at the C-terminal end of PTH, since glutamine is the C-terminal amino acid.

Recent evidence presented by Cohn *et al.* (7) has indicated that a basic protein labeled before PTH upon exposure to [ $^{14}\text{C}$ ]amino acid *in vitro* can be found after chromatography of parathyroid extracts on carboxymethyl-cellulose columns; tests indicate that it has biological and immunological activity (7). They have suggested that it is a biosynthetic precursor to PTH. The properties of that protein are consistent with the properties of proPTH described in this paper.

There are at least two cleavage events involving parathyroid hormone between its initial biosynthesis and its ultimate metabolism. The first of these is an intracellular cleavage, as described in this paper. There has been some confusion about the second cleavage of PTH that results in the presence of one or more fragments of PTH in the peripheral circulation. Sherwood *et al.* (8) and Sizemore *et al.* (9), on the basis of *in vitro* studies, suggest that the second cleavage occurs in the cell at the same time as secretion of the hormone; they consider PTH to be a precursor to the circulating hormone. Contrary to this proposal, recent studies performed *in vivo* have shown that the hormone secreted from the gland is identical in molecular size and immunochemical characteristics with the intact PTH stored in the gland. After secretion into the circulation, the hormone is cleaved into one or more frag-

ments that can be distinguished from the intact hormone by altered immunoreactivity (18). This "double cleavage" has naturally posed a semantic problem as to which molecule should be considered the prohormone. By analogy with insulin, we suggest that the intracellular precursor to PTH described in the paper is properly designated as proPTH. It is not known whether the second cleavage produces the predominant biologically active species in the peripheral circulation, or whether it is simply the first step in the catabolism and inactivation of PTH.

The data presented in the paper lend further support to the previously reported observation that certain human parathyroid adenomas appear to secrete an immunoreactive PTH of a higher molecular weight than the 84 amino-acid hormone (18). It seems likely that human PTH also is synthesized as a precursor of higher molecular weight, and that it may be secreted by tumors of the parathyroids in a manner similar to the secretion of proinsulin by islet cell tumors (19).

The discovery of proinsulin was the first demonstration that a biosynthetic precursor to a polypeptide hormone existed (3). Since then, evidence has been reported suggesting the existence of precursors of higher molecular weight for other hormones (4-6). The function and significance of prohormones are unknown; they may simply be an intermediate in the continuing molecular evolution of proteins in vertebrates. On the other hand, the emerging generality of prohormones for polypeptide hormones, and the specific mechanisms required for cleavage, strongly suggest some function for the prohormone. In the case of other nonhormonal proteins, such as the pancreatic proteases, the proenzyme is inactive and thus prevents self-digestion (20). After reduction of disulfide bonds, proinsulin renatures to an active form more efficiently than does insulin (21). This observation suggests that the connecting peptide in proinsulin insures the proper folding necessary for the disulfide bridges of insulin to form. However, there seems to be no reason for PTH to be synthesized in an inactive form, since an active form is stored in the gland, and furthermore PTH contains no cysteines that are required for the formation of disulfide bridges as in insulin. Thus, the extra amino acids may play some role in the post-synthetic transport and packaging of the hormone in the cell, or perhaps the conversion of proPTH to PTH provides an additional point for control of the secretion of PTH.

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1. Niall, H. D., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G. & Potts, J., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1586-1588.
2. Brewer, H. B., Jr. & Ronan, R. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1862-1869.
3. Steiner, D. F., Cunningham, D., Spigelman, L. & Aten, B. (1967) *Science* **157**, 697-700.
4. Noe, B. D. & Bauer, G. E. (1971) *Endocrinology* **89**, 642-651.
5. Yalow, R. S. & Berson, S. A. (1971) *Biochem. Biophys. Res. Commun.* **44**, 439-445.
6. Berson, S. A. & Yalow, R. S. (1971) *Gastroenterology* **60**, 215-222.
7. Cohn, D. V., MacGregor, R. R., Chu, L. J. H. & Hamilton, J. W. (1972) in *Proc. Fourth Parathyroid Conference*, ed. Talmage, R. V. (Excerpta Medica), in press.
8. Sherwood, L. M., Rodman, J. S. & Lundberg, W. B. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1631-1638.
9. Sizemore, G. W., Oldham, S. B., Fisher, J. A. & Arnaud, C. D. (1971) *J. Clin. Invest.* **50**, 85 abstr.
10. Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature* **195**, 281-283.
11. Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462-477.
12. Potts, J. T., Jr. & Deftos, L. J. (1969) in *Duncan's Textbook of Metabolism*, ed. Bondy, P. K. (W. B. Saunders Co., Philadelphia), 6th Ed. chap. 19, pp. 904-1082.
13. Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959) *J. Biol. Chem.* **234**, 2897-2900.
14. Keutmann, H. T., Aurbach, G. D., Dawson, B. F., Niall, H. D., Deftos, L. J. & Potts, J. T., Jr. (1971) *Biochemistry* **10**, 2779-2786.
15. Potts, J. T. Jr., Aurbach, G. D., Sherwood, L. M. & Sandoval, A. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 1743-1751.
16. Nolan, C., Margoliash, E., Peterson, J. D. & Steiner, D. F. (1971) *J. Biol. Chem.* **246**, 2780-2795.
17. Yip, C. C. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1312-1315.
18. Habener, J. F., Powell, D., Murray, T. M., Mayer, G. P. & Potts, J. T., Jr. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2986-2991.
19. Melani, F., Ryan, W. G., Rubenstein, A. H. & Steiner, D. F. (1970) *N. Eng. J. Med.* **283**, 713-719.
20. Neurath, H. (1957) *Advan. Protein Chem.* **12**, 319-386.
21. Steiner, D. F. & Clark, J. L. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 622-629.
22. Dayhoff, M. O. (ed.) (1969) *Atlas of Protein Sequence and Structure* V. 4, (National Biomedical Research Found., Silver Spring, Md.), Vol. 4.