Enzymic Processing of Proparathyroid Hormone by Cell-Free Extracts of Parathyroid Glands†

Joel F. Habener,*,‡ Howard T. Chang, and John T. Potts, Jr.

ABSTRACT: We examined, in cell-free extracts of parathyroid glands, the proteolytic activities exerted on radiolabeled preproparathyroid and proparathyroid hormone and a chemically synthesized hexapeptide consisting of the peptide fragment proteolytically removed from the NH_2-terminal end of proparathyroid hormone to give parathyroid hormone. Radiolabeled peptides in extracts of parathyroid gland slices were analyzed by thin-layer chromatography, and immunoreactive peptides were measured with a radioimmunoassay that detects both hexapeptide and proparathyroid hormone. The findings were: (1) extracts of parathyroid gland contain trypsin-like activity that preferentially cleaves the Arg–Ala bond joining the proparathyroid hormone specific hexapeptide to parathyroid hormone without additional cleavages in the sequence of parathyroid hormone itself; (2) this trypsin-like activity, found in all the subcellular fractions, was more concentrated in the particulate fractions than in the cell sol; (3) the trypsin-like activity could be inhibited by ethylenediaminetetraacetate but not by tosyl-L-lysine chloromethyl ketone and soybean trypsin inhibitors, indicating that the activity is probably different from pancreatic trypsin; (4) although the trypsin-like cleavage alone is sufficient to give parathyroid hormone, the hexapeptide sequence removed is further modified by the carboxypeptidase B like activity, suggesting that this dual enzymic activity may be universally involved in the processing of prohormone precursors; (5) no radiolabeled fragments of the hexapeptide could be identified in the extracts, suggesting that the remaining tri- or tetrapeptides may be rapidly further degraded in the parathyroid gland; and (6) no activity that converted radiolabeled pre-proparathyroid hormone to either proparathyroid hormone or parathyroid hormone was found in the gland extracts. These results support previous observations that the conversion of pre-proparathyroid hormone to proparathyroid hormone is dependent on translation, and further suggest that conformational or other kind of alteration in the structure of preproparathyroid hormone prevents the action of the proparathyroid hormone converting enzymes.

Parathyroid hormone (PTH)[1] is synthesized via two biosynthetic precursors that are modified posttranslationally by successive proteolytic cleavages within the parathyroid cell (Habener, 1976; Habener et al., 1976a, 1977). The initial product synthesized is a 115 amino acid polypeptide, preproparathyroid hormone (Pre-ProPTH) (Kemper et al., 1974, 1976a) that is rapidly converted within 1 min of its synthesis to proparathyroid hormone of 90 amino acids by proteolytic removal of the NH_2-terminal sequence of 25 amino acids (Habener et al., 1976a). After a delay of 15 min, proparathyroid hormone (ProPTH) is further modified to the final product, parathyroid hormone (PTH), of 84 amino acids by removal of the NH_2-terminal sequence of 6 amino acids (hexapeptide), Lys-Ser-Val-Lys-Lys-Arg (Cohn et al., 1972; Kemper et al., 1972; Hamilton et al., 1974; Jacobs et al., 1974; Habener, 1976; Habener et al., 1976a). The initial cleavage process is thought to occur at or near the site of synthesis of the polypeptide in the rough endoplasmic reticulum, with the second cleavage occurring in the Golgi apparatus during the sequential segregation and translocation of the hormone within subcellular organelles (Chu et al., 1974; Habener, 1976; Habener et al., 1976a, 1977).

Because of the likelihood that both cleavage processes involve specific proteases located within the subcellular transport pathway for the hormone, we have been interested in the characterization and in the eventual isolation of the enzymic activities responsible for the proteolytic processing of the PTH precursors. In addition, we wish to determine the metabolic fates of the peptide sequences that are cleaved from the precursors during their proteolytic modifications in the parathyroid gland. Such information should provide insights into the nature and regulation of the cellular events involved in the transport and secretion of parathyroid hormone.

In studies reported previously, we showed that dilute pancreatic trypsin will specifically convert ProPTH to PTH without the occurrence of other cleavages within the sequence of PTH (Goltzman et al., 1976). Although we had already shown that there need be no enzymic activity required other than that of a single trypsin-like enzyme for conversion of ProPTH to PTH (Goltzman et al., 1976), several factors indicated to us that the proteolytic processes involved in the cellular conversion of ProPTH to PTH might involve carboxypeptidase B activity, as well as trypsin-like activity. Firstly, it was shown that the specific conversion of proinsulin to insulin requires the combined actions of trypsin and carboxypeptidase B (Kemmler et al., 1971, 1973; Steiner, 1976). Secondly, evidence derived from the known primary structures of other polypeptide hormones presumed to be prohormones, such as the proglucagon fragment (Tager and Steiner, 1973) and β lipotropin (Chrétien and Li, 1967), and also of proalbumin (Russell and Geller, 1975) indicate that both trypsin-like and carboxypeptidase B like activities could be involved in their

† From the Endocrine Unit, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114. *Received February 17, 1977.* Studies were supported in part by grants from the United States Public Health Service and the John A. Hartford Foundation.

‡ Investigator, Howard Hughes Medical Institute.

[1] Abbreviations used: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; Pre-ProPTH, pre-proparathyroid hormone; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TLCK, tosyl-L-lysine chloromethyl ketone; STI, soybean trypsin inhibitor; Tris, tris(hydroxymethyl)aminomethane; hexapeptide, −6 – −1 (minus 6 through minus 1), the NH_2-terminal sequence of the proparathyroid hormone; ProPTH-(−6 – +12) and ProPTH-(−6 – +34), synthetic peptides consisting of prohormone hexapeptide through PTH residues 12 and 34, respectively.

cellular conversion to the final products. Finally, the carboxyl-terminal sequence of the ProPTH-specific hexapeptide, bProPTH-(−6 - −1), removed by trypsin consists of three basic amino acids, Lys-Lys-Arg (Hamilton et al., 1974; Jacobs et al., 1974), a suitable substrate for the actions of carboxypeptidase B.

We have analyzed cell-free extracts prepared from parathyroid glands for evidence of the combined actions of tryptic and carboxypeptidase B like activities during the conversion of biosynthetically radiolabeled ProPTH to PTH, specifically for evidence of carboxypeptidase B like activity in the degradation of the prohormone-specific hexapeptide. To aid our studies we used chemically synthesized hexapeptide to develop a radioimmunoassay that detects both ProPTH and the ProPTH-specific hexapeptide and have used these assays for analyses of ProPTH and hexapeptide levels in extracts of parathyroid glands.

The results of our studies indicate that, although in the parathyroid cell tryptic cleavage alone is sufficient to convert ProPTH to PTH, carboxypeptidase B like activity further modifies the hexapeptide sequence of ProPTH removed by the trypsin-like activity. The results support the possibility that a dual enzymic action is involved universally in the cellular proteolytic processing of prohormones.

Materials and Methods

Incubations of Parathyroid Gland Slices with Radioactive Amino Acids. Bovine parathyroid glands were obtained at the time of slaughter and were chilled immediately in ice-cold Earle's balanced salt solution. Approximately 100 mg of slices prepared from the glands was preincubated with shaking for 30 min at 37 °C in 2 mL of Eagle's minimum essential medium modified appropriately by omission of certain amino acids that were added supplementally as the radioisotopically labeled forms. In incubations carried out for the preparation of radiolabeled ProPTH and PTH, the media were supplemented with 5% calf serum (Grand Island Biological Co.), and calcium concentration was adjusted to 1.8 mM. For the attempted identification of ProPTH-specific peptide fragments in tissue and media, the calf serum was omitted, and the calcium concentration was adjusted to 0.5 mM. Magnesium concentration in all media was 0.8 mM. All media were equilibrated with a mixture of 5% CO_2 and 95% air before incubation. After the preincubation period of 30 min, radioactive amino acids were added to the media, and the incubations were continued as follows.

(a) For Preparation of Radiolabeled PTH. [^{14}C]Leucine (320 mCi/mM, 5 μCi/mL, New England Nuclear) was added, and incubation was continued for 3 h.

(b) For Preparation of Radiolabeled ProPTH. [4,5-^{3}H]Leucine (42 Ci/mM, 20 μCi/mL) was added and incubation was continued for 20 min.

(c) For Attempted Preparation of Radiolabeled ProPTH-Specific Peptides. Either [4,5-^{3}H]lysine (52 Ci/mM, 20 μCi/mL) or combinations of [^{14}C]lysine and [^{3}H]serine (10 μCi/mL) or of [^{14}C]lysine and [^{3}H]valine (15 μCi/mL) were added, and the incubations were continued for 90 to 180 min. Incubation of gland slices was terminated by removal of media and addition of 4 to 5 mL of ice-cold Earle's balanced salt solution to the incubation vessels. The slices were immediately frozen in preparation for extraction and isolation of radiolabeled proteins.

Isolation of Radiolabeled PTH, ProPTH, and Pre-ProPTH. Parathyroid gland slices that had been incubated with radioactive amino acids for the times indicated above were extracted with 8 M urea–0.2 N HCl, and radiolabeled ProPTH and PTH were isolated by electrophoresis on 10% discontinuous polyacrylamide gels containing 8 M urea and 0.1 M potassium acetate (urea–acetate gels) as described previously (Kemper et al., 1972; Habener and Potts, 1975). [^{35}S]Methionine-labeled Pre-ProPTH was biosynthesized by translation of parathyroid RNA prepared from hyperplastic human parathyroid tissue and from bovine parathyroid glands in a heterologous cell-free system derived from wheat germ (Kemper et al., 1974; Habener et al., 1975a). The radioactive products of the cell-free translations were separated by electrophoresis on 10% polyacrylamide gels containing 8 M urea–0.1 M phosphate–0.1% sodium dodecyl sulfate (urea–$NaDodSO_4$ gels), and radiolabeled Pre-ProPTH was isolated by extraction from the gel as described previously (Kemper et al., 1974, 1976a).

Preparations of Cell-Free Extracts and Subcellular Fractions of Parathyroid Tissues. Bovine parathyroid glands were homogenized either directly without prior incubation or after incubation of slices in vitro with [^{3}H]leucine for 20 min. For assay of carboxypeptidase B activity, 500 mg of whole parathyroid glands was homogenized in a motor-driven Teflon–glass homogenizer in 5 mL of 0.2 M sodium bicarbonate (pH 8.3). The supernatant obtained after centrifugation of the homogenate for 10 min at 5000g was used in the assay. Subcellular fractions of parathyroid gland slices that had been incubated with [^{3}H]leucine for 20 min were prepared by homogenization of 100 mg of the slices in 2 mL of 0.25 M sucrose, 50 mM Tris (pH 7.4), 5 mM $MgCl_2$ (sucrose–Tris–magnesium buffer) followed by successive centrifugations at 800g for 5 min, 10 000g for 15 min, and 105 000g for 60 min. The pellets obtained by the centrifugations were resuspended in the original volumes of sucrose–Tris–magnesium buffer.

A cell-free extract of human parathyroid tissue was prepared by homogenization of 200 mg of hyperplastic (primary chief-cell hyperplasia) tissue obtained at the time of surgery in 2 mL of sucrose–Tris–magnesium buffer (Habener et al., 1975a). A supernatant fraction was prepared from the homogenate by centrifugation at 10 000g for 15 min.

In attempts to identify endogenously synthesized prohormone peptide, extracts were made of parathyroid gland slices incubated with [^{3}H]lysine or mixtures of [^{14}C]lysine and [^{3}H]serine or [^{3}H]valine by homogenization of the tissues in 0.15 N acetic acid–0.1% NP-40 detergent (Shell Oil Co.), followed by removal of cellular debris by centrifugation at 10 000g for 15 min.

Incubations of Radiolabeled ProPTH and Pre-ProPTH in Cell-Free Extracts. ^{14}C-Labeled ProPTH, 1500–2000 cpm, isolated by electrophoresis on polyacrylamide gels, was added to 0.2-mL aliquots of the various subcellular fractions prepared from bovine parathyroid gland slices previously incubated for 20 min with [^{3}H]leucine. The mixtures were incubated for 30–120 min at 37 °C. [^{3}H]Leucine-labeled human ProPTH prepared from human parathyroid tissue pulse-labeled for 20 min and [^{35}S]methionine-labeled Pre-ProPTH prepared by electrophoresis of the cell-free products synthesized in response to translation of bovine and human parathyroid RNAs in a cell-free extract of wheat germ were separately added to 0.2-mL aliquots of clarified homogenates of bovine parathyroid gland and human hyperplastic parathyroid gland tissue, respectively, and were incubated at 37 °C for 10 min. After the incubations, 0.5 mL of 8 M urea–0.2 N HCl was added, and the radioactive proteins were precipitated by addition of cold 10% trichloroacetic acid. The acid-insoluble proteins were dissolved in 0.2 N NaOH, reprecipitated in 10% Cl_3CCOOH, and lyophilized to dryness. The dried Cl_3CCOOH powders were extracted with 8 M urea–0.15 N acetic acid, and ra-

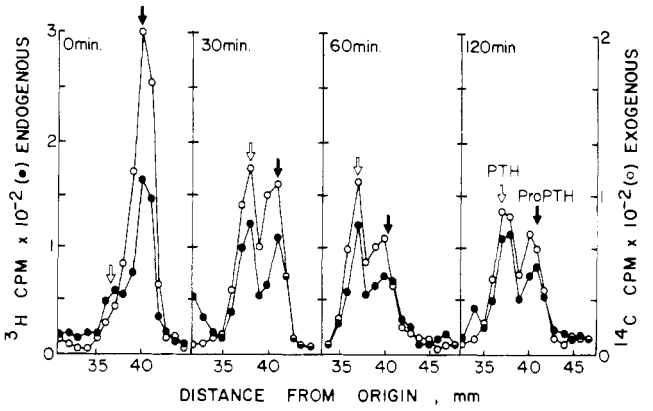

FIGURE 1: Polyacrylamide gel electrophoresis showing conversion of ProPTH to PTH during incubation of endogenous ^{3}H-labeled (●) and exogenous ^{14}C-labeled (○) ProPTH in resuspended particulate sedimenting between 1000*g* and 10 000*g* prepared from extract of bovine parathyroid glands. See Materials and Methods for details of the experiment. Only the regions of the polyacrylamide gels containing PTH and ProPTH are shown.

dioactive proteins were analyzed by electrophoresis on urea–acetate gels (Habener and Potts, 1975).

Development of a Radioimmunoassay for bProPTH-(−6–−1). An octadecapeptide consisting of a continuous copolymer of the ProPTH-specific hexapeptide sequence bProPTH-(−6–−1) (-Lys-Ser-Val-Lys-Lys-Arg)$_3$ was synthesized using solid-phase methods as described previously (Tregear et al., 1974). Rabbits were immunized with the octadecapeptide (50 μg per rabbit) using the method described by Vaitukaitis et al. (1971). Sera were obtained by ear puncture at biweekly intervals after immunization and were tested for ability to bind a ^{125}I-labeled synthetic ProPTH peptide [+1-serine,+13-tyrosine]bProPTH-(−6 - +13) as described previously (Habener et al., 1976b). Binding activity appeared in the sera of 1 of 10 rabbits by 16 weeks after initial immunization. A competitive-binding radioimmunoassay was prepared using one of the antisera at a dilution of 1:500, 5000–10 000 cpm of radioiodinated tracer, buffer consisting of 0.02 M phosphate (pH 6.0), 10% heat-inactivated calf serum, and 10 mM EDTA. Standard solutions used in the assay consisted of the synthetic ProPTH peptides: bProPTH-(−6 - −1), bProPTH-(−6 - +12), and bProPTH-(−6 - +34); synthetic PTH peptides bPTH-(1–12) and bPTH-(1–34); and the native hormones bProPTH-(−6 - +84) and bPTH-(1–84) prepared as described previously (Habener et al., 1976b). Assay volumes were 0.5 mL, incubations were carried out at 4 °C for 3 to 6 days under nonequilibrium conditions, and separation of antibody-bound from free radioactivity was accomplished using dextran-coated charcoal (Habener and Potts, 1976b).

ProPTH in extracts of the parathyroid gland slices was measured specifically by a radioimmunoassay (antiserum R-254) that detects only ProPTH and not hexapeptide or PTH (Habener et al., 1974a).

Preparation of a Carboxypeptidase B Digest of the ProPTH-Specific Hexapeptide. One microgram (1 μL of a 1 mg/mL solution in 0.001 N HCl) of carboxypeptidase B (Worthington Biochemicals) was added to 50 μg of synthetic ProPTH hexapeptide in a volume of 50 μL of 0.2 N ammonium bicarbonate. The solution was incubated at 23 °C for 60 min, and during that time 10-μL aliquots were withdrawn and added to 1 μL of 5 N acetic acid. The aliquots were frozen and subsequently analyzed by radioimmunoassay and by thin-layer electrophoresis. Larger amounts of the enzymic digest at 30 min of incubation were prepared periodically to use as markers for the electrophoretic analyses of radiolabeled extracts of parathyroid glands. Control incubations were also performed without addition of carboxypeptidase B and with varying concentrations (6.6–66.0 mM) of 2,2′-bipyridine (Eastman Chemicals).

Incubations of ProPTH-Specific Hexapeptide with Serum and with Cell-Free Extracts of Bovine Parathyroid Glands. ProPTH-specific hexapeptide (bProPTH-(−6- −1)), 750 μg, was incubated for 20 min at 23 °C with 135 μL of clarified extract of bovine parathyroid glands in 0.2 M ammonium bicarbonate (pH 8.5). Aliquots of 20 μL were withdrawn and acidified by putting them in tubes containing 2 μL of acetic acid. Hexapeptide, 375 μg, was added to 135 μL of calf serum (Grand Island Biological) and was incubated at 37 °C for 30 min. Aliquots of 20 μL were withdrawn during the incubation and added to tubes containing 2 μL of 80% Cl_3CCOOH. Cl_3CCOOH-insoluble material was pelleted by centrifugation at 8000*g* for 2 min, and the supernatant was removed and transferred to small tubes. During all of the incubations described above, control incubations were conducted without addition of tissue extract or of serum. Aliquots of the incubation mixtures were analyzed by radioimmunoassay and by electrophoresis on thin-layer plates.

Thin-Layer Electrophoresis of Digests of Hexapeptide. Aliquots, 5–10 μL, of the digests prepared by incubation of hexapeptide with carboxypeptidase B extract of parathyroid glands or calf serum, and acetic acid extracts of parathyroid gland slices prepared after incubations with radioactive lysine, serine, or valine were spotted on thin-layer plates (Silica Gel GF, Analtech, Inc.). The samples were electrophoresed for 2.5 h in 0.05 M sodium barbital buffer. The plates were dried, the peptide spots were developed with ninhydrin spray, and the radioactivity in 5-mm-wide bands of the silica removed from the plates was measured by scintillation spectroscopy.

Gel Filtration and Radioimmunoassay of Extracts of Parathyroid Glands. Urea-HCl extracts of a bovine parathyroid gland and a human parathyroid adenoma (0.1 mL) were filtered on a column (0.6 × 30 cm) of Bio-Gel P-2 (100–200 mesh, Bio-Rad). The elution buffer consisted of 0.02 M phosphate (pH 6.0), 10 mM EDTA, and 2% heat-inactivated calf serum. Fractions, 0.6 mL, were collected, and 10–20-mL aliquots of each fraction were assayed in quadruplicate in the radioimmunoassay for hexapeptide. For control studies, 100 μg of hexapeptide in 0.1 mL of 8 M urea–0.2 N HCl, and also a comparable volume of 8 M urea–0.2 N HCl by itself, were gel filtered and the fractions were collected and analyzed by radioimmunoassay.

Results

The conversion of ProPTH to PTH by subcellular fractions prepared from bovine parathyroid gland slices that had been pulse-labeled for 20 min with [^{3}H]leucine and to which ^{14}C-labeled ProPTH was added was evaluated by electrophoresis on polyacrylamide gels. Figure 1 shows a time course for the conversion of ProPTH to PTH by the resuspended pellet obtained after centrifugation of the nuclear-free supernatant (1000*g* supernatant) at 10 000*g*. Table I shows the results obtained upon analyses of the prohormone converting activity in the other three subcellular fractions: 800*g* pellet, 105 000*g* pellet, and the 105 000*g* supernatant. Activity was found in all fractions analyzed, but, on the basis of activity per milligram of protein in the fractions, the activity was relatively greater in the particulate fractions. No differences were observed between the rates or degree of conversion of endogenous ^{3}H-labeled ProPTH compared with exogenous ^{14}C-labeled ProPTH. The converting activity in the glandular fractions

TABLE I: Subcellular Distribution of ProPTH Converting Activity.

Subcellular fractions[a]	Incubation time[b] (min)	Protein per fraction (mg)	Radiolabeled PTH and proPTH by electrophoresis[c] (Endogenous) [^{3}H]PTH (cpm)	(Endogenous) [^{3}H]ProPTH (cpm)	(Exogenous) [^{14}C]PTH (cpm)	(Exogenous) [^{14}C]ProPTH (cpm)	Converting act. expressed as formation of radiolabeled PTH (Endogenous) [^{3}H]PTH (cpm/mg of protein)	(cpm/ fraction)	(Exogenous) [^{14}C]PTH (cpm/mg of protein)	(cpm/ fraction)
Particulates										
800*g*	0		129	394	33	308				
	60	3.20	366	204	152	137	740	2370	372	1190
10 000*g*	0		140	339	33	308				
	60	1.92	207	205	136	125	349	670	536	1030
105 000*g*	0		222	534	33	308				
	60	2.28	313	303	129	92	399	910	421	960
Sum of particulates:								3950		3180
Supernatant										
105 000*g*	0		151	377	33	308				
	60	7.80	342	202	278	179	245	1910	314	2450

[a] Fractions prepared by differential centrifugation of homogenate from 20 parathyroid glands after incubation with [^{3}H]leucine for 30 min. Each fraction was resuspended in 2.0 mL of sucrose–Tris–magnesium buffer. [b] Subcellular fractions (0.2-mL aliquots) incubated at 37 °C after addition of approximately 400 cpm of [^{14}C]leucine-labeled ProPTH. [c] Electrophoresis on urea–acetate polyacrylamide gels.

appeared to be notably specific for the conversion of prohormone to hormone, inasmuch as the amounts of radiolabeled ProPTH plus PTH remained relatively constant during the incubations. The extent of non-specific degradation of ProPTH and/or of PTH was no more than 15–20%. The rate of conversion observed (50% conversion in 30 min) is considerably slower than that seen during pulse-chase studies using intact gland slices (50% conversion in 5–10 min) (Habener et al., 1974b; Kemper et al., 1975). We attribute the failure to localize the converting activity of ProPTH to a single subcellular fraction to difficulties in the preparation of homogeneous subcellular fractions from the parathyroid glands free of contamination by the other fractions. Nonetheless, qualitatively we have confirmed the observations reported previously that extracts of parathyroid glands contain trypsin-like activity that will selectively convert ProPTH to PTH (Cohn et al., 1972; Habener and Potts, 1975; Habener et al., 1977; MacGregor et al., 1976). Additionally, we have evaluated the effects of some enzyme inhibitors, particularly those that are known to inhibit trypsin activity, on the converting activity of ProPTH in the 10 000*g*-particulate fraction from the parathyroid extracts (Table II). Of the three agents tested, only EDTA inhibited the conversion reaction; the more-common inhibitors of pancreatic trypsin, TLCK and soybean trypsin inhibitor, were without effect. We had previously found EDTA to be the most effective agent for the inhibition of ProPTH conversion by serum (Habener et al., 1976b). Thus, the prohormone converting activity in the gland extract appears to be different from that of pancreatic trypsin.

In attempts to determine the metabolic fate of the ProPTH-specific hexapeptide both in the parathyroid cell and in the blood under the assumption that it may be secreted from the cell along with PTH, we developed a radioimmunoassay for the detection of the ProPTH hexapeptide (see Materials and Methods). One of ten rabbits developed antibodies that bound a radioiodinated synthetic fragment of ProPTH, and the binding could be inhibited competitively by unlabeled hexapeptide, as well as by ProPTH and by peptides of ProPTH containing the hexapeptide sequence (Figure 2). This radioimmunoassay for ProPTH readily detects immunoreactivity

TABLE II: Effects of Trypsin Inhibitors and of Calcium on the Conversion of ProPTH to PTH by 10 000*g* (post-800*g*) Particulate Fraction of Parathyroid Gland Extracts.

	% conversion ProPTH to PTH[a] [^{3}H]PTH[b] (endogenous)	[^{14}C]PTH[c] (exogenous)
Control	50[d]	52
TLCK (60 μg/mL)	50	55
STI (10 μg/mL)	57	45
EDTA (10 mM)	16	27
Calcium (5 mM)	65	52

[a] Percent of PTH originally present as ProPTH appearing after a 60-min incubation. [b] Parathyroid gland slices were pulse-labeled with [^{3}H]leucine for 20 min. [c] Approximately 1500 cpm of [^{14}C]leucine-labeled ProPTH was added to the particulate fractions. [d] Values are averages of duplicate experiments.

in extracts of parathyroid glands, but when the levels of immunoreactivity detected were compared with those levels determined by a second radioimmunoassay using an antiserum that recognizes only ProPTH peptides containing the intact Arg–Ala bond joining the hexapeptide to PTH (Habener et al., 1974a), the values for ProPTH were the same. The means ± SEM for five glands were: hexapeptide assay 34.1 ± 5.7, ProPTH-specific assay 38.3 ± 4.9, and PTH assay 531 ± 7.9 pmol/mg of tissue protein. Furthermore, analysis by gel filtration and radioimmunoassay of extracts of both bovine and human parathyroid glands showed little if any immunoreactivity in the fractions where hexapeptide elutes from the column (Figure 3). If the hexapeptide were present in intact form in the parathyroid gland extracts, there should have been 10–15 times more immunoreactivity (in moles) found by the assay that detects both ProPTH and hexapeptide than by the assay that detects only ProPTH, inasmuch as we have shown previously that the ratio of PTH to ProPTH is approximately 15:1 (Habener et al., 1976b), and one expects to find 1 mol of hexapeptide for each mol of PTH.

It seemed plausible that one explanation for the failure to

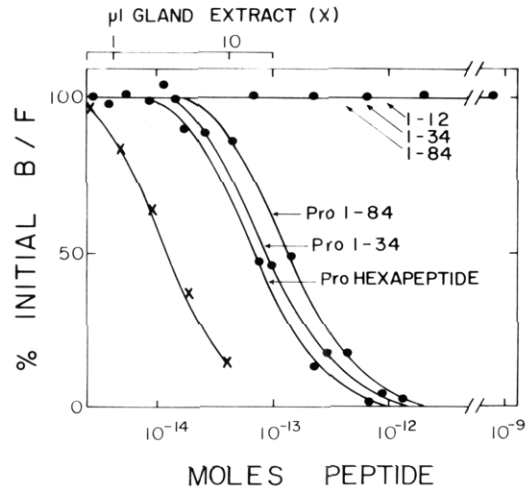

FIGURE 2: Competitive displacement curves in a radioimmunoassay using antiserum produced by immunization with a synthetic octadeca copolymer of ProPTH hexapeptide.(×) = μL of 8 M urea–0.2 N HCl extract of a bovine parathyroid gland. 1–84 and Pro 1–84 indicate native bovine PTH and ProPTH, respectively. 1–12, 1–34, and prohexapeptide are all chemically synthesized peptide fragments of the hormone and prohormone. Ordinate scale denotes measure of bound-to-free ratio of tracer based on ratio obtained with no peptide present (100%). Further details of the procedure are described under Materials and Methods.

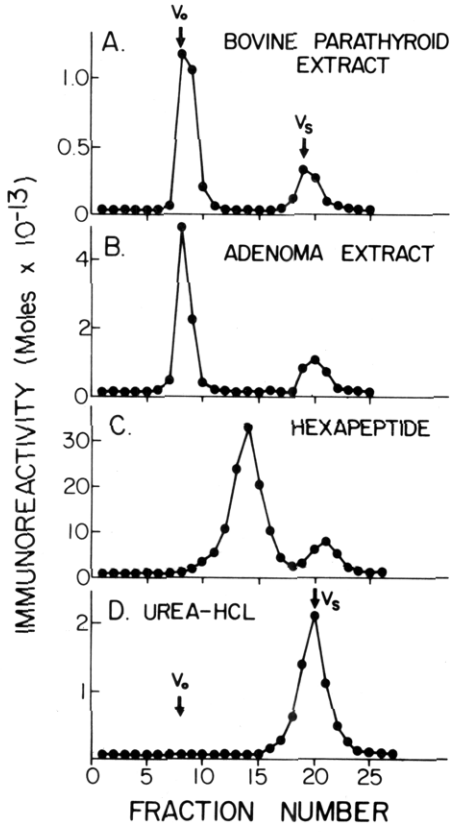

FIGURE 3: Gel filtration (Bio-Gel P-2) and immunoassay of extracts of parathyroid glands: (A) bovine gland; (B) human adenoma; (C) synthetic ProPTH hexapeptide; and (D) extraction solvent (urea–HCl) using radioimmunoassay for hexapeptide. V_0 and V_s mark void and salt volumes of the column. Peak at fractions 18–20 is due not to immunoreactivity but to inhibition of antigen–antibody binding in the assay by the urea–HCl in the extraction solvent.

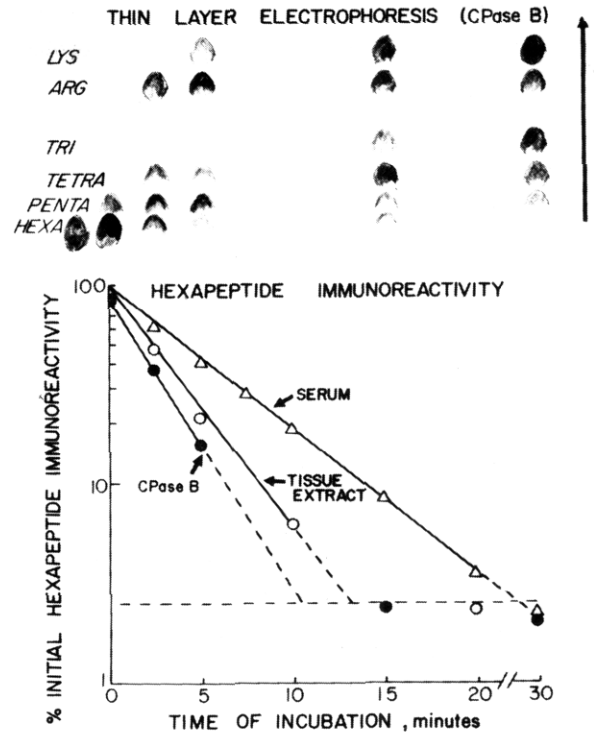

FIGURE 4: Comparison of appearance of degradation products by thin-layer electrophoresis with loss of immunoreactivity by hexapeptide radioimmunoassay during incubation of hexapeptide with carboxypeptidase B (●), extract of bovine parathyroid gland (○), and bovine serum (△). Copy of electrophoretogram depicted is that obtained with products formed by carboxypeptidase B; similar electrophoretic patterns were obtained with products from the incubations with parathyroid tissue extract and with serum. Horizontal dashed line denotes approximate limit of detection in the radioimmunoassay.

detect hexapeptide by the radioimmunoassay might be that the hexapeptide is further modified in the parathyroid gland by enzymic removal of one or more of the three basic amino acids from the carboxyl terminus of the peptide, rendering it unreactive in the radioimmunoassay. Such carboxypeptidase B like activity was shown to modify the carboxyl terminus of the C peptide of proinsulin, as well as of the B chain of insulin (Steiner, 1976). Indeed, treatment of synthetic hexapeptide with carboxypeptidase B, or for that matter with extracts prepared from parathyroid glands or with serum, resulted in a progressive fall in immunoreactivity of the hexapeptide that corresponded with the successive release from the hexapeptide of the C-terminal amino acids arginine, lysine, and lysine, and the appearance of the pentapeptide, tetrapeptide, and tripeptide, respectively, as determined by thin-layer electrophoresis (Figure 4). The release of the basic amino acids from the hexapeptide during incubation in the gland extract was completely inhibited in the presence of 0.06 M 2′,2′-bipyridine (50% inhibition at 0.03 M concentration) but was not inhibited by 50 mM EDTA (data not shown). 2′,2′-Bipyridine is a known inhibitor of carboxypeptidase B and acts by chelation of the zinc ion required for activity of the enzyme (Folk et al., 1960). Thus, parathyroid extract contains carboxypeptidase B activity that rapidly modifies the prohormone hexapeptide, leaving the tripeptide Lys-Ser-Val.

We next attempted to identify the tripeptide or intermediate tetra- or pentapeptides in media from or extracts of parathyroid gland slices that had been incubated in vitro with [^{3}H]lysine or with combinations of [^{3}H]serine and [^{14}C]lysine or [^{3}H]valine and [^{14}C]lysine. Extracts of the radioactively labeled gland slices were prepared either in acetic acid or in acetic acid plus NP-40 detergent. Aliquots of the extracts were subjected to electrophoresis on thin-layer plates simultaneously with added hexapeptide that had been partially digested with carboxypeptidase B. A representative result is shown in Figure

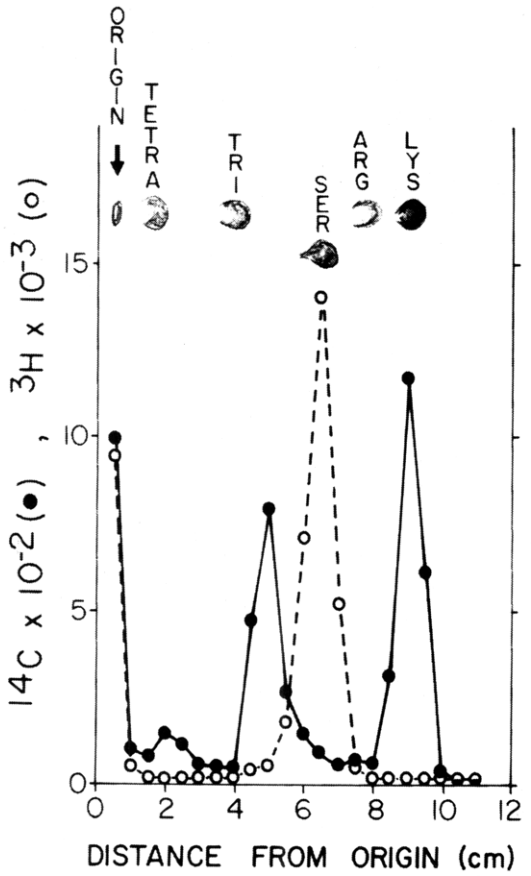

FIGURE 5: Thin-layer electrophoresis on silica gel of an acetic acid extract of bovine parathyroid gland slices that had been incubated for 90 min with [^{14}C]lysine and [^{3}H]serine. Hexapeptide, 50 mg, partially digested with carboxypeptidase B was added to the gland extract before electrophoresis. Spots show ninhydrin-positive material. Plot shows radioactivity through silica gel assayed in 0.5-cm bands through the region of ninhydrin-positive material.

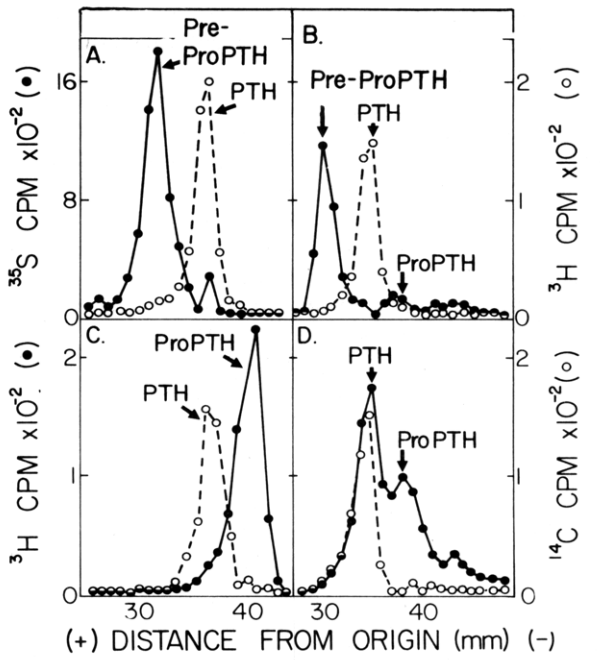

FIGURE 6: Failure of conversion of Pre-ProPTH to either ProPTH or to PTH during incubation in cell-free homogenate prepared from hyperplastic human parathyroid tissue. Analyses are by electrophoresis on urea–acetate polyacrylamide gels of Pre-ProPTH labeled with [^{35}S]methionine (A, B) and for comparison of ProPTH labeled with [^{3}H]methionine (C, D) before (A, C) and after (B, D) incubation for 10 min at 25 °C in homogenate. [^{3}H]Methionine-labeled PTH (A, B) or [^{14}C]leucine-labeled PTH (C, D) was added as a marker to the homogenate before the incubation. Only the regions of the polyacrylamide gels containing measurable radioactivity are shown.

5. Three bands of radioactivity corresponding to the lysine label were consistently observed. The more rapidly migrating band is lysine; neither of the two bands that migrated more slowly than lysine corresponded in mobility to any of the peptide fragments of the hexapeptide, particularly to the tripeptide Lys-Ser-Val or the tetrapeptide Lys-Ser-Val-Lys. Upon further analyses, we determined that the radioactive bands contained over ten times more radioactivity than could theoretically be present in the aliquot of the extract that was electrophoresed. This determination was based on the amounts of radiolabeled PTH contained in the extracts as determined by electrophoresis on polyacrylamide gels and the calculation that the tripeptide and tetrapeptide would contain at most one or two lysines, respectively, compared with 9 lysines in PTH. In addition, we observed these same bands upon thin-layer electrophoresis of [^{14}C]lysine that had been incubated in conditioned media (media obtained after incubation of parathyroid gland slices) but not in fresh media. We believe, therefore, that these bands represent biochemical modifications of lysine and are not peptide fragments of the hexapeptide. This conclusion is further substantiated by the finding that none of these bands contained substantial amounts of either [^{3}H]serine (Figure 6) or of [^{3}H]valine (not shown), as would be expected if they were the tri- or tetrapeptides of the ProPTH hexapeptide. Thus, efforts to positively detect peptide fragments of the hexapeptide formed by action of carboxypeptidase B were inconclusive.

At present, we have less information about the enzymic processes involved in the cellular cleavage of Pre-ProPTH. ^{35}S-labeled Pre-ProPTH was prepared by isolation from polyacrylamide gels of the major product synthesized in response to the translation of parathyroid mRNA in a cell-free system derived from wheat germ (Kemper et al., 1974; Habener et al., 1975a). No conversion of the Pre-ProPTH to either ProPTH or PTH could be demonstrated upon incubation of the prehormone in extracts of parathyroid tissue under conditions in which ProPTH was readily converted to PTH (Figure 6). Repeated attempts to convert radiolabeled Pre-ProPTH to ProPTH or PTH using various subcellular fractions (10 000*g* and 105 000*g* particulate fractions and 105 000*g* supernatant) prepared both from bovine and human parathyroid tissues were unsuccessful.

Discussion

Several lines of evidence derived from these and prior studies indicate that the proteolysis of ProPTH in the parathyroid gland involves the combined actions of trypsin-like and carboxypeptidase B like activities. In earlier studies, it was shown that dilute pancreatic trypsin will cleave specifically the arginine–alanine bond joining the ProPTH-specific hexapeptide to the NH_2 terminus of PTH (Goltzman et al., 1976). In these present studies we have confirmed results reported recently (MacGregor et al., 1976) that extracts of parathyroid glands contain trypsin-like activity that will similarly convert ProPTH to PTH. Moreover, the finding that this activity is not inhibited by the inhibitors of pancreatic trypsin, TLCK and soybean trypsin inhibitor, but is inhibited by chelation of metal ions with EDTA, indicates that the trypsin-like activity in the parathyroid cell probably is an enzyme that is different from pancreatic trypsin, a conclusion shared also by MacGregor et al. (1976).

We have extended the earlier observations on the enzymic conversion of ProPTH to PTH to include an analysis of the fate of the ProPTH hexapeptide after its removal from PTH by tryptic activity. It appears that the hexapeptide is further modified in the parathyroid gland by the action of an exopeptidase with the specificity of carboxypeptidase B, resulting in the sequential removal of one or more of the basic residues from the COOH terminus of the hexapeptide. The most-convincing evidence in support of an action of carboxypeptidase B comes from the results of the radioimmunoassay studies. The levels of immunoreactivity in extracts of parathyroid glands, measured by a radioimmunoassay that readily detects both ProPTH and the hexapeptide, were no higher than those measured by a second radioimmunoassay that detects only ProPTH with hexapeptide covalently attached to the NH_2-terminal sequence of PTH. Moreover, the detection of hexapeptide by radioimmunoassay fell rapidly when the synthetic hexapeptide was treated with carboxypeptidase B, resulting in the sequential removal of Arg-Lys-Lys from the COOH terminus of the hexapeptide. Although these observations lend additional support to our conclusions, the enzymic activity found in cell-free extracts of the tissue may not reflect the specific enzymic activities expressed in the intact tissue. An example of the lack of specificity in the location of these two enzyme activities is the finding that serum contains both trypsin- and carboxypeptidase-like activities that cleave ProPTH and the hexapeptide of ProPTH.

Although efforts to localize the ProPTH cleavage activities within specific subcellular fractions were only partially successful, we did, however, find a relative enrichment of converting activity in the particulate fractions of the cell homogenate, as did MacGregor et al. (1976).

Radioactive labeling studies failed to reveal a carboxypeptidase B modified fragment of the hexapeptide, i.e., a tripeptide Lys-Ser-Val, either in the parathyroid tissues or released into the media. Such a fragment may have escaped detection by the analytical methods that we used. Estimates, however, of the theoretical amounts of radiolabeled peptide fragment that would be formed from ProPTH indicated that the fragment should have been detectable if present in molar amounts equivalent to those of PTH. It is possible that such a small peptide may be rapidly metabolized within the parathyroid cell and thus not be incorporated with PTH into the secretory vesicles or secreted from the cell. This is in contrast to the fate of the comparable C peptide removed from proinsulin. The C peptide is conserved in the β-cell granule and is secreted in equimolar amounts with insulin (Rubenstein et al., 1969; Steiner et al., 1971).

The identification and characterization of the two biosynthetic precursors of PTH, as well as of the precursors of an increasing number of polypeptide hormones and nonhormonal secretory proteins (Habener et al., 1977; Steiner, 1976), have led to the recognition that there exists a distinct class of biosynthetic precursors termed preproteins, or prehormones, that are structurally and functionally separate from the class of proproteins or prohormones (Habener et al., 1977). This distinction is based upon the rapidity of the cleavages during the process of biosynthesis, the location within the cellular transportation pathway at which the cleavage occurs, and, presumably, upon the enzyme specificities involved in the cleavages. Little information is available as yet concerning the nature of the enzymic activities involved in the conversions of the preproteins to the intermediate proproteins, or directly to the authentic final protein products, as appears to be the case with the prehormonal forms of growth hormone (Sussman et al., 1976), prolactin (Evans and Rosenfeld, 1976; Maurer et al., 1976), and placental lactogen (Boime et al., 1975). It is known, however, that the primary amino acid sequences of several of the preproteins, pre-proparathyroid hormone (Kemper et al., 1976a), pre-proinsulin (Chan et al., 1976), pre-L-chains (Burstein et al., 1976; Schechter and Burstein, 1976), and presecretory proteins of the exocrine pancreas (Devillers-Thiery et al., 1975), are uniformly hydrophobic. Moreover, studies in vitro in cell-free systems indicate that the specific cleavage activities reside in the membranous fraction of the cell (microsomal membranes) and that the cleavages are translation dependent (Blobel and Dobberstein, 1975; Habener et al., 1975b; Szczesna and Boime, 1976). Only polypeptide chains undergoing synthesis are cleaved; completed preproteins isolated and added to the membrane-containing systems remain intact. These observations suggest that growing polypeptides are specifically sequestered within the membranous elements that contain the proteolytic activities.

In view of the above observations on the enzymic conversion of prehormones and preproteins by microsomal membrane fractions, it is not surprising that we were unable to demonstrate specific cleavage of Pre-ProPTH to ProPTH by incubations of Pre-ProPTH added to cell-free extracts of parathyroid tissue. We did not expect to find, however, that extracts of parathyroid tissues that contain endoplasmic reticular membranes and other intact subcellular organelles that readily convert preformed ProPTH to PTH did not convert any Pre-ProPTH to PTH.

The failure to observe a direct conversion is unexplained, but we presume that the presence of the amino-terminal sequence of Pre-ProPTH must inhibit the normally rapid and selective action of the trypsin-like enzyme present in parathyroid tissue on the Arg-Ala site of the ProPTH, perhaps owing to secondary structural constraints imposed on the polypeptide by the hydrophobic amino-terminal sequence of the Pre-ProPTH or to binding of the Pre-ProPTH to membranous particulate elements in the cellular extracts. Preproparathyroid hormone is, of course, rapidly converted to tryptic fragments by conventional doses of pancreatic trypsin (1%) in vitro (Kemper et al., 1974, 1976a,b; Habener et al., 1975a). What is lacking is the selective cleavage of the Arg-Ala site accomplished by dilute solutions of trypsin (Goltzman et al., 1976) or by parathyroid gland slices during the biosynthesis of PTH (Kemper et al., 1972).

The cumulative evidence indicates that combined trypsin-like and carboxypeptidase B like activities are involved universally in the cellular processing of polypeptide prohormones and proproteins into their respective products (Figure 7). The action of carboxypeptidase B is not required in the formation of the parathyroid hormone itself, inasmuch as the basic residues at the site of the trypsin-like initial cleavage are located on the prohormone-specific hexapeptide and not on the hormone. This mechanism, illustrated in Figure 7 as type A, is probably also characteristic of the conversion of proalbumin to albumin (Russell and Geller, 1975) and of progastrin to gastrin (Gregory and Tracy, 1972). The other type of prohormone conversion process (type B) exemplified by glucagon (Tager and Steiner, 1973) in Figure 7 involves further processing of the hormonal fraction by carboxypeptidase after initial tryptic cleavage, leaving the prohormone-specific peptide unmodified. Both types of processing appear to be characteristic of proinsulin (type C) (Kemmler et al., 1971; Steiner, 1976).

Continued efforts toward the cellular localization and isolation of the enzyme activities involved in the conversion of prohormones, such as those of ProPTH to PTH, should lead to a more complete understanding of the biochemical and

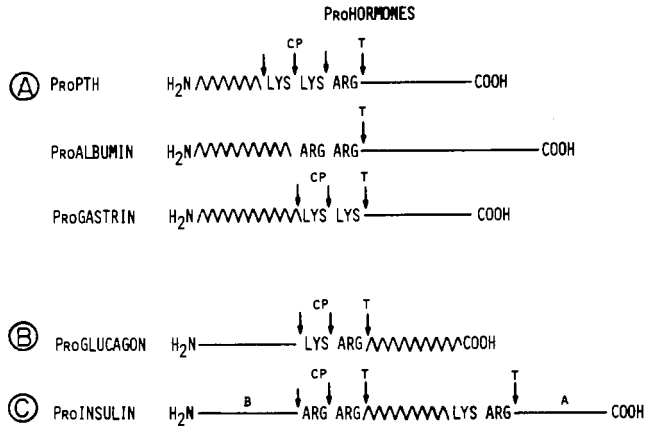

FIGURE 7: Schematic diagrams of the primary structures of several different prohormones. (A) Class of prohormones where tryptic-like cleavage alone is sufficient to give the authentic hormonal product. (B) ProGlucagon fragment where combined tryptic-like and carboxypeptidase-like cleavages are required to produce the final product, glucagon. (C) ProInsulin, an example where both tryptic-like alone and combined tryptic-like and carboxypeptidase-like activities are involved in the conversion of the prohormone to insulin.

cellular processes responsible for the translocation and the posttranslational proteolytic modification of the prohormone precursors generally.

Acknowledgments

We thank G. W. Tregear for preparation of the synthetic hormonal peptides, T. D. Stevens for laboratory assistance, and L. B. Fred for editorial help.

References

Blobel, G., and Dobberstein, B. (1975), *J. Cell Biol. 67,* 835.

Boime, I., Boguslawski, S., and Caine, J. (1975), *Biochem. Biophys. Res. Commun. 62,* 103.

Burstein, Y., Kantor, F., and Schechter, I. (1976), *Proc. Natl. Acad. Sci. 73,* 2604.

Chan, S. J., Keim, P., and Steiner, D. F. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1964.

Chrétien, M., and Li, C. H. (1967), *Can. J. Biochem. 45,* 1163.

Chu, L. L. H., MacGregor, R. R., Hamilton, J. W., and Cohn, D. V. (1974), *Endocrinology 95,* 1431.

Cohn, D. V., MacGregor, R. R., Chu, L. L. H., Kimmel, J. R., and Hamilton, J. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1521.

Devillers-Thiery, A., Kindt, T., Scheele, G., and Blobel, G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 5016.

Evans, G. A., and Rosenfeld, M. G. (1976), *J. Biol. Chem. 251,* 2842.

Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. (1960), *J. Biol. Chem. 235,* 2272.

Goltzman, D., Callahan, E. N., Tregear, G. W., and Potts, J. T., Jr. (1976), *Biochemistry 15,* 5076.

Gregory, R. A., and Tracy, H. J. (1972), *Lancet 2,* 797.

Habener, J. F. (1976), *Ciba Found. Symp. 41,* 197.

Habener, J. F., Kemper, B. W., Potts, J. T., Jr., and Rich, A. (1974b), *Endocr. Res. Commun. 1,* 239.

Habener, J. F., Kemper, B. W., Potts, J. T., Jr., and Rich, A. (1975a), *J. Clin. Invest. 56,* 1328.

Habener, J. F., Kemper, B. W., Potts, J. T., Jr., and Rich, A. (1975b), *Biochem. Biophys. Res. Commun. 67,* 1114.

Habener, J. F., Kemper, B. W., Rich, A., and Potts, J. T., Jr. (1977), *Recent Prog. Horm. Res. 33* (in press).

Habener, J. F., and Potts, J. T., Jr. (1975), *Methods Enzymol. 37,* 345.

Habener, J. F., and Potts, J. T., Jr. (1976a), in Handbook of Physiology, Section 7, Vol. VII, Greep, R. O., and Astwood, E. B., Section Ed., Aurbach, G. D., Volume Ed., Geiger, S. R., Executive Ed., Washington, D.C., American Physiological Society, p 313.

Habener, J. F., and Potts, J. T., Jr. (1976b), in Hormones in Human Blood: Detection and Assay, Antoniades, H. N., Ed., Cambridge, Mass., Harvard University Press, p 551.

Habener, J. F., Potts, J. T., Jr., and Rich, A. (1976a), *J. Biol. Chem. 251,* 3893.

Habener, J. F., Stevens, T. D., Tregear, G. W., and Potts, J. T., Jr. (1976b), *J. Clin. Endocrinol. Metab. 42,* 520.

Habener, J. F., Tregear, G. W., Stevens, T. D., Dee, P. C., and Potts, J. T., Jr. (1974a), *Endocr. Res. Commun. 1,* 1.

Hamilton, J. W., Niall, H. D., Jacobs, J. W., Keutmann, H. T., Potts, J. T., Jr., and Cohn, D. V. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 653.

Jacobs, J. W., Kemper, B. W., Niall, H. D., Habener, J. F., and Potts, J. T., Jr. (1974), *Nature (London) 249,* 155.

Kemmler, W., Peterson, J. D., and Steiner, D. F. (1971), *J. Biol. Chem. 246,* 6786.

Kemmler, W., Steiner, D. F., and Borg, J. (1973), *J. Biol. Chem. 248,* 4544.

Kemper, B. W., Habener, J. F., Ernst, M. D., Potts, J. T., Jr., and Rich, A. (1976a), *Biochemistry 15,* 15.

Kemper, B. W., Habener, J. F., Mulligan, R. C., Potts, J. T., Jr., and Rich, A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3731.

Kemper, B. W., Habener, J. F., Potts, J. T., Jr., and Rich, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 643.

Kemper, B. W., Habener, J. F., Potts, J. T., Jr., and Rich, A. (1976b), *Biochemistry 15,* 20.

Kemper, B. W., Habener, J. F., Rich, A., and Potts, J. T., Jr. (1975), *Endocrinology 96,* 903.

MacGregor, R. R., Chu, L. L. H., and Cohn, D. V. (1976), *J. Biol. Chem. 251,* 6711.

Maurer, R. A., Stone, R., and Gorski, J. (1976), *J. Biol. Chem. 251,* 2801.

Rubenstein, A. H., Clark, J. L., Melani, F., and Steiner, D. F. (1969), *Nature (London) 224,* 697.

Russell, J. H., and Geller, D. M. (1975), *J. Biol. Chem. 250,* 3409.

Schechter, I., and Burstein, Y. (1976), *Biochem. Biophys. Res. Commun. 68,* 489.

Steiner, D. F. (1976), in Peptide Hormones, Parsons, J. A., Ed., London, Macmillan, p 49.

Steiner, D. F., Cho, S., Oyer, P. E., Terris, S., Peterson, J. D., and Rubenstein, A. H. (1971), *J. Biol. Chem. 246,* 1365.

Sussman, P. M., Tushinski, R. J., and Bancroft, F. C. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 29.

Szczesna, E., and Boime, I. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1179.

Tager, H. S., and Steiner, D. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2321.

Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., O'Riordan, J. L. H., and Potts, J. T., Jr. (1974), *Hoppe-Seyler's Z. Physiol. Chem. 355,* 415.

Vaitukaitis, J. L., Robbins, J. B., Nieschlag, E., and Ross, G. T. (1971), *J. Clin. Endocrinol. Metab. 33,* 988.