Edman Degradation of Radioiodinated Parathyroid Hormone: Application to Sequence Analysis and Hormone Metabolism in Vivo†

Gino V. Segre,*,‡ Hugh D. Niall,§ Robert T. Sauer,¶ and John T. Potts, Jr.

ABSTRACT: A general method for preparation and controlled chemical degradation of radioiodinated parathyroid hormone was developed for both investigating amino acid sequence and analyzing the metabolism of the hormone in vivo. Sequence analysis of radioiodinated parathyroid hormone was used to determine the positions of tyrosyl and histidyl residues in hormones from several species. Degradation of intact and enzymically digested human parathyroid hormone showed histidyl residues at positions 9, 14, 32, and 63 of the sequence, but indicated that tyrosine was not present within human parathyroid hormone. Although the method is limited to detection of tyrosyl and histidyl residues, important aspects of the overall primary structure can be deduced, particularly between homologous hormones, using very small quantities of material. The sensitivity of the method is 10^7 higher than conventional chemical techniques. The metabolism of bovine parathyroid hormone was studied in dogs. The high specific activity attainable with radioiodinated preparations permitted evaluation, by the microsequencing technique, of the metabolic fate of physiologic concentrations of hormone. The heterogeneity of circulating parathyroid hormone observed by many workers was shown to be due, at least in part, to the cleavage of the hormone after entry into the circulation. Cleavage occurs in one or more extravascular sites. The nature of the cleavage could be deduced from the radioiodinated fragments produced; Edman degradation revealed fragments with residues 34, 37, 41, and 43 as the NH_2-terminal amino acid. These results leave unanswered the precise nature of the cleavage process, but do suggest that, inasmuch as no cleavages occur nearer the NH_2 terminus than residue 33, an NH_2-terminal fragment containing the necessary structure for biologic activity (shown in structure–function studies, using synthetic peptides, to be residues 2 through 27) may be produced within peripheral tissues in vivo by the process of hormone metabolism. Sequence analysis of radioactive polypeptides shows promise as a general method for the study of the metabolism of these compounds.

In a previous report, we described the use of sequence analysis of radioiodinated human parathyroid hormone to determine the positions of histidyl residues within the primary structures of the NH_2-terminal portion of the molecule (Niall et al., 1974). The success of this approach suggested that several issues concerning polypeptides could be addressed using this technique; accordingly, the methods were better defined and applied to further analyses of structure and, in addition, to analysis of hormone metabolism in vivo.

In the present studies, intact and enzymatically digested preparations of microquantities of radioiodinated parathyroid hormones, including human parathyroid hormone, were subjected to sequence analysis to determine certain chemical features. These included the positions of tyrosyl and histidyl residues throughout the molecule and the overall homology of human (still incompletely sequenced), bovine, and porcine hormones.

Second, this method was employed to study the metabolism of physiologic concentrations of parathyroid hormone in dogs by analysis of the radiolabeled fragments present in the plasma at various times after injection of intact hormone. The metabolism of parathyroid hormone and the heterogeneity of its circulating forms are subjects of current widespread interest (Habener et al., 1971, 1972; Canterbury and Reiss, 1972; Segre et al., 1972, 1974; Goldsmith et al., 1973; Silverman and Yalow, 1973; Arnaud et al., 1974; Fischer et al., 1974; Canterbury et al., 1975; Hruska et al., 1975; Neuman et al., 1975). Our principal aims were: (1) to examine the metabolic products of intact bovine parathyroid hormone in dogs to see whether they are similar to the forms of endogenous hormone found in the circulation, (2) to determine whether, after injection of intact hormone intravenously, those forms of the hormone that, upon gel filtration, elute later than intact hormone are actually hormone fragments and not intact hormone itself with an altered conformation, and, most important, if cleavage occurs, to (3) define, chemically, the exact location of the bonds within the hormone molecule that undergo proteolytic digestion during peripheral metabolism.

Materials and Methods

Preparation of Parathyroid Hormones. Intact bovine, human, and porcine parathyroid hormones were purified by gel filtration and ion-exchange chromatography as previously described (Niall et al., 1970; Keutmann, 1971; Woodhead et al., 1971; Keutmann et al., 1974; Sauer et al., 1974).

Preparation of Parathyroid Hormone Fragment 53–84. Fragment 53–84 of each hormone was prepared by tryptic digestion of highly purified native hormone after reversibly blocking the ϵ-amino groups of lysine residues as previously described (Sauer et al., 1974). The human and porcine fragments were made together. Intact porcine parathyroid hor-

† From the Endocrine Unit, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114. *Received November 29, 1976.* This work was supported in part by Grants AM 11794 and 04501 from the National Institute of Arthritis, Metabolism, and Digestive Diseases of the National Institutes of Health, by a grant from the John A. Hartford Foundation, Inc., and by Contract NAS 9-11011 from the National Aeronautics and Space Administration.

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§ Present address: Howard Florey Institute, University of Melbourne, Parkville, Victoria 3052, Australia.

¶ Present address: Department of Biochemistry, Harvard University, Cambridge, Mass. 02138.

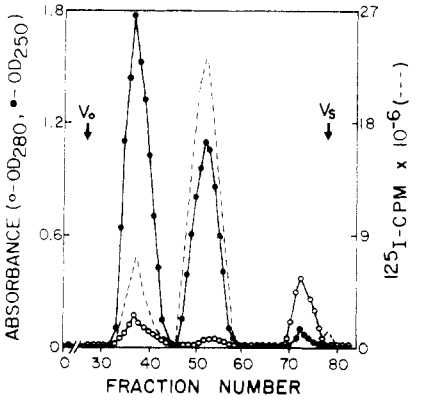

FIGURE 1: Sephadex G-50 column chromatography of a tryptic digest of maleoylated ^{125}I-labeled human and unlabeled porcine parathyroid hormones. The maleoyl groups account for the high absorbance at 250 nm. V_0 and V_s mark the void and salt volumes, respectively.

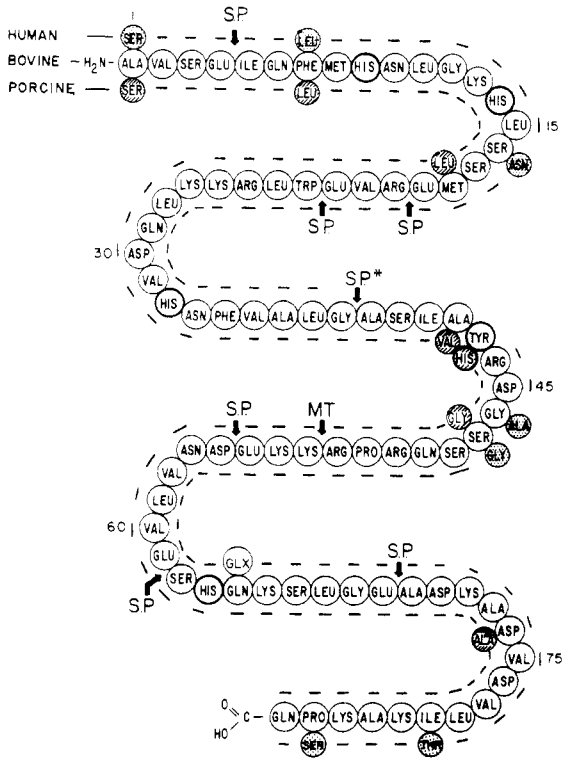

FIGURE 2: The sequences of bovine, human, and porcine parathyroid hormones are shown for reference. Sites of cleavage of the maleoylated hormones with trypsin (MT) and of the native hormones with staphylococcal protease (S.P.) that are pertinent to the Edman degradations performed are shown. Additional MT cleavages occur adjacent to arginine residues at positions 20, 25, and 44. S.P.* indicates the unexpected cleavage between residues 38 and 39.

mone, 0.6 μmol, was added to 50 pmol of intact human parathyroid hormone after the latter had been iodinated with ^{125}I. The porcine hormone acted as both carrier for the human preparation and substrate for the enzymatic digestion. Unreacted reagents of the iodination procedure were removed by gel filtration on a column (2.0 × 50 cm) of Bio-Gel P-2, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.) that was equilibrated and eluted with 0.14 M ammonium acetate, pH 4.9. After lyophilization, the two hormones were dissolved in 0.2 M sodium borate and treated with maleic anhydride. The maleoylated hormones were separated from unreacted reagents and salt by gel filtration (Sauer et al., 1974). Optical density of the eluate tubes was read at 250 and 280 nm using the Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif.), and the radioactivity was determined using a dual-channel, γ-well spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Fractions comprising the peak eluting at the void volume were pooled and lyophilized. The maleoylated hormones were digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin (Worthington Biochemical Corp., Freehold, N.J.) for 2 h at 37 °C in 0.2 M trimethylamine acetate, pH 8.2, using an enzyme-substrate ratio of 1/100. The digest was gel filtered on a column (1.2 × 140 cm) of Sephadex G-50, superfine (Pharmacia, Uppsala, Sweden). Eluate fractions (2.2 mL) were monitored by measuring the absorbance at 250 and 280 nm and by counting the ^{125}I (Figure 1). Fractions comprising the first major peak, which contains the 53–84 fragment (Niall et al., 1970; Sauer et al., 1974), were pooled and lyophilized. The maleoyl groups were removed by incubating the peptides in 1 N formic acid for 45 min at 80 °C.

Bovine parathyroid hormone fragment 53–84 was prepared by identical methods, except that procedures relating to iodination and the monitoring of radioactivity were omitted.

Parathyroid Hormone Fragments Prepared Using Staphylococcal Protease. Staphylococcal protease (generously provided by Dr. G. Drapeau, Dept. of Microbiology, University of Montreal, Montreal, Canada) was used to prepare a mixture of hormone fragments (Figure 2). Before digestion with the enzyme, the human hormone (50 pmol) and bovine hormone (50 pmol and 10 nmol) were iodinated with ^{125}I, ^{131}I, and ^{127}I, respectively. The hormones were then combined, and reagents of the iodination reaction were removed by gel filtration on a column of Bio-Gel P-2 (see above). Fractions comprising the peak eluting at the void volume were pooled and lyophilized. Digestion was performed at the enzyme–substrate ratio of 1/20 in 0.05 M ammonium acetate, pH 4.0, for 24 h at 37 °C. The digest was then relyophilized. For additional studies, 0.4 μmol of unlabeled bovine hormone was also treated with staphylococcal protease, using procedures identical to those above.

Preparation of Synthetic Parathyroid Hormone Fragments. Bovine hormone fragment 1–34 and subfragments 1–13, 1–21, 1–26, 1–27, 1–28, 1–30, 14–34, and 19–34 were synthesized by solid-phase methods and purified by gel filtration and ion-exchange chromatography as previously described (Tregear et al., 1973).

Quantitation and Characterization of the Hormone Preparations. Amino acid composition and precise quantitation of the bovine hormone and all synthetic and natural fragments of the bovine hormone used in characterizing the antisera used in the radioimmunoassays were determined by amino acid analysis after both acid and enzymatic hydrolysis (Niall et al., 1970; Keutmann et al., 1971).

The biologic activity of the bovine hormone used in the studies performed in dogs was assessed in vitro by the activation of rat renal cortical adenylyl cyclase (Marcus and Aurbach, 1969) and in vivo by the increase in serum calcium in the chick (Parsons et al., 1973); potency was 3000 and 2500 MRCU[1]/mg, respectively.

[1] Abbreviations used are: Pth, phenylthiohydantoin; Tyr(I), iodotyrosine; Tyr(I_2), diiodotyrosine; His(I), iodohistidine; C assays, radioimmunoassays using antisera that specifically recognize determinants in the COOH-terminal portion of the hormone sequence; N assays, radioimmunoassays using antisera that specifically recognize determinants in the NH_2-terminal portion of the hormone sequence; MC fragments, fragments comprising the middle and COOH-terminal portions of the hormone sequence; MRCU, Medical Research Council Units (Great Britain).

Iodination. The hormones and hormone fragments were iodinated with $Na^{125}I$, $Na^{131}I$ (New England Nuclear, Boston, Mass.), and $K^{127}I$ (Allied Chemicals, Morristown, N.J.), by modifications of the methods of Hunter and Greenwood (1962), at room temperature in 0.2 M sodium phosphate, pH 7.4, using a molar ratio of hormone/I^-/chloramine-T (Eastman Chemical, Rochester, N.Y.)/sodium metabisulfite (Mallinckrodt, St. Louis, Mo.) of 1/3.4/940/4700. To ensure complete and uniform labeling, twice these concentrations of I^- and chloramine-T were used to iodinate the human and bovine hormones, and preparations of the human hormone used in the studies of primary structure were also iodinated using 0.2 M sodium phosphate/8 M urea, pH 7.4, as the buffer. Sodium metabisulfite was added after 30 s to stop the reaction. Unlabeled or ^{127}I-labeled parathyroid hormone was then added to carry the microquantities of hormone to be used in the chemical studies. The yield of the phenylthiohydantoin (Pth) amino acid derivatives recovered during sequence analysis of the carrier also served as a measure of the efficiency of the repetitive Edman reaction. After the carrier was added, these radiolabeled preparations were purified on columns of Bio-Gel P-2 (see below). The radiolabeled hormone used in the metabolic studies was purified by methods previously described (Segre et al., 1975).

Specific activity was estimated in samples of the radiolabeled hormones precipitated with 10% trichloroacetic acid. In addition, the specific activity of the ^{125}I-labeled preparations was determined by radioimmunoassay using bovine ^{131}I-labeled hormone as tracer and the homologous preparation as standard. Values were 200–300 mCi/mg for bovine ^{125}I-labeled hormone, 150–300 mCi/mg for bovine ^{131}I-labeled hormone, and 150–250 and 200–300 mCi/mg for the two human ^{125}I-labeled hormone preparations. The specific activity was not influenced by labeling in the presence of 8 M urea. The specific activity of the ^{131}I-labeled hormones used to mark the elution positions during gel filtration of plasma samples was not determined.

Procedure for Analysis of Parathyroid Hormone Metabolism in Vivo. Studies were performed in healthy mongrel dogs weighing 14–18 kg, anesthetized by intravenous injection of sodium methohexital (Brevital) (Eli Lilly and Co., Indianapolis, Ind.). Two V-9 poly(vinyl chloride) catheters (Bolab Inc., Derry, N.H.) were inserted through incisions in the right and left external jugular veins and advanced into the right atrium and superior vena cava, respectively.

Labeled or unlabeled bovine hormone was injected rapidly (10–30 s) into the right atrium. Ten–fifteen-mL samples from the superior vena cava were collected in heparinized tubes before and at various time intervals up to 4 h after injection. Blood volume was maintained by replacing the removed blood with an equal volume of normal saline.

The heparinized samples were immediately centrifuged at 3000 rpm for 5–10 min at 4 °C. To avoid repeated freezing and thawing, the plasma from each sample was placed in several vials, quick-frozen in acetone–dry ice, and stored at −70 °C. Each vial was thawed only once and then discarded.

In four studies, bovine ^{125}I-labeled parathyroid hormone in 0.1 N acetic acid/20% acetone/1% acetic acid (v/v) was injected. In two of these, the dose was approximately 1 μg, and in the other two, approximately 10 μg. All samples were analyzed by gel filtration (see below), and the radioactivity in each fraction was evaluated. Selected fractions from the experiments using the larger dose of labeled hormone were further analyzed by automated sequence analysis and thin-layer chromatography or by paper chromatography.

The protocol was essentially the same in studies of the metabolism of unlabeled, biologically active, bovine hormone, except that 600 μg of the preparation was injected in 0.1 N acetic acid. Samples were analyzed by gel filtration and sequence-specific radioimmunoassays.

In addition, bovine ^{125}I-labeled hormone, 2×10^6 cpm (approximately 4 ng), and unlabeled bovine hormone (1 μg) were added to separate tubes containing 0.5 mL of heparinized dog plasma and incubated for 6 h at 37 °C. Samples from each were chromatographed by gel filtration with the appropriate ^{131}I-labeled column markers and analyzed according to the methods described below.

Gel Filtration of Plasma Samples. Gel filtration of all plasma samples was carried out on columns (1.2 × 70 cm) of Bio-Gel P-100, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.) at 4 °C with an eluting buffer of 0.05 M sodium barbital and human plasma, pH 8.6. Fraction size was 0.8 mL. Samples from experiments using unlabeled hormone were chromatographed with an eluting buffer consisting of 0.05 M sodium barbital/10% (v/v) human plasma. This corresponds to the diluent used in the radioimmunoassays, and, except when measuring immunoreactive hormone in the fractions in or near the void volume of the column, this permitted different volumes of the eluted fractions to be added to the assay without changing the final protein concentration. One set of control tubes without added antisera was thus adequate to correct for any nonspecific effects due to alterations of the immunologic properties of the tracer in samples taken from most of the fractions. For samples taken from the fractions in or near the void volume of the column, in which the plasma protein concentration varied and was higher, individual controls were used. To avoid interference during sequence analysis by this excess of protein content, samples from studies using labeled hormone were chromatographed with the same buffer, but with only 1% (v/v) human plasma as carrier.

Several duplicate samples from experiments using bovine ^{125}I-labeled hormone were additionally evaluated by chromatography on columns of Bio-Gel A-0.5m, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.), at 26 °C using an eluting buffer at 6 M guanidine hydrochloride (Heico Laboratories, Delaware Water Gap, Pa.)/0.1 M ammonium acetate/1% (v/v) human plasma, pH 6.5. Before application to the columns, these samples, which included radioiodinated hormone after incubation in 0.5 mL of heparinized dog plasma and several plasma samples collected from dogs after hormone injection, were made 6 M in guanidine hydrochloride by incubation with the solid salt for 2 h at 26 °C. The radioiodinated hormone and the samples were chromatographed, respectively, on columns measuring 1.2 × 70 and 2.0 × 85 cm. Fraction sizes were 0.75 and 1.4 mL respectively.

After gel filtration, each fraction was counted in a γ-well spectrometer to determine the profiles of the various ^{131}I-labeled column markers and the ^{125}I-labeled samples, calculated after appropriate correction for ^{131}I. Recovery of ^{125}I and immunoreactive hormone ranged from 84 to 93% for the former and 79 to 96% for the latter.

To remove nonvolatile salts from the samples before sequence analysis, selected fractions were pooled and chromatographed on columns (2.0 × 45 cm) of Bio-Gel P-2, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.), at 4 °C using an eluting buffer of 0.1 M ammonium acetate/1% (v/v) human plasma adjusted to pH 4.0 with glacial acetic acid. Separation of the high concentration of guanidine required columns measuring 2.5 × 90 cm. All radioactivity appeared in the void volume of the column with a recovery of 91 to 96%.

The radioactive fractions were then pooled and lyophilized.

Edman Degradations. Automated Edman degradations were performed in a Model 890 "Sequencer" (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) using a single-coupling, double-cleavage program (Edman and Begg, 1967). Lyophilized samples were dissolved in trifluoroacetic acid. All plasma samples were subjected to automated sequence analysis for at least 25 cycles, and several were sequenced for 46 cycles. Reagents and solvents were obtained from Beckman Instruments. The mixture of peptides resulting from staphylococcal protease digestion of unlabeled bovine hormone was degraded, manually, by modifications (Sauer et al., 1974) of the three-stage method of Edman (Edman, 1960).

The amino acid derivatives (anilinothiazolinones) were collected in 3 mL of 1-chlorobutane and dried under nitrogen. In the degradations of porcine hormone fragment (53–84) and of the staphylococcal protease digest of bovine hormone, 1,4-butanedithiol (1:20,000) was added to protect the labile Pth derivative of serine. In studies using radioiodinated hormone, ^{127}I-containing Pth derivatives of iodotyrosine (Tyr(I)), diiodotyrosine (Tyr(I_2)), and iodohistidine (His(I)) were added. His(I) was synthesized by the method of Brunnings (1947) using the modifications of Savoie et al. (1973). Tyr(I) and Tyr(I_2) were purchased from Mann Research Lab., New York, N.Y., and Pierce Chemicals, Inc., Rockford, Ill., respectively. The Pth derivatives were prepared by the methods of Edman (1950). The anilinothiazolines were converted to the stable Pth derivatives by incubating in 0.2 mL of 1 N HCl for 10 min at 80 °C (Ilse and Edman, 1963). After twice extracting with 0.8 mL of ethyl acetate, approximately 70% of the Pth derivatives of [^{125}I]iodohistidine and [^{131}I]iodohistidine remained in the aqueous phase. The Pth derivatives of [^{125}I]iodotyrosine and [^{125}I]diiodotyrosine (and [^{131}I]iodotyrosine and [^{131}I]diiodotyrosine) were extracted into the organic phase with an approximate efficiency of 95%. The phases were separated and dried under nitrogen. Aliquots were then chromatographed on silica-gel GF glass plates (20 × 20 cm; 250 μm) (Uniplate, Analtech, Inc., Newark, N.J.) using two solvent systems: ethylene dichloride/acetic acid (30/7) (Edman, 1970) and chloroform/methanol/heptafluorobutyric acid (140/60/1) (Schlesinger et al., 1975). The positions of the ^{127}I-containing Pth derivatives of histidine and tyrosine were visualized under ultraviolet light, and ^{125}I and ^{131}I were identified by autoradiography. Radioactive areas were coincident with the ^{127}I-containing standards, indicating that, under these iodination conditions, only tyrosine and histidine were labeled. The individual radioactive areas were removed and counted in a γ-well spectrometer. Results of the studies of hormone metabolism are corrected, assuming a repetitive yield of 96% at each cycle of the Edman degradation. Data from the chemical studies are not corrected.

Identification and quantitation of the Pth-amino acid derivatives of porcine hormone fragment 53–84 and of the mixture of peptides resulting from staphylococcal protease digestion of bovine hormone were performed by gas–liquid chromatography (Pisano and Bronzert, 1969) using a Beckman Model 45 gas chromatograph. The Pth-tyrosine in the bovine hormone treated with staphylococcal protease was also identified by thin-layer chromatography on glass plates (see above) using ethylene dichloride/acetic acid (30/2) in order to resolve completely Pth-tyrosine from Pth-histidine.

The radioactive fractions that eluted at the salt volume of the column after gel filtration of the plasma samples were pooled. Samples taken from these pools were cochromatographed with ^{127}I-containing Tyr(I), Tyr(I_2), His(I), and NaI, all of which served as both carrier and standards, on Whatman 3MM (W & R Balston, Ltd., England) filter paper for 18–24 h using two solvent systems: butanol/glacial acetic acid/water (15/1/4) and collidine/2 N acetic acid (8/2) (Covelli and Wolff, 1966). The iodinated amino acids were identified with ninhydrin, and the paper strips, cut into 1-cm sections from the origin to the solvent front, were counted in a γ-well spectrometer. Recovery of total radioactivity in the two solvent systems was 97 and 93%, respectively. In addition, recovery of ^{125}I, [^{125}I]iodotyrosine, and [^{125}I]diiodotyrosine (Amersham/Searle Corp., Arlington Heights, Ill.) was 95, 97, and 99% in the butanol/acetic acid/water system and 90, 96, and 95% in the collidine/2 N acetic acid system.

Radioimmunoassays. Radioimmunoassays, using 3-day equilibrium incubation conditions, were performed by methods previously described (Segre et al., 1972, 1975). Highly purified bovine hormone was used as standard and, after iodination with ^{125}I, the same preparation was used as tracer. A partial identification of the portions of the hormone recognized by the antibody populations of the two anti-bovine parathyroid hormone antisera used in these studies, GP-1 and GP-133, and the methods used to restrict antigenic recognition to selected portions of the hormone molecule were reported previously (Segre et al., 1972, 1975). In brief, the antigenic determinants recognized by each antiserum were defined by the inhibition of binding of bovine ^{125}I-labeled hormone tracer to the antiserum by bovine hormone fragments 1–34 and 53–84, and by subfragments of the 1–34 fragment: 1–13, 1–21, 1–26, 1–27, 1–28, 1–30, 14–34, and 19–34. Both GP-1 and GP-133 contain antibody populations that recognize multiple determinants. After preincubation with an excess of bovine hormone fragment 1–34, the remaining antibodies in both GP-1 and GP-133 recognize the same or different major antigenic determinant(s) that are included in the 53–84 region of the hormone. After preincubation with an excess of bovine hormone fragment 53–84, the remaining antibody populations of the two antisera bind to different major NH_2-terminal determinants; GP-1 recognizes a determinant that includes some or all of the 14–27 region, whereas GP-133 binds to a determinant that includes some or all of the 31–34 region.

Results

Chemical Studies

Edman Degradation of Radioiodinated Bovine and Human Parathyroid Hormone. Preparations containing 4 pmol of ^{131}I-labeled bovine and ^{125}I-labeled human parathyroid hormone, each with 150 nmol of unlabeled bovine hormone, were combined and subjected to 65 cycles of Edman degradation. Peaks of both ^{125}I and ^{131}I, in a ratio of approximately 9:1, were seen at cycles 9, 14, and 32 of the degradation (Figure 3A). In addition, a peak of ^{131}I was seen at cycle 43. Pth-[^{125}I]iodohistidine and Pth-[^{131}I]iodohistidine were identified at cycles 9, 14, and 32 (Figure 3B), and Pth-[^{131}I]iodotyrosine and Pth-[^{131}I]diiodotyrosine were identified at cycle 43 by thin-layer chromatography (Figure 3C). No iodotyrosyl derivatives labeled with ^{125}I were seen during the degradation. Because the Pth derivative of [^{131}I]iodohistidine was not detected in the bovine hormone at cycle 63, a known histidine residue, the degradation was discontinued after 65 cycles. Approximately 10% of the total radioactivity introduced into the sequenator was recovered as the Pth derivatives of [^{131}I]iodotyrosine and [^{131}I]diiodotyrosine after 43 cycles of degradation. This is consistent with the 94% repetitive yield at each cycle of degradation found for the unlabeled hormone

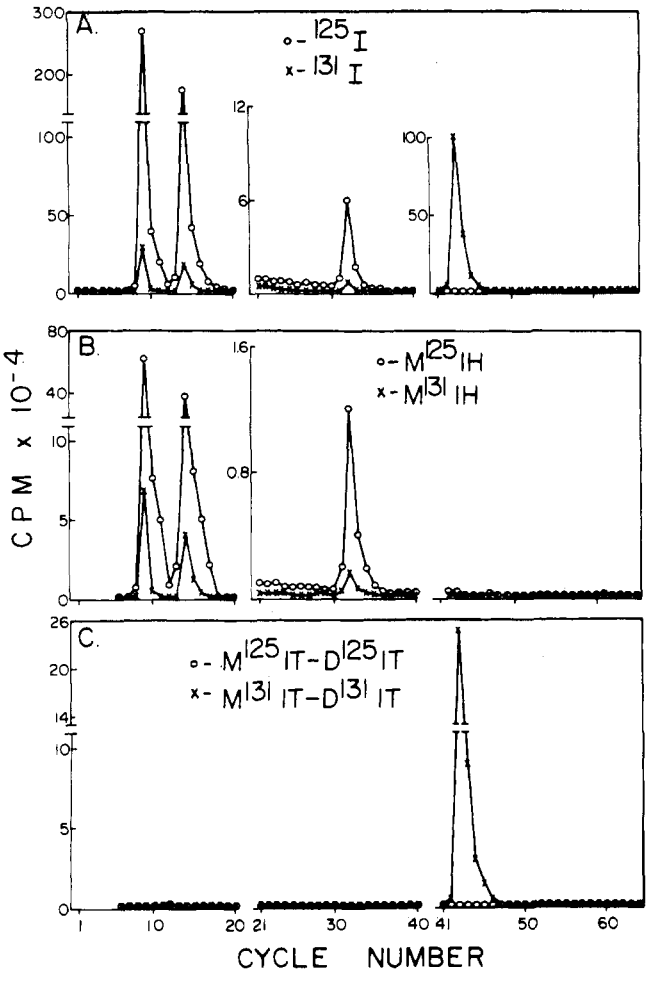

FIGURE 3: Radioactivity released during sequencing of intact ^{125}I-labeled human and ^{131}I-labeled bovine parathyroid hormones. (A) Iodide (I) radioactivity; (B) specific iodohistidyl (MIH) radioactivity; (C) specific iodotyrosyl (MIT, DIT) radioactivity. The results shown in Figures 3, 4, and 5 are from degradations of human parathyroid hormone after iodination with ^{125}I in the absence of urea and using the higher concentration of iodide and chloramine-T. Iodination in the presence of urea did not alter the distribution of the label.

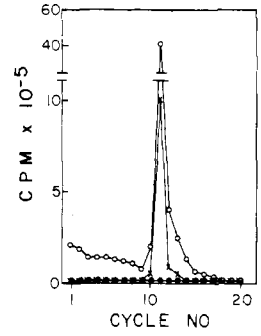

FIGURE 4: Radioactivity released during sequencing of the earliest eluting radioactive peak seen in Figure 1, known to be fragment 53–84 of the hormones; ^{125}I (O), [^{125}I]iodohistidyl radioactivity (×), [^{125}I]iodotyrosyl and [^{125}I]diiodotyrosyl radioactivity (●).

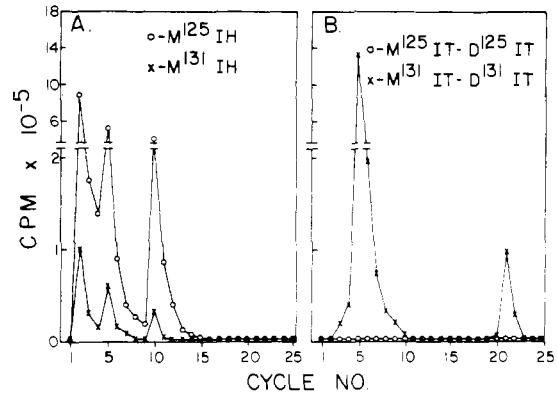

FIGURE 5: Radioactivity released during sequencing of ^{125}I-labeled human and ^{131}I-labeled bovine hormones after digestion with staphylococcal protease. (A) Specific iodohistidyl (MIH) radioactivity. (B) Specific iodotyrosyl (MIT, DIT) radioactivity.

and suggests that the iodotyrosyl derivatives do not undergo a significant degree of deiodination under the conditions of the Edman reactions. However, if one assumes that the histidines at positions 9, 14, and 32 are equally labeled initially, their estimated repetitive yield is 85%, suggesting that iodohistidyl residues undergo some deiodination under these experimental conditions.

Edman Degradation of Fragments 53–84 of ^{125}I-Labeled Human and Porcine Parathyroid Hormones. To determine whether histidine is present at position 63 in the human hormone, as it is in both bovine and porcine hormones, the first-eluting peak after Sephadex G-50 chromatography of the tryptic digestion of maleoylated porcine hormone (600 nmol) and ^{125}I-labeled human hormone (50 pmol), the peak corresponding to the 53–84 fragment (Figure 1), was studied after the maleoyl groups were removed. At cycle 11 of the automated degradation, ^{125}I in large quantities was released and identified by thin-layer chromatography as the Pth derivative of [^{125}I]iodohistidine (Figure 4). Pth-histidine was readily identified at the same cycle, reflecting the presence of this residue at position 63 in the porcine hormone.

Edman Degradation of Fragments of Human ^{125}I- and Bovine ^{131}I-Labeled Parathyroid Hormone Generated by Staphylococcal Protease Digestion. A portion of the fragment mixture generated by staphylococcal protease that represented approximately 10 pmol of human ^{125}I-labeled hormone and bovine ^{131}I-labeled hormone and 20 nmol of bovine ^{127}I-labeled hormone was subjected to automated sequence analysis. ^{125}I was released in large quantities at cycles 2, 5, and 10. ^{131}I was released in large quantities at cycle 5, with lesser amounts at cycles 2, 10, and 21. No release of ^{125}I above background was seen at cycle 21. The ^{125}I and the ^{131}I at cycles 2 and 10 were identified by thin-layer chromatography as the Pth derivatives of [^{125}I]iodohistidine and [^{131}I]iodohistidine, respectively (Figure 5). The ^{131}I at cycle 21 consisted exclusively of the Pth derivatives of [^{131}I]iodotyrosine and [^{131}I]diiodotyrosine, whereas the ^{131}I at cycle 5 was a mixture. Iodotyrosyl derivatives comprised 95% of the ^{131}I, and the iodohistidyl derivatives accounted for the remaining 5% (Figure 5). At cycles 2, 5, and 10, the ratio of ^{125}I- to ^{131}I-iodohistidyl derivatives was approximately 9:1.

The release of iodotyrosyl radioactivity after 5 cycles of Edman degradation suggested that bovine ^{131}I-labeled hormone had been cleaved between glycine and alanine, residues 38 and 39. To determine whether this unexpected phenomenon was related to iodination of the peptide, 0.4 μmol of unlabeled bovine hormone was digested with staphylococcal protease, and a portion of the mixture was subjected to manual Edman degradation for 6 cycles. Pth-tyrosine was clearly identified at cycle 5 by both gas–liquid and thin-layer chromatography. Although it is difficult to quantitate the yields accurately after several degradative cycles of a mixture, an estimate can be

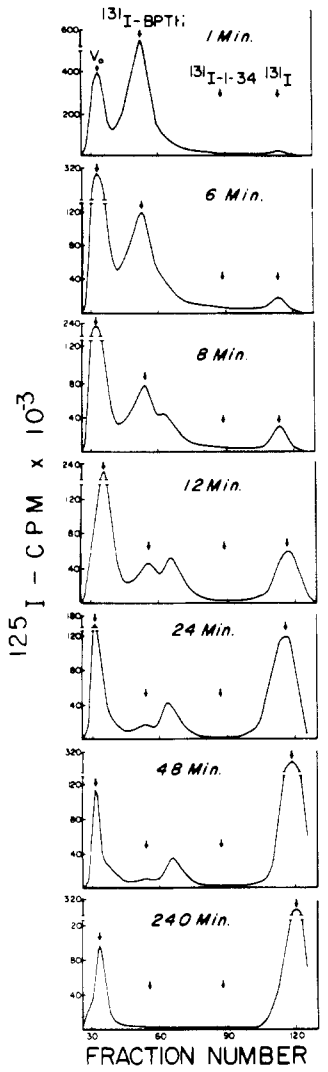

FIGURE 6: Bio-Gel P-100 gel-filtration profile of ^{125}I in 3-mL samples taken at various time intervals after injection of ^{125}I-labeled bovine hormone. The eluting buffer is 0.05 M barbital/1% (v/v) human plasma, pH 8.6. In this, as well as in Figure 10, the elution position of fractions within the void volume (V_0) and the elution positions of intact hormone, bovine parathyroid hormone fragment 1-34, and ^{131}I are indicated by the arrows.

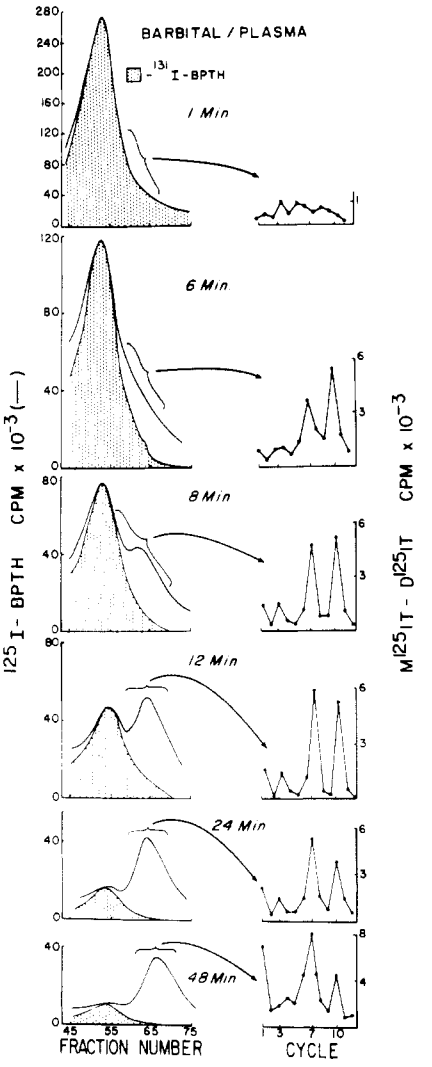

FIGURE 7: On the left side are shown the second and third peaks initially plotted in Figure 6. The shaded area represents the exact elution position of ^{131}I-labeled bovine hormone. On the right side is shown the specific iodotyrosyl radioactivity released at each cycle of the Edman degradation for each pool of fractions. For details see text.

made by assessing, at each cycle, the recovery of a Pth derivative that is easily quantitated and that occurs uniquely in one of the peptides in the mixture. Estimates show the following recoveries: cycle 1, 162 nmol of isoleucine (residue 1 in fragment 5-19); cycle 2, 194 nmol of leucine (residue 2 in fragments 22-38 and 23-55); and cycle 3, 92 nmol of valine (residue 3 in fragment 56-61) and 45 nmol of isoleucine (residue 3 of fragment 39-55). It appears, therefore, that although cleavage between glycine and alanine at positions 38 and 39 of the bovine hormone is incomplete, staphylococcal protease cleaves 20-50% of the peptide at this site, a figure consistent with estimates of the cleavage efficiency based on the studies of the iodinated bovine hormone.[2]

Physiologic Studies

Metabolism of Bovine ^{125}I-Labeled Parathyroid Hormone in Dogs. Figure 6 shows the gel-filtration profiles of ^{125}I given by plasma samples taken at various times after injection of bovine ^{125}I-labeled hormone. Chromatography of the samples from the four experiments gave virtually identical gel-filtration patterns, and the results of sequence analysis of plasma samples from the two experiments in which the higher dose of hormone was used were also nearly the same. Therefore, data from only one experiment are presented graphically.

Four radioactive peaks are seen. The first elutes at the position of the void volume of the column. The second coincides with the ^{131}I-labeled bovine hormone and presumably represents intact hormone. A third peak is first appreciated at 6 min as a late-eluting shoulder of the intact-hormone peak. By 12 min it is discrete and as large as the peak of intact hormone. The last peak, which coelutes with the ^{131}I column marker, is virtually absent in the 1-min sample, but increases for the first 48 min of the experiment. The 48- and 240-min samples have essentially the same concentration of this material.

The second and third peaks shown in Figure 6 are replotted on the left half of Figure 7. The shaded area represents the bovine ^{131}I-labeled hormone. On the right half of Figure 7 is shown the specific iodotyrosyl radioactivity at each cycle of sequence analysis of the pooled fractions, which are indicated

[2] In unpublished observations, R. T. Sauer and H. T. Keutmann found that intact porcine parathyroid hormone and fragment 26-44 of bovine parathyroid hormone also underwent proteolysis between residues 38 and 39 when treated with staphylococcal protease.

TABLE I: Analysis of the Radioactivity Eluting at the Salt Volume after Gel Filtration of Plasma Samples from Dogs after Injection of Bovine ^{125}I-Labeled Parathyroid Hormone.

Time sample taken (min)	Solvent System	% Distribution of Radioactivity				
		Origin	MIH[a]	MIT + DIT[b]	I[c]	Unidentified[d]
12	B/A/W[e]	3	2	37	56	2
	C/A[f]	2	2	35	61	0
24	B/A/W	2	1	30	65	2
	C/A	2	1	32	65	0
48	B/A/W	1	0	15	84	0
	C/A	3	1	14	82	0
240	B/A/W	0	0	4	96	0
	C/A	0	0	4	96	0

[a] Monoiodohistidine. [b] Sum of monoiodotyrosine and diiodotyrosine. [c] Iodide. [d] Radioactivity that neither remained at the origin nor migrated with the standards. [e] Butanol/acetic acid/water (15/4/1). [f] Collidine/2 N acetic acid (8/2).

by the brackets. In the 1-min sample, the profiles of radioactivity of the sample and column marker coincide. Sequence analysis of the pool taken from the region of the gel profile where fragments of the hormone may be expected to be present shows the release of very little iodotyrosyl radioactivity that was distributed randomly throughout the cycles of degradation.

By 6 min, the profiles of radioactivity of the ^{125}I-labeled sample and the bovine ^{131}I-labeled hormone marker are different, and, at successive times in the experiment, this distinction becomes more marked. Sequence analysis of the fractions pooled from the region of the late-eluting peak at 6 min shows that the greatest release of iodotyrosyl radioactivity occurs at cycle 10, with a lesser increase at cycle 7. In samples taken later, there is a relative increase in the iodotyrosyl radioactivity released at cycle 7, so that by 12 min release at this cycle has become dominant and remains so for the duration of the experiment. In addition, in these later samples there is a significant release of iodotyrosyl radioactivity after 3 cycles and particularly after 1 cycle of degradation. By 48 min, the latter is almost as prominent as that seen after 7 cycles. The appearance of iodotyrosyl radioactivity at cycles 10, 7, 3, and 1 corresponds, respectively, with fragments whose NH_2-terminal positions are residues 34, 37, 41, and 43.

The nature of the radioactivity eluting at the salt volume after gel filtration was studied by paper chromatography. The appropriate fractions of the samples taken at 12, 24, 48, and 240 min after injection were separately pooled. The percent of total radioactivity in samples from each pool that migrated at positions corresponding to the standards is shown in Table I. At all times, at least 95% of the radioactivity can be identified as either free iodide or as ^{125}I incorporated in a single amino acid residue.

Several samples taken at different times after injection were also chromatographed on Bio-Gel A-0.5m with an eluting buffer of 6 M guanidine hydrochloride/0.1 M ammonium acetate/1% (v/v) human plasma, pH 6.5. These studies, using severe denaturing conditions, were performed to determine: (1) whether fragments of the hormone were eluting at the positions of the void volume, (2) whether the multiple cleavage products seen could have resulted from events occurring during gel filtration in the barbital buffer system, and (3) whether any cleavage occurred between residues 1 and 33, by analyzing fractions obtained after gel filtration in a system in which the molecular weight of the peptides is accurately reflected by their elution position.

Figure 8 shows the results of one of these studies in which a 12-min sample was studied by gel filtration with guanidine as the eluting buffer. When compared with the profile of radioactivity given by a duplicate sample chromatographed using barbital, a striking decrease in the radioactivity eluting in the void volume is seen, which is matched by an increase in the peak coeluting with the bovine ^{131}I-labeled hormone. Thus, it appears that most, if not all, of the radioactivity that had been present in the void volume is intact hormone.

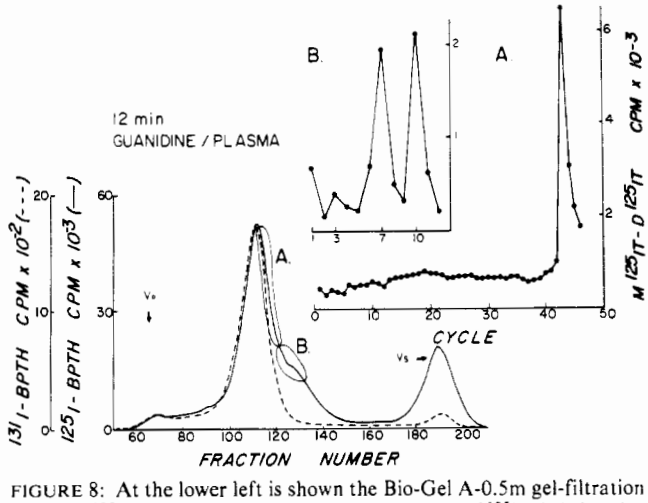

FIGURE 8: At the lower left is shown the Bio-Gel A-0.5m gel-filtration profile of ^{125}I in a sample taken 12 min after injection of ^{125}I-labeled bovine hormone (—) cochromatographed with ^{131}I-labeled bovine hormone (- - -). The eluting buffer is 6 M guanidine hydrochloride/0.1 M ammonium acetate/1% (v/v) human plasma, pH 6.5. At the upper right is shown the specific iodotyrosyl radioactivity released at each cycle of the Edman degradation of pools A and B. For details see text.

In pool A, one would expect to find fragments representing cleavages occurring closer to the NH_2 terminal than position 33. When pool A was subjected to Edman degradation, the pattern of release of iodotyrosyl radioactivity was indistinguishable from that seen on sequence analysis of intact hormone and showed a marked increase in release of radioactivity only at cycle 43.[3] The pattern of release of iodotyrosyl radioactivity seen following Edman degradation of pool B is indistinguishable from that seen when a duplicate sample eluted in the barbital system was analyzed. These findings indicate that the multiple cleavage products seen are generated in vivo

[3] The iodotyrosine released at cycles 44–46 of the degradation probably represents a minor degree of asynchrony in the degradation of intact hormone (Edman and Begg, 1967; Smithies et al., 1971; Niall, 1973).

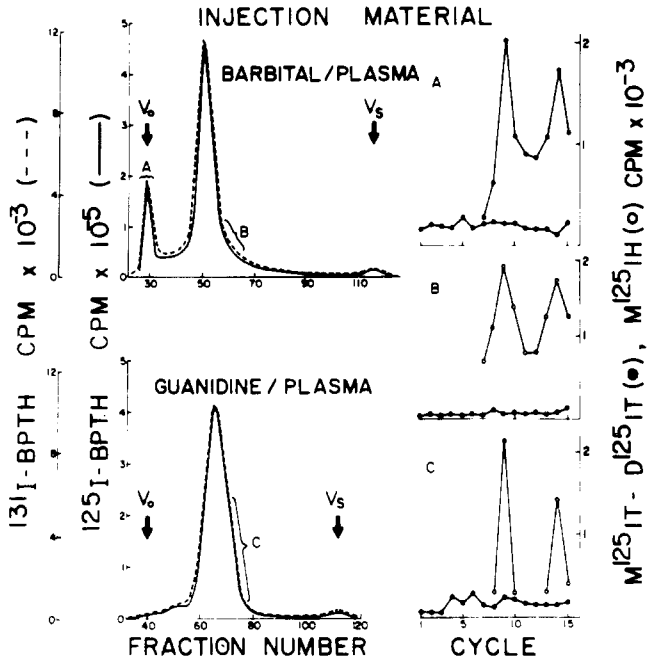

FIGURE 9: On the left half is shown the gel-filtration profile of ^{125}I in the ^{125}I-labeled bovine hormone used in the dog studies (—) cochromatographed with ^{131}I-labeled bovine hormone (- - -) after filtration in the two chromatographic systems. On the right half is shown the specific iodotyrosyl (●-●) and iodohistidyl (○-○) radioactivity released at each cycle of the Edman degradation of pools A, B, and C.

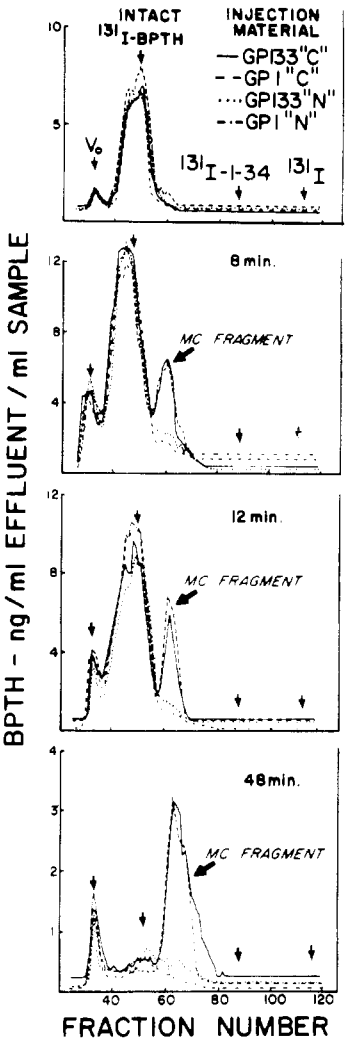

FIGURE 10: Bio-Gel P-100 gel-filtration profiles of immunorcactivity in the bovine hormone injected and in representative samples taken at various time intervals after injection of the hormone. The eluting buffer is 0.05 M Veronal/10% (v/v) human plasma, pH 8.6.

and are not due to cleavage occurring during gel filtration in barbital. They also exclude the possibility that other labeled fragments of the hormone might have eluted in unexpected positions, such as in the void volume of the column, in this system.

To test whether fragments of the hormone could have resulted from cleavage during the iodination procedure or perhaps were there already in the preparation used, the bovine ^{125}I-labeled hormone used in the dog studies was chromatographed on both gel-filtration systems. The regions of the profile in which fragments might be expected to appear, including the fractions in the void volume, were pooled and subjected to Edman degradation. There was no significant release of iodotyrosyl radioactivity at any of the first 15 cycles of degradation (Figure 9). Thus, the iodinated hormone used in these studies appears to be free of contaminating fragments. The appearance of iodohistidyl radioactivity at cycles 9 and 14 is consistent with the known location of histidine at these positions, and serves as an important control for the degradation.

Metabolism of Unlabeled Bovine Parathyroid Hormone in Dogs. Samples collected at various time intervals after intravenous injection of 600 μg of unlabeled hormone into dogs were chromatographed on Bio-Gel P-100, and all fractions were simultaneously assayed using GP-1 and GP-133 alternately preincubated with excess concentrations of 1–34 or 53–84 fragment. The first panel of Figure 10 shows that the hormone used for one of these studies elutes at a position coincident with the intact hormone marker and is recognized equally well in all four radioimmunoassay systems. A small peak consisting of less than 9% of the total immunoreactivity elutes at the void volume. The hormone is free of any demonstrable contamination with fragments.

The remaining panels of Figure 10 show the column profiles given by samples taken at increasing time intervals after injection of the hormone. The data are graphed in terms of concentration per milliliter of plasma applied to the column. At 4 min, no fragments are apparent. By 8 min, there is a late-eluting immunoreactive peak recognized by the two antisera specific for the COOH-terminal portion of the hormonal sequence (C assay), but not by either of the two antisera blocked with 53–84 fragment, which would limit recognition to the NH_2-terminal portion of the sequence (N assay). Between 12 and 24 min, this late-eluting peak becomes the dominant form of immunoreactive hormone in the circulation, and, by 48 min, intact hormone has virtually disappeared. The quantitation of the immunoreactivity in this late-eluting peak is probably accurate, inasmuch as virtually identical concentrations are detected in both of the C assays. In addition, when samples from a pool of fractions comprising this late-eluting peak were assayed in these two systems, the slope of inhibition of tracer binding given by serial dilutions of the sample was identical with that given by the bovine hormone standard.

Incubation in Vitro of Bovine ^{125}I-Labeled and Unlabeled Parathyroid Hormone. One microgram of bovine hormone and 2×10^6 cpm (approximately 4 ng) of bovine ^{125}I-labeled hormone were incubated, separately, in 0.5 mL of heparinized dog plasma for 6 h at 37 °C. After gel filtration with appropriate column markers, fractions were evaluated by either radioim-

munoassay or counting of ^{125}I. There was no evidence for the conversion of intact hormone to a late-eluting peak other than the appearance of 6% of the radioactivity at the salt volume in the study using labeled hormone.

Discussion

Certain characteristics of the primary structure of human parathyroid hormone were defined using sequence analysis of the radioiodinated hormone and its enzymatically generated fragments. Foremost among the advantages of this approach is its sensitivity; because histidyl and tyrosyl residues can be radioiodinated to high specific activity, they can be detected readily in the degradation of only picomoles of bovine parathyroid hormone. The amount of radioactivity detected indicates that these residues could be identified readily using less than 0.4 pmol of hormone. This sensitivity represents a 10^5 improvement over the high-sensitivity technique recently reported by Jacobs and Niall (1975), which in turn represented a 10^2-fold increase in sensitivity over conventional automated Edman procedures.

Although the method is limited, of course, to determining the positions of iodinated residues, it has proved useful both in locating peptide bonds that are susceptible to attack by specific enzymes, and, in particular, in comparing the primary structures of a closely homologous series of peptides. The simultaneous digestion, degradation, and analysis of microquantities of these hormones or their fragments, each labeled with a different radioactive form of iodide, serve to control the chemical steps. Because iodination can be performed in 8 M urea, the conformation of the peptide molecules is unlikely to interfere with labeling of certain tyrosyl and histidyl residues, which might be poorly accessible in neutral, aqueous buffers and thereby might limit the general utility of the technique.

This approach has been useful in examining the nature of residue 43 in the human hormone, and also in addressing the question of whether a tyrosine-containing isohormonal form of the hormone is present. (Compositional analysis had shown 0.15 mol of tyrosine/mol of peptide (Keutmann et al., 1974).) Iodotyrosyl derivatives were not found at cycle 43 in the human hormone, nor were they detected in any of the degradations of enzymically cleaved human parathyroid hormone. Inasmuch as ^{131}I-labeled iodotyrosine was easily discernible after 43 cycles of degradation of the bovine hormone, we could conclude that position 43 in the human hormone is not tyrosine. It is also unlikely that position 43 of the human hormone is histidine because, whereas iodohistidine was readily detected after 32 cycles of degradation of the intact hormone, it was not detected at cycle 43, or at cycle 21 (corresponding to position 43) in the mixture analysis after digestion of the hormone with staphylococcal protease. However, bovine hormone which contains a tyrosyl residue at position 43 was used as the control in these experiments. Therefore, although the results suggest that histidine is not present at position 43 of the human hormone, the possibility remains that failure to detect iodohistidine at this position of the human hormone (Figure 3) could be due to the known lability of iodohistidine. In addition, the absence of iodotyrosyl radioactivity during sequence analysis of the human hormone, in spite of the high sensitivity of the method for detecting this derivative, virtually excludes the possibility of a tyrosine-containing isohormonal form of the human hormone.

Finally, sequence analysis of the radioiodinated human parathyroid hormone and its fragments permitted assignment of histidyl residues to positions 9, 14, 32, and 63 of the sequence. These assignments have been confirmed by direct chemical analyses (Niall et al., 1974; Keutmann et al., 1975).

Microsequencing of radioiodinated parathyroid hormone has been especially helpful for analyzing the metabolism of the hormone in vivo. Previous reports have shown that endogenous parathyroid hormone in the general circulation is heterogeneous, consisting of intact hormone and at least one other, apparently smaller, hormonal form. The smaller forms are distinguishable from the intact hormone because they are retarded upon gel filtration and they have altered immunologic properties; the dominant late-eluting peak contains antigenic determinants within the COOH-terminal portion of the intact hormone, but lacks determinants within the NH_2-terminal portion (Habener et al., 1971, 1972; Canterbury and Reiss, 1972; Segre et al., 1972, 1974, 1975; Goldsmith et al., 1973; Silverman and Yalow, 1973; Arnaud et al., 1974; Fischer et al., 1974).

Despite extensive study, the nature and significance of the multiple immunoreactive forms of parathyroid hormone in plasma remain unsettled. We have addressed several of these issues. The first concerns the origin of the late-eluting forms of the hormone found in plasma. Our earlier studies of immunoreactive parathyroid hormone in parathyroid effluent and peripheral circulation indicated that these late-eluting forms arise from metabolism of intact hormone after secretion (Habener et al., 1971, 1972; Segre et al., 1972). More recently, however, the observation that hormone fragments may disappear from the circulation in individuals with renal failure at a rate less than 1% of that of intact hormone led to the suggestion that hormone fragments may be secretory products of the gland that, although secreted in small quantities, became the dominant circulating forms of the hormone because of their extraordinarily slow removal from blood (Silverman and Yalow, 1973). Since intact hormone and its fragments are incompletely separated upon gel filtration, and also cross-react, immunologically, it is impossible to exclude contamination of exogenously administered intact hormone by a few percent of hormone fragments.

A second major issue is whether the late-eluting forms of the hormone represent actual cleavage products of intact hormone, or merely intact hormone with an altered conformation which accounts for the changes in immunochemical and physical-chemical properties. The data using sequence-specific radioimmunoassays suggested that the single tyrosyl residue, located at position 43 of the bovine hormone, should be contained within the material comprising this late-eluting peak. Therefore, if cleavage of the hormone does occur, the precise location of cleavage could be defined by counting the number of cycles of degradation required for release of iodotyrosine during sequence analysis of this late-eluting material.

This latter issue is critical to an understanding of the biological significance of the heterogeneity of the hormone in blood. The minimum sequence required for biological activity consists of an intact sequence of 26 residues at the NH_2 terminus, extending from the valine at position 2 to the lysine at position 27 (Tregear et al., 1973). Therefore, hormone cleavage within the 2–27 sequence would result not only in biologically inactive fragments comprising the middle and COOH-terminal portions of the sequence (MC fragments), but also in biologically inactive NH_2-terminal fragments. Alternatively, if cleavage occurs COOH terminal to position 27, the NH_2-terminal fragments, if otherwise chemically unmodified, could be biologically active. Such an NH_2-terminal fragment could then be a form of the hormone with a spectrum of biological

activities different from that of the intact molecule.

Direct structural analysis by microsequencing of the radioiodinated peptides shows that the late-eluting forms of the hormone are generated after entry into the circulation and cannot be labeled fragments inadvertently injected with intact hormone. This can be seen by examining the injected material and analyzing the changes in concentration of the various hormone peaks with time after injection into animals. Concentrations of less than 0.1% of fragments would have been readily detected in the injected preparation by sequence analysis of the material eluting either in the trailing portion of the gel-filtration profile of the intact-hormone peak or in the void-volume peak (Figure 9), but none were seen. Two additional considerations confirm that the MC fragments originated de novo from metabolism of intact hormone in vivo. There is an absolute increase in the concentration of the late-eluting forms of the hormone for the first 20 min after injection (Figure 7). Second, relative to the concentration of radiolabeled intact hormone, the concentration of the MC fragments is 16% at 6 min and 100% at 12 min (Figure 7), respectively, but could have been no greater than 0.1% at time 0. Thus, in the absence of peripheral metabolism, the relative concentrations of intact hormone and the fragments found at 6 and 12 min could only be explained by a fall in the intact-hormone concentration to less than 1% by 6 min and to 0.1% by 12 min with respect to the concentration at time 0. Such a disappearance rate is several orders of magnitude greater than that actually observed (Figure 7). Hence, the results clearly establish that parathyroid hormone undergoes metabolism to fragments after entry into the circulation, and that peripheral metabolism must account for some, if not all, of the hormone fragments seen in plasma.

Cleavage of exogenously administered hormone occurs between residues 33 and 34 and at other sites that are all COOH terminal to position 34. Thus, because the critical sequence required for biological activity, 2–27, appears to be intact, it is possible that NH_2-terminal hormonal fragments may be generated that are biologically active (Canterbury et al., 1973, 1975).

Studies of the metabolism of unlabeled hormone (Figure 10) not only confirm the results seen with radiolabeled hormone, but also are essential to validate the physiological significance of conclusions based on the analysis of the metabolic fate of the labeled hormone, since the latter partially loses biologic potency during iodination, presumably because of oxidation of the methionine residues (Rasmussen and Craig, 1962; Tashjian et al., 1964; Potts et al., 1966). The finding that cleavage of radiolabeled hormone occurs between residues 33 and 34 is consistent with the observation that the N assays do not detect the MC fragments, whereas the C assays do (Figure 10). Furthermore, the overall rates of disappearance of intact hormone and appearance and disappearance of the MC fragments are similar by the two methods (Figures 7 and 10).

The use of radiolabeled hormone and the microsequencing technique offers several other advantages for metabolic studies. The high specific activity of the radioiodinated preparations permits analysis of circulating concentrations of hormone that closely approach those found endogenously (Sherwood et al., 1966; Segre et al., 1972). For example, after distribution in a volume of 1400 mL (found in our study), 1 and 10 μg of radiolabeled parathyroid hormone represent initial plasma concentrations of 0.7 and 7 ng/mL, respectively.

Second, the radiochemical method circumvents the difficulties encountered in quantitating hormone fragments by radioimmunoassay, especially when these fragments are assayed using antisera raised against the intact molecule. Because the immunoreactivity of the fragments and the intact hormone may differ with a particular antiserum, assurance that quantitation is accurate requires that multiple antisera be employed and that the displacement curves of the sample and standard hormone be shown to be parallel in each assay. Because the radiochemical method does not depend on the immunochemical integrity of the fragments being measured, it avoids the use of these cumbersome and time-consuming examinations.

Third, when efforts are undertaken to isolate the enzymes from the tissues that are responsible for proteolysis of parathyroid hormone, the precise cleavage pattern, as defined by sequencing of the radiolabeled peptides, is the equivalent of an enzyme assay during purification of the proteases.

Many issues concerning the metabolism of parathyroid hormone remain unresolved. Our studies establish, unequivocally, that the hormone undergoes proteolytic metabolism in organs or tissues, but not in plasma. It is not clear, however, to what extent this peripheral metabolism accounts for the heterogeneity of the hormone in plasma, or whether secretion by the parathyroid glands of hormone fragments (Silverman and Yalow, 1973; Flueck et al., 1976) also contributes. The physiologic significance of hormone metabolism and the details of the cleavage process are not clear. Although the single late-eluting peak seen on gel filtration appears to be homogeneous when assessed by sequence-specific radioimmunoassays, sequence analysis shows it to consist of several distinct peptides. The mechanisms responsible for this microheterogeneity, which may involve both endopeptidases and exopeptidases, and its possible significance are unknown. Perhaps individual organs contribute specific fragments to, or clear specific fragments from, the circulation. The concentration of any specific fragment in the general circulation would, therefore, reflect the different rates at which these fragments are generated, released, and subsequently cleared and/or converted to other fragments. Because these questions concerning the metabolism of the hormone require exact definition of the fragments, they may be best resolved by application of the microsequencing techniques.

Our findings suggest that the approaches described, using radioiodinated hormone, may prove useful for analyzing proteolysis and metabolism of other peptides hormones.

Acknowledgments

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