Metabolism of Parathyroid Hormone by Kupffer Cells: Analysis by Reverse-Phase High-Performance Liquid Chromatography†

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ABSTRACT: Parathyroid hormone (PTH) undergoes rapid proteolysis in the liver, which results in the appearance of multiple COOH-terminal fragments in plasma. Using reverse-phase high-performance liquid chromatographic (HPLC) techniques, we have shown that biologically active bovine PTH (bPTH) internally labeled with [^{3}H]tyrosine is, like ^{125}I-labeled bPTH, rapidly metabolized by isolated rat Kupffer cells in vitro to multiple COOH-terminal fragments that are chemically identical with those previously found in plasma after metabolism in vivo. Quantitation of specific carboxyl fragments in crude mixtures is achieved rapidly by direct HPLC analysis and is as precise as that achieved by Edman degradation. In addition, several different carboxyl fragments with identical NH_2 termini were resolved, revealing a complexity not apparent in previous studies employing direct Edman degradation of such mixtures. Parallel studies with [[^{35}S]Met]bPTH show the generation, by the Kupffer cells in vitro, of several labeled NH_2-terminal fragments which undergo rapid further degradation in vitro. Thus, hepatic metabolism of PTH by Kupffer cells proceeds by an initial endopeptidase cleavage within the hormonal sequence in a manner compatible with the generation of biologically active NH_2 fragments.

The metabolism of parathyroid hormone (PTH) and its potential physiologic importance have been subjects of intensive study since the heterogeneity of circulating immunoreactive hormone was first described (Berson & Yalow, 1968). Although numerous studies have confirmed that the cleavage of PTH by peripheral tissues results in the rapid appearance in plasma of COOH-terminal fragments (Segre et al., 1972, 1974, 1976; Hruska et al., 1975; Neuman et al., 1975), the relation of this metabolism to the control of biologically active hormone concentrations in plasma remains unclear. Sequential Edman degradation of radioiodinated peptides isolated from the plasmas of animals receiving injections of bovine parathyroid hormone (bPTH) labeled with ^{125}I has identified several specific COOH-terminal fragments; the two major hormonal fragments result from proteolysis between positions 33 and 34 and between 36 and 37 of the intact hormonal sequence (Segre et al., 1976, 1977). On the other hand, NH_2-terminal fragments(s)—expected products of endopeptidase-mediated cleavage(s)—have not been consistently observed in vivo by immunochemical analysis (Segre et al., 1972; Canterbury et al., 1973; Fischer et al., 1974; Habener et al., 1976; Goltzman et al., 1980).

Whereas both the liver and, to a lesser extent, the kidneys appear to contribute to the clearance of intact PTH from plasma, the liver generates most, if not all, of the circulating COOH-terminal fragments derived from the metabolism of injected bPTH (Segre et al., 1981a). Studies employing isolated rat hepatic cells have shown that Kupffer cells account for the bulk of the hepatic metabolism of both unlabeled and ^{125}I-labeled bPTH and, moreover, that these cells generate fragments in vitro that are chemically identical with those previously found in the plasma of rats or dogs into which ^{125}I-labeled bPTH was injected (Segre et al., 1981b).

Further progress in investigation of the mechanism(s) and interpretation of the biologic significance of hepatic metabolism of PTH has been slowed by methodologic difficulties relating to the inadequate resolution or poor recovery of conventional protein-separation methods: the need for repetitive, laborious, sequential Edman degradation for precise chemical identification of hormonal fragments, and the properties of available radioactive PTH probes which either lack biologic activity, contain prosthetic groups, or have limited site(s) of incorporation of radiolabel. In an effort to circumvent these limitations, we have employed reverse-phase HPLC for both rapid, high-yield purification of biologically active, biosynthetically labeled bPTH and direct analysis of the multiple closely related hormonal fragments resulting from PTH metabolism in vitro.

Materials and Methods

Radioactive PTH. Internally labeled bPTH was prepared by incubation of bovine parathyroid gland slices at 37 °C with [^{3}H]tyrosine or [^{35}S]methionine (New England Nuclear Co.) in protein-free Eagle's minimum essential medium (MEM) supplemented with nonessential amino acids but lacking the amino acid corresponding to the radiolabel (Habener & Potts, 1975). The incubations were terminated after 4 h by addition to the medium of cold trichloroacetic acid (Cl_3CCOOH) at a final concentration of 10% Cl_3CCOOH. The resulting precipitate was washed twice with cold 10% Cl_3CCOOH, the pellet was redissolved in 1.5 mL of 8 M urea/20% acetonitrile/0.1% trifluoroacetic acid (TFA) in water, and the solution was applied to the HPLC. The specific activity of the purified bPTH tracer was approximately 1000 dpm/ng.

Radioiodination of purified bPTH-(1–84) was performed by a modification of the method of Hunter and Greenwood, as previously described (Segre et al., 1977). When the labeled hormone was lyophilized, redissolved in 20% acetonitrile/0.1% TFA, and purified by HPLC as described for internally labeled hormone, three major peaks of radioiodinated peptide were typically observed, the first of which eluted just before, and the remaining two after, unlabeled bPTH-(1–84). Further characterization of these labeled species is in progress, but preliminary studies suggest that they differ with respect to oxidation and iodination status. No apparent qualitative or quantitative differences in the metabolism of these three probes by isolated Kupffer cells have been observed. The studies reported here were performed with that form of ^{125}I-labeled bPTH which eluted last from the HPLC column.

High-Performance Liquid Chromatography. Reverse-phase HPLC was conducted by using a water/acetonitrile/TFA solvent system (Bennett et al., 1977) and a modular system

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incorporating Waters Co. M-6000 and M-45 pumps, UK-6 injector, 660 solvent programmer, and μBondapak C_{18} steel columns (0.4 × 30 cm) operated at ambient temperature, pressures of 1000–2500 psi, and flow rates of 1–2 mL/min. The two limit solvents, 0.1% TFA in water and 60% acetonitrile in 0.1% TFA, were filtered (0.45 μm, Millipore Co.), degassed, and then mixed by the two pumps in proportions determined by the solvent programmer. All water for HPLC was deionized and prefiltered through C_{18} Sep-Paks (Waters Co.), acetonitrile was spectral/LC grade (Merck Co.), and TFA ("sequenator grade", Pierce Co.) was redistilled in glass. Samples were typically dissolved in 20% acetonitrile/0.1% TFA and centrifuged (3000*g* for 10 min) before injection. Fractions were collected directly into plastic counting vials, into borosilicate glass tubes, or, during preparation of the hormone, into glass tubes containing 0.05 mL of 0.5% heat-inactivated bovine albumin (lot 30F-0214, Sigma Co.). Radioactive iodine was determined on a Packard Auto-Gamma spectrometer, and β radioactivity was measured on a Packard Tri-Carb 3350 spectrometer after addition of 4 mL of toluene-based scintillant (Scint-A, Packard Co.) to fractions or aliquots. No significant differences in solvent quenching of ^3H or ^{35}S were found across the range of solvent strengths comprising the gradient(s). The elution of standard bPTH-(1–84) was monitored at 206 nm (Uvicord S, LKB Co.) after injection of 1–5 μg of highly purified hormone provided by Dr. H. T. Keutmann.

Peptide Sequencing. Peptides were applied in 100% TFA to a Beckman 890-C sequencer and degraded for at least 20 cycles with a single-coupling, single-cleaving program (Edman & Begg, 1967). The repetitive yield per cycle was estimated by calculating the recovery of phenylthiohydantoin-derivatized amino acids of sperm whale myoglobin, which was added to each sample before degradation. The repetitive yields averaged 93%. Sequenator fractions (cycles) in 1 mL of butyl chloride were either counted directly (^{125}I) or added to 10 mL of liquid scintillation fluid for detection of β emitters. The radioactivity recovered at each cycle was corrected for background radioactivity, repetitive yield (assuming equivalence with that of myoglobin), initial coupling efficiency, and isotopic decay (as necessary). In the case of samples of unchromatographed incubation medium, the overall recovery of radioactivity was corrected for that fraction of applied radioactivity associated with intact hormone (and thus not detectable within 20 cycles of degradation). This was estimated from independent analysis of duplicate samples by gel filtration or HPLC.

Studies of Methionine Oxidation. Aliquots of lyophilized, HPLC-purified [[^3H]Tyr]bPTH and [[^{35}S]Met]bPTH (50000 cpm) were dissolved in 0.2 mL of 2% hydrogen peroxide, incubated at 37 °C for 30 min, diluted to 1 mL with cold water, and lyophilized. Subsequent analysis by HPLC revealed that the oxidation was over 90% complete, as shown by the disappearance of radioactivity from the elution position of the bPTH standard. Control and peroxide-oxidized tracers were then incubated for 24 h at 25 °C with cyanogen bromide (25 mg/mL) in 0.1 N HCl, lyophilized twice, redissolved in 20% acetonitrile/0.1% TFA, and counted for radioactivity.

Bioassay Systems. To determine the effect of HPLC purification on the biological activity of internally labeled bPTH, we prepared a pool of [[^3H]Tyr]bPTH of low specific activity by addition of 150 μg of purified bPTH to 300000 dpm of HPLC-purified [[^3H]Tyr]bPTH. Approximately half of this hormone (80 μg) was subjected to the two-step HPLC purification as described under Results, and the remainder was lyophilized directly. The HPLC-purified hormone was then dispensed to separate tubes in aliquots of 5 μg (10000 dpm) and lyophilized. Five micrograms each of HPLC-purified [[^3H]Tyr]bPTH, unchromatographed [[^3H]Tyr]bPTH (as above), and fresh bPTH-(1–84) was dissolved in 0.01 M acetic acid and further diluted over the range of 10^{-9}–10^{-6} M. The following two bioassays were used: the generation of cyclic AMP by confluent monolayers of clonal rat osteosarcoma cells (17/2, provided by Dr. G. Rodan) and the release of ^{45}Ca from neonatal mouse calvaria. So that bone-cell cyclic AMP stimulation could be assayed, hormone was diluted with phosphate-buffered saline (PBS) containing glucose (100 mg/dL), isobutylmethylxanthine (2 mM), and bovine albumin (2 mg/mL). Diluted hormone (or control solution) was added simultaneously (0.3 mL/well) to 16-mm multiwell plates (Costar Co.) containing cells that had been grown to confluence in Ham's F-12 medium with antibiotics and 10% fetal calf serum (lot A193719, Gibco) and washed twice with PBS just before hormone addition. Incubations were performed in quadruplicate at 37 °C for 15 min and terminated by immersion of the plates in liquid nitrogen. The aqueous phase was subsequently removed by evaporation over steam, and the dried residue was scraped into 1 mL of 0.05 M sodium acetate, pH 6.2, with a Teflon policeman. Aliquots of the 1000*g* supernatants of these extracts were then assayed directly for cyclic AMP by radioimmunoassay (Collaborative Research Co. kit). Appropriate controls were included for the effect of cell extract on tracer "damage" in the absence of antibody, and the results were expressed as picomoles of cyclic AMP per 16-mm well.

To study bone-resorbing activity, we diluted the hormone preparations with Dulbecco's modified Eagle's medium (DMEM) with 5% heat-inactivated horse serum (lot A891023, Gibco) and 1% antibiotic/antimycotic solution (Gibco). Half-calvaria from 5-day-old mice (CD-1 strain, Charles River Co.) were obtained as previously described (Bringhurst & Potts, 1981) 2 days after subcutaneous administration of $^{45}CaCl_2$ to each animal (5–10 μCi/mouse). The bones were precultured in 35-mm plastic dishes (Costar Co). in 2 mL of the above medium (DMEM) on a rocking platform at 60 oscillations/min in a 37 °C incubator under 5% CO_2 in air. After 24 h, the medium was discarded and replaced with 1.5 mL of the same medium containing hormone or the 0.01 M acetic acid vehicle. At 24 and 48 h after the time of hormone addition, 1 mL of fresh medium (without hormone) was added to each culture. After 72 h, the bones were removed, rinsed in saline, placed in 20-mL glass scintillation vials containing 2 mL of 2 N HCl, and hydrolyzed in an autoclave for 30 min. Aliquots of culture medium (1 mL) were transferred to separate scintillation vials containing 10 mL of liquid scintillation fluid for determination of released radioactivity. Calvarial hydrolysates were counted similarly, and, after correction for differences in quenching, the percentage of total ^{45}Ca released from each bone during the 72-h culture period was calculated. Results are expressed as the mean ± standard error of the mean (SEM) of the percentage ^{45}Ca released for each treatment group of six bones.

Isolated Hepatic Cell Incubations. Kupffer cells were isolated after collagenase digestion (Worthington Co., lot 40C190) of excised livers from CD-1 rats (150–250 g, Charles River Co.) as previously described (Segre et al., 1981b). In most experiments, Kupffer cells were harvested magnetically after the animals had received intravenous injections of colloidal iron (Wincek et al., 1975). Some experiments were conducted with purified mixed sinusoidal lining cells, obtained by differential centrifugation of collagenase-dispersed hepatic

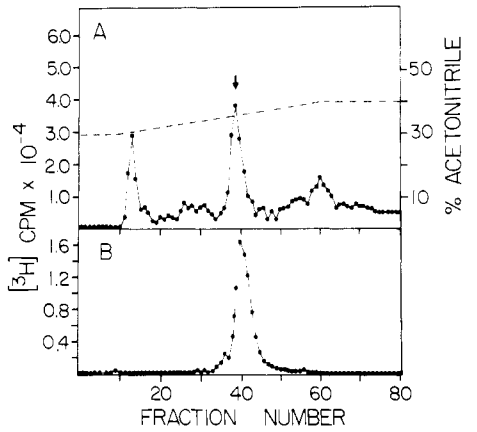

FIGURE 1: Purification by HPLC of biosynthetically labeled [[^{3}H]-Tyr]bPTH. (A) The redissolved 10% Cl_3CCOOH precipitate of medium from a 4-h incubation of [^{3}H]tyrosine (5 mCi) with parathyroid gland slices was applied to a μBondapak C_{18} column equilibrated in 30% acetonitrile/0.1% TFA and eluted at 2.0 mL/min with a linear gradient of 30–40% acetonitrile/0.1% TFA over 15 min (---). Fractions were collected at 18-s intervals, and aliquots (10 μL) were withdrawn for determination of radioactivity. (B) Radioactivity eluting in the position (↓) of standard bPTH (fractions 37–43) in (A) was pooled, lyophilized, redissolved in 1.5 mL of 20% acetonitrile/0.1% TFA, applied to the same column equilibrated in 30% acetonitrile/0.1% TFA, and eluted as above.

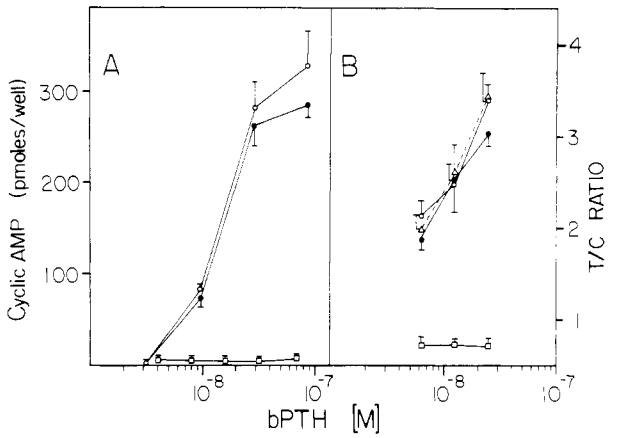

FIGURE 2: Biological activity of [[^{3}H]Tyr]bPTH purified by HPLC. (A) Cyclic AMP production by confluent monolayers of rat osteosarcoma cells was measured after 15 min of exposure to hormone at 37 °C. Responses are shown for unpurified [[^{3}H]Tyr]bPTH before HPLC (○), for HPLC-chromatographed [[^{3}H]Tyr]bPTH (●), and for equivalent dilutions of dried HPLC solvent and albumin (□). Points depict the mean ± SEM for each group of four wells. (B) Bone resorption (^{45}Ca release) by neonatal mouse calvaria in organ culture was measured 72 h after addition of hormone. The treated to control ratios (mean ± SEM) for groups of six bones are shown for bPTH-(1–84) standard (○), [[^{3}H]Tyr]bPTH incubated in HPLC solvent before lyophilization (△), [[^{3}H]Tyr]bPTH purified by HPLC (●), and equivalent dilutions of HPLC solvent and albumin (□).

cell suspensions (Tolleshaug et al., 1977), of which approximately 20% are Kupffer cells, as verified by direct peroxidase staining (Wisse, 1974). In both types of cell preparations, the contamination by hepatocytes was routinely less than 5% on microscopical examination, and viability of the hepatocyte fraction was over 90% as assessed by exclusion of trypan blue.

The washed cells were incubated with HPLC-purified radioactive bPTH [(0.02–2.0) × 10^6 cpm/tube] at concentrations of (0.5–5.0) × 10^6 total cells/mL in polypropylene tubes containing a total volume of 0.25 mL of MEM supplemented with 0.2% bovine albumin. Tubes were gassed with a mixture of 5% CO_2/95% O_2 and placed in a shaking metabolic incubator at 37 °C. Incubations were terminated by transfer of the tubes to an ice bath and addition of 0.55 mL of cold saline. After centrifugation at 300g for 5 min (Beckman TJ-6 centrifuge), the cell-free supernatants were transferred to plastic microfuge tubes (1.5 mL) containing 0.2 mL of 90% acetonitrile/1% TFA in water, frozen, and stored at −70 °C until further analysis.

Results

Purification of Radioactive bPTH. Biosynthetically prepared bPTH, internally labeled with [^{3}H]tyrosine or [^{35}S]-methionine, was purified directly by HPLC. The resolubilized crude Cl_3CCOOH precipitate of the parathyroid gland incubation medium was applied directly to an analytical reverse-phase column eluted with a linear gradient of 30–40% acetonitrile in 0.1% TFA (Figure 1A). The peak of radioactivity eluting in the position of standard bPTH (at 12 min) was pooled and rechromatographed under the same conditions (Figure 1B) to remove minor contaminants. The recovery of total radioactivity from the columns under these conditions was routinely greater than 90%, of which 10–15% eluted in the position of standard bPTH during the inital chromatographic step.

The purity of the radioactive bPTH that appeared to be homogeneous by HPLC was directly assessed by microsequence analysis of [^{35}S]methionine-labeled hormone. Over 93% of the applied radioactivity in [[^{35}S]Met]bPTH preparations was recovered at cycles 8 and 18, in accordance with the known sequence of the bovine hormone (AVSEIQF*M*HNLGKHLSS*M*ERVEWLRKKLQOVH-NFVALGASIAYRDGSSQRPRKKEONVLVESHQKS-LGEAOKADVDVLIKAKPQ) (Niall et al., 1970). In addition, rechromatography of the final purified tracer after storage at −70 °C in air routinely yielded a single peak that coeluted with standard bPTH, which indicates that the tracer is stable under these conditions for at least 1 month.

Bovine PTH purified by HPLC retained full biologic activity when compared with unchromatographed bPTH in the cyclic AMP assay (potency ratio = 1.01; 95% confidence limits = 0.79 and 1.14) (Figure 2A) and was equipotent, in the mouse calvarial resorption assay, with the unchromatographed preparation and with fresh bPTH (potency ratio = 0.91; 95% confidence limits = 0.51 and 1.41) (Figure 2B). Lyophilized solvent blanks, prepared from HPLC effluent and albumin, had no effect in either system.

Oxidation of the methionine residues of bPTH is known to result in loss of biologic activity (O'Riordan et al., 1974). Accordingly, as an additional test for modifications of PTH that might alter biologic activity, we determined the oxidation state of the methionines in the HPLC-purified, internally labeled bPTH. Lyophilization of the reaction mixture of [[^{35}S]Met]bPTH with cyanogen bromide (CNBr) resulted in the loss of 91% of the original radioactivity, a finding consistent with the release of volatile methyl [^{35}S]thiocyanate during CNBr cleavage at unoxidized methionine residues. As expected, CNBr treatment of [[^{35}S]Met]bPTH oxidized by hydrogen peroxide resulted in only 4% loss of total ^{35}S (Gross, 1967).

Metabolism of bPTH by Kupffer Cells. Isolated Kupffer cells were incubated with ^{125}I-labeled bPTH for 60 min, and the medium was subsequently analyzed by gel filtration chromatography or reverse-phase HPLC. As previously reported (Segre et al., 1981b), sequential Edman degradation of the pooled fractions comprising the fragment peak, which elutes just after intact PTH during Bio-Gel P-100 chromatography, confirmed the presence of multiple fragments corresponding to cleavages between positions 33 and 34, 36 and

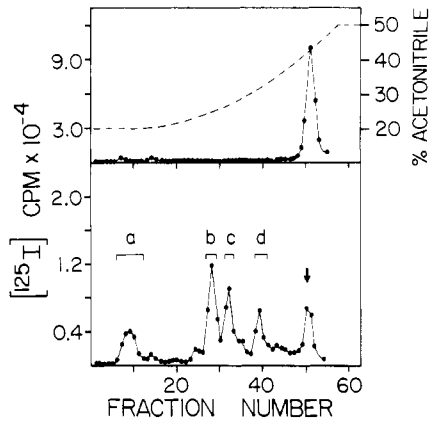

FIGURE 3: Metabolism of ^{125}I-labeled bPTH by isolated Kupffer cells as analyzed by HPLC. Radioiodinated bPTH (2×10^6 cpm/mL) was incubated for 60 min with medium alone (upper panel) or isolated Kupffer cells (10^6 cells/mL) (lower panel). An aliquot (80 μL) of medium was diluted to 1 mL with 20% acetonitrile/0.1% TFA, clarified by centrifugation, and applied directly to a μBondapak C_{18} column equilibrated in 20% acetonitrile/0.1% TFA. Radioactive peptides were eluted with a concave gradient (curve 7, Waters programmer) of 20–50% acetonitrile/0.1% TFA over 15 min at 2.0 mL/min (---). Fractions (0.3 min) were collected into glass tubes containing albumin (see Materials and Methods) for determination of radioactivity. Intact ^{125}I-labeled bPTH eluted in the position of standard bPTH (↓). Fractions comprising the indicated peaks (lower panel) were separately pooled, as shown, and lyophilized.

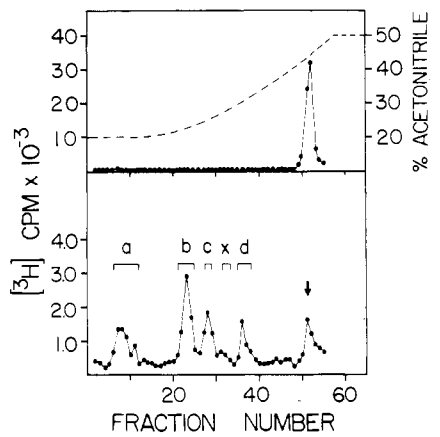

FIGURE 4: Metabolism of [[^{3}H]Tyr]bPTH by isolated Kupffer cells as analyzed by HPLC. [[^{3}H]Tyr]bPTH (0.8×10^6 cpm/mL) was incubated with medium alone (upper panel) and isolated Kupffer cells (10^6 cells/mL) (lower panel) for 60 min, and the medium was subsequently analyzed by HPLC, as described in legend to Figure 3. Fractions were pooled, as indicated, and lyophilized.

37, and 40 and 41 of intact ^{125}I-labeled bPTH. Analysis of an identical aliquot of this incubation medium by HPLC (Figure 3), however, demonstrated resolution of the individual hormonal fragments, with at least four distinct peaks eluting earlier than intact ^{125}I-labeled bPTH.

The metabolism of internally labeled, biologically active bPTH by isolated Kupffer cells was similar to that observed with ^{125}I-labeled bPTH, although the intact hormone and the ^{3}H-labeled fragments consistently eluted from the HPLC columns earlier than did their ^{125}I-labeled counterparts. Thus, analysis of medium from a 60-min incubation of [[^{3}H]Tyr]-bPTH with Kupffer cells (Figure 4) again demonstrated multiple peaks of radioactivity, including one minor peak (peak x) that was not a consistent finding in all experiments but is included here to illustrate the resolution provided by HPLC.

Incubation of ^{125}I- or ^{3}H-labeled bPTH in medium alone (upper panels, Figures 3 and 4) revealed no proteolysis of the hormone in the absence of cells. In at least four additional

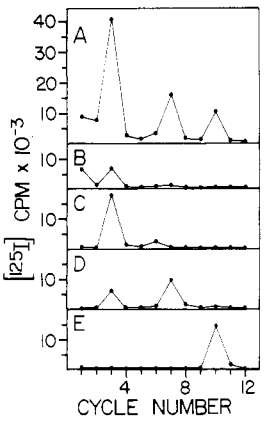

FIGURE 5: Sequential Edman degradation of ^{125}I-labeled bPTH fragments separated by HPLC. Unchromatographed incubation medium and the four major ^{125}I-labeled fragment peaks previously resolved by HPLC (Figure 3) were separately analyzed by sequential Edman degradation. Values on the ordinates indicate the radioactivity recovered at each cycle of degradation after preliminary corrections, as described under Materials and Methods. (A) Unchromatographed medium; (B) peak a; (C) peak b; (D) peak c; (E) peak d.

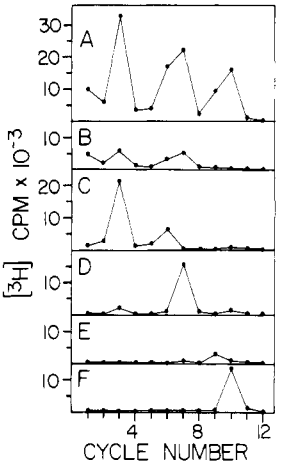

FIGURE 6: Sequential Edman degradation of [[^{3}H]Tyr]bPTH fragments separated by HPLC. Unchromatographed medium and the five major peaks previously resolved by HPLC (Figure 4) were separately degraded. Ordinates are labeled as in Figure 5. (A) Unchromatographed medium; (B) peak a; (C) peak b; (D) peak c; (E) peak x; (F) peak d.

experiments with each tracer (not shown), the recovery of radioactivity applied to the HPLC column was consistently over 90%, and the retention times of individual fragment peaks were highly reproducible (±0.3 min) for the same solvent batch and column.

The chemical identities of the ^{125}I- and ^{3}H-labeled bPTH fragments resolved by HPLC were studied by microsequence analysis of lyophilized fractions comprising the various peaks, pooled as indicated in Figures 3 and 4. Similar results were obtained with both ^{125}I- and ^{3}H-labeled fragments. Thus, Edman degradation of peaks b and d (Figure 5C,E and Figure 6C,F) indicated that these were each nearly homogeneous, corresponding to COOH-terminal fragments with NH_2 termini at Ile^{41} (peaks b) and Phe^{34} (peaks d), respectively, of the intact hormone. Peak b also contained a small amount of peptide with an NH_2 terminus at Gly^{38} of bPTH, which was not a consistent feature of all experiments. The major component of peak c (Figures 5D and 6D) was a fragment terminating at Leu^{37}, seven cycles from the tyrosine at position 43, although some fragment with an NH_2 terminus at Ile^{41} was also present in amounts consistent with incomplete resolution of peaks b and c. In the experiment with [[^{3}H]Tyr]bPTH shown in

Figures 4 and 6E, peak x was found to consist almost entirely of a fragment terminating at Val^{35}, with a small amount of Leu^{37}-terminal fragment consistent with overlap from peak c. The nonretained peak a commonly contained a mixture of small amounts of fragments with NH_2 termini at Leu^{37}, Gly^{38}, and Ile^{41}; the earlier elution position of these fragments indicates that they are distinct from other fragments with the same NH_2 termini in peaks b and c. Radioactivity recovered at cycle 1 during degradation of peak a may represent Tyr^{43}-terminal fragment(s) or, alternatively, overlap from the preliminary wash cycle of shorter, relatively hydrophilic COOH-terminal fragments or free [^{3}H]tyrosine, or both.

Comparison of the sequence analyses of unchromatographed incubation medium (top panels, Figures 5 and 6) with those of individual fragment peaks revealed no evidence of spontaneous cleavage of hormone or fragments during the HPLC procedure. Several fragments with identical NH_2 termini were resolved, however, revealing a complexity not previously appreciated from direct sequence analysis of unchromatographed media or of mixed fragment peaks after gel filtration of such media. Thus, peaks a, b, and c (Figure 5B–D and Figure 6B–D) each contain at least one fragment with an NH_2 terminus at Ile^{41} of intact bPTH. Whereas the Ile^{41}-terminal fragment in HPLC peak c could be a consequence of incomplete resolution from the larger peak b, an Ile^{41}-terminal fragment was also present in peak a, which is well resolved from peak c. Similar considerations apply to the Leu^{37}-terminal fragments in peaks a and c. These fragments with common NH_2 termini presumably differ in length at their COOH termini, or they may reflect unique chemical modifications within the same hormonal fragment(s). Further characterization of these peptides is in progress.

To evaluate the quantitative precision of HPLC in the direct analysis of COOH-terminal fragments in crude incubation media, we compared the data derived from HPLC with those from microsequence analysis of the same incubation media from one experiment. This comparison was limited partly by the fact that, in some instances, several fragments with the same NH_2 termini were resolved during HPLC, as discussed above. This multiplicity of fragments with the same NH_2 termini could not, of course, be appreciated by direct sequence analysis of unchromatographed medium. In general, peptides with NH_2 termini at Tyr^{43}, Ile^{41}, and Gly^{38} of bPTH, corresponding to release of radioactivity at sequenator cycles 1, 3, and 6, respectively, were confined mainly to HPLC peaks a and b. Thus, for the purpose of the comparison, we took the combined fraction of total medium radioactivity recovered in HPLC peaks a and b as representative of the sum of the fractions of total radioactivity recovered at cycles 1, 3, and 6 during Edman degradation of the same (unchromatographed) medium. Otherwise, as established by the results in Figures 5 and 6, HPLC peaks c, x, and d were presumed to correspond to fragments with NH_2 termini at Leu^{37}, Val^{35}, and Phe^{34}, respectively, of intact bPTH (cycles 7, 9, and 10). The results of these comparisons show that the fragment compositions of crude media as predicted by HPLC agree well with the results of direct Edman degradation of the same samples (Figure 7).

When media from incubations of mixed sinusoidal lining cells with [[^{35}S]Met]bPTH were analyzed by HPLC under the conditions already employed for the optimal resolution of COOH-terminal fragments, multiple NH_2-terminal fragments were clearly demonstrated. Thus, as shown in Figure 8, at least three major NH_2-terminal fragments appear in the medium within 10 min after the addition of tracer to the cell

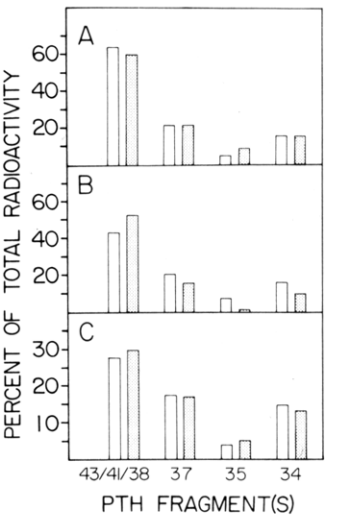

FIGURE 7: Comparison of the bPTH fragment composition of incubation media as predicted by HPLC and by direct microsequence analysis. The percentages of total medium radioactivity represented in individual COOH-terminal fragments with the indicated NH_2 termini were calculated from the results of direct Edman degradation of medium from each of three separate incubations with Kupffer cells (stippled bars). These estimates were then compared with those obtained by HPLC of duplicate aliquots of the same media (open bars), using the peak assignments and equivalences outlined in the text. The designation "43/41/38" refers to the combined radioactivity represented in fragments with NH_2 termini at positions Tyr^{43}, Ile^{41}, and Gly^{38} of bPTH, as discussed in the text. (A) [[^{3}H]Tyr]bPTH incubation (60 min); (B) ^{125}I-labeled bPTH incubation (60 min); (C) ^{125}I-labeled bPTH incubation (30 min).

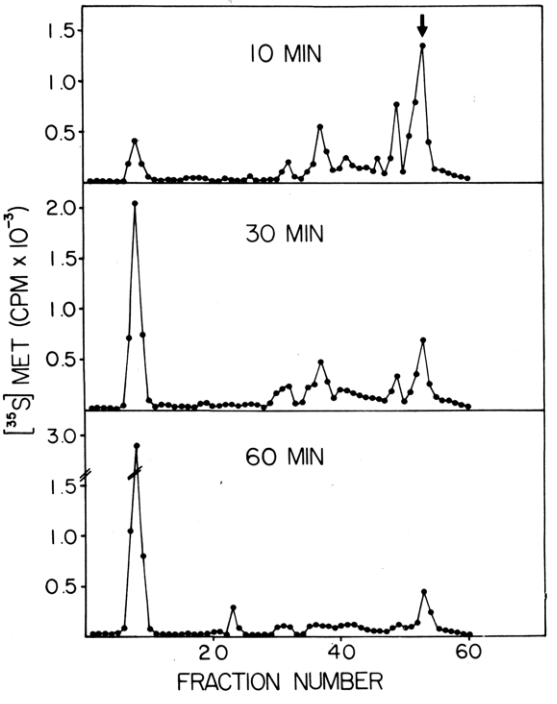

FIGURE 8: Metabolism of [[^{35}S]Met]bPTH by isolated Kupffer cells, as analyzed by HPLC. Isolated sinusoidal lining cells (5×10^6/mL) were incubated with [[^{35}S]Met]bPTH (8×10^4 cpm/mL) for the indicated periods. The cell-free media were subsequently analyzed by HPLC as described in the legend to Figure 3. The results of a control incubation for 60 min without cells (not shown) were similar to those seen with [[^{3}H]Tyr]bPTH (Figure 4, top panel), and the arrow indicates the elution position of unmetabolized PTH.

suspensions. Unlike the COOH-terminal fragments, however, these NH_2-terminal fragments appeared to undergo rapid further degradation to peptides (or free [^{35}S]methionine) that were no longer retained by the HPLC columns under the

elution conditions employed. Thus, at concentrations of 5×10^6 cells/mL, intact [[^{35}S]Met]bPTH and all early NH_2-terminal fragments had been completely degraded to nonretained material within 60 min, at which time large amounts of COOH-terminal fragments resulting from proteolysis of [[^{3}H]Tyr]bPTH persisted in the medium (Figure 4).

Discussion

Reverse-phase HPLC has been widely employed in the purification and analysis of peptides and proteins (Bennett et al., 1977; McMartin & Purdon, 1978; O'Hare & Nice, 1979; Regnier & Gooding, 1980) and for separation of radioactive (Hudson & McMartin, 1980) and immunoreactive (Bennett et al., 1981) products of hormonal metabolism in vivo. The present results illustrate the utility of reverse-phase HPLC in studies of the mechanism(s) of proteolysis of PTH. Chemical identification of the fragments generated during PTH metabolism has been hampered by the need for repetitive sequential Edman degradations because the closely related peptides cannot be resolved by gel filtration or electrophoresis. Additional limitations are related to available hormonal probes: analysis by radioimmunoassay of unlabeled hormone (Canterbury et al., 1973; Segre et al., 1974, 1977; Hruska et al., 1975; Martin et al., 1976; Bennett et al., 1981) has not permitted precise chemical definition, and radioactive PTH labeled with ^{125}I (Sammon et al., 1973; O'Riordan et al., 1974) or [^{3}H]acetamidate (Zull & Repke, 1972) contains prosthetic groups not present in the native hormone and also offers limited sites for incorporation of radioactivity. As recently shown for human parathyroid hormone (hPTH) by Bennett et al. (1981), we have found that the biological activity of bPTH is preserved during reverse-phase HPLC. We have further shown that internally labeled bPTH may be obtained in high yield with greater than 93% radiochemical purity by direct HPLC purification of crude parathyroid gland incubation medium. Resolution by reverse-phase HPLC of individual internally labeled carboxyl fragments of bPTH permits direct identification of the fragments generated during hormonal metabolism without the need for repetitive Edman degradations. Moreover, accurate quantitation of hormonal fragments may be accomplished directly from the HPLC chromatogram.

The present observations on the metabolism of biologically active, unmodified, radioactive bPTH by isolated rat Kupffer cells confirm the results of previous studies with radioiodinated hormone (Segre et al., 1981b) and considerably extend our knowledge of the nature of the enzymes responsible for the proteolytic degradation of the hormone. Incubations of [[^{3}H]Tyr]bPTH and ^{125}I-labeled bPTH with these cells produce similar patterns of hormonal fragments, and, in both cases, sequence analysis verifies the predominance of cleavages between residues 33 and 34, 36 and 37, and 40 and 41 of the intact hormone. A similar pattern of COOH-terminal fragments was observed in blood after in vivo administration of ^{125}I-labeled bPTH to normal or nephrectomized, but not hepatectomized, rats (Segre et al., 1981a). Taken together, these results suggest that hepatic macrophages generate the majority of the circulating COOH-terminal fragments of bPTH.

Earlier findings indicating that peripheral metabolism of intact PTH was accompanied by a predominance of COOH-terminal PTH fragments in blood as metabolism proceeded (Segre et al., 1972; Canterbury et al., 1973; Fischer et al., 1974) were consistent with at least two mechanisms of hormone proteolysis in the liver. In one case, initial attack by one or more aminopeptidases might rapidly degrade the NH_2-terminal portion of PTH (thereby destroying the biologically active NH_2-terminal region of the molecule) and subsequently release the degraded hormone with only its middle and carboxy regions intact. The multiplicity of end groups detected and the appearance, with time, of fragments with NH_2-terminal residues located in the middle region of the molecule were consistent with such exopeptidase (NH_2-peptidase) attack, although no carboxyl fragments representing cleavage more NH_2 terminal than residue 33 were detected. An alternate mechanism would be initial cleavage by one or more endopeptidases within the peptide sequence, with liberation of both NH_2- and COOH-terminal fragments (Segre et al., 1981b). Only the latter mechanism would permit the generation of NH_2-terminal fragments that might be active on distant target tissues such as bone or kidney. The present studies provide chemical evidence that the initial cleavage of biologically active bPTH by isolated hepatic macrophages is indeed mediated by one or more endopeptidases directed at the peptide sequence bounded by residues 33 and 43 of intact bPTH. This conclusion is supported by the demonstration of both NH_2- and COOH-terminal fragments in media from incubations of internally labeled bPTH with Kupffer cells and by the fact that at no time during the incubations are significant amounts of ^{3}H- or ^{125}I-labeled tyrosine associated with hormonal fragments larger than bPTH-(34–84). We cannot exclude the additional, subsequent action of amino- or carboxypeptidases (which, in fact, seems likely), but such cleavages are clearly not major mechanisms of initial hormonal degradation by Kupffer cells. The preferentially rapid further degradation of NH_2-terminal fragments in the Kupffer cell system mirrors in vivo observations on the disproportion between circulating NH_2- and COOH-terminal fragment immunoreactivity (Segre et al., 1981a). Nevertheless, whereas, in vitro, Kupffer cell incubations constitute a closed system providing no egress of generated fragments, it remains possible that, in vivo, some intact, biologically active NH_2-terminal fragments may escape further degradation in the liver to enter the general circulation.

The present results are most compatible with single or multiple initial endopeptidic cleavage(s), followed by further degradation of NH_2- and possibly COOH-terminal PTH fragments by enzymatic mechanisms of unknown specificity. The resolution by HPLC of several different COOH-terminal fragments possessing the same NH_2 termini indicates that the metabolism of bPTH in vivo may be more complex than could be detected by previous studies (Segre et al., 1981a,b), which relied exclusively upon Edman degradation of fragments isolated by gel filtration. Alternatively, these more hydrophilic (possibly smaller) fragments may reflect further modification of initially generated fragments that occurs only in vitro because of the "closed" nature of the incubation system. Similarly, the several NH_2-terminal fragments detected in Kupffer cell medium may reflect the action of multiple endopeptidases or a single such cleavage followed by digestion by one or more exopeptidases, or both. With the resolution provided by HPLC, it should now be possible to isolate and sequence individual labeled NH_2-terminal fragments and to further elucidate the mechanisms responsible for their degradation. Finally, the ability to rapidly resolve and directly analyze the individual fragments of bPTH will greatly facilitate the study of ionic, hormonal, or other influences on the rates of cleavage at specific sites within the bPTH sequence, the purification of the responsible enzyme(s), and the design of synthetic peptides directed toward inhibition of one or more of these specific proteolytic events.

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