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A Biologically Active Amino-Terminal Fragment of Bovine Parathyroid Hormone Prepared by Dilute Acid Hydrolysis†

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ABSTRACT: Early work with bovine parathyroid hormone extracted from gland tissue with hot hydrochloric acid had shown evidence for a number of small, biologically active fragments arising from cleavages within the peptide chain during extraction. Introduction of improved extraction methods and purification of the 84-amino acid native parathyroid polypeptide has provided the opportunity to investigate these smaller active peptides under controlled conditions. Recovery of biological activity was studied in preparations of hormone treated with 0.03 N HCl at 110° for varying time periods; hydrolysis times of 6 hr or less provided satisfactory yields of active peptides. Native hormone was then treated on a preparative scale for 4 hr and the resulting peptides were identified; most arose from cleavages of the molecule at the 6 aspartic acid residues. A fragment comprising residues 1–29 of the parathyroid hormone sequence, generated by cleavage of aspartic acid at position 30, was isolated from the reaction product by gel filtration and ion-exchange chromatography. This amino-terminal peptide possessed significant biological potency by both *in vivo* and *in vitro* assay methods, and represents the shortest biologically active fragment thus far obtained from the parathyroid hormone molecule.

Parathyroid hormone (BPTH)[1] was first extracted from bovine parathyroid glands by Collip (1925) by a technique using hot hydrochloric acid. Evidence rapidly accumulated, however, that use of these conditions for extraction was accompanied by considerable damage to the extracted peptide, in part through cleavages within the polypeptide chain (Handler *et al.*, 1954; Aurbach *et al.*, 1958).

Ultimately, effective extraction methods, including use of phenol (Aurbach, 1959a) and, later, urea–cysteine–hydrochloric acid (Rasmussen *et al.*, 1964), were developed which employed milder conditions than the hot acid method. Application of these extraction procedures avoided the problem of cleavages during extraction and permitted the eventual isolation of the intact 84-residue parathyroid polypeptide.

Meanwhile, the earlier evidence for the presence of smaller,

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[1] Abbreviations used are: BPTH, bovine parathyroid hormone; CMC, carboxymethylcellulose.

biologically active hormone subfragments in hot acid extracts stimulated continued interest in their chemical nature. Only comparatively recently, however, did our understanding of the mechanism of the peptide bond cleavages during exposure of polypeptides to heated acidic solutions become sufficiently advanced (Tsung and Fraenkel-Conrat, 1965) to suggest a systematic approach.

Recently, availability of preparations of bovine parathyroid hormone I completely free from trace contaminants and minor variants of the hormone (Keutmann *et al.*, 1971), as well as definitive sequence information (Niall *et al.*, 1970; Brewer and Ronan, 1970; Potts *et al.*, 1971a), have made possible the characterization of these fragments.

This report describes the isolation and characterization of a 29-residue amino-terminal fragment obtained following treatment of bovine parathyroid hormone I with 0.03 N hydrochloric acid. This fragment, while only one-third the length of the native molecule, possesses significant biological activity as measured by both *in vitro* and *in vivo* assay systems.

Materials and Methods

Hormone Preparations. Purified bovine parathyroid hormone I was prepared from the trichloroacetic acid precipitate (Wilson Laboratories, Chicago, Ill.) using Sephadex G-100 gel filtration and ion-exchange chromatography on carboxymethylcellulose (CMC) in 8 M urea, as described by Keutmann *et al.* (1971).

Treatment with Dilute Acid. A series of experiments was first carried out to determine the optimal duration of time for hydrolysis. In the initial study, 18.5 mg of BPTH was dissolved in 10 ml of 0.03 N HCl made 1:2000 (v/v) in mercaptoethanol (redistilled; Eastman Organic Chemicals, Rochester, N. Y.) to avoid oxidation of methionine; 1.0-ml aliquots (1.85 mg of BPTH) were placed in 15 × 90 mm glass tubes and heated to 110° in an evacuated desiccator for 3, 6, 9, 12, and 15 hr, respectively. HCl (200 ml, 0.03 N) had been introduced into the bottom of the desiccator to minimize evaporation from the tubes. The samples were lyophilized for bioassay promptly after removal from the desiccator. The zero-time sample was directly lyophilized without heating.

Several additional aliquots of 2.5–5.0 mg of hormone were subsequently treated similarly for 6 and 9 hr for confirmatory bioassays and preliminary compositional studies.

Following completion of the time-course studies, larger scale preparative hydrolyses were carried out using 10- to 30-mg aliquots of hormone. The BPTH was dissolved in 0.03 N HCl (again containing 1:2000 mercaptoethanol) to a concentration of 10 mg/ml. The solution was introduced into a 2 × 15 cm thick-walled glass tube which was evacuated, sealed, and heated to 110° for 4 hr (the time period does not include the time required for return of the heated preparation of hormone to room temperature following removal from the oven). The treated preparation was lyophilized and redissolved in 3 ml of 0.1 N acetic acid. Precisely measured aliquots were removed, lyophilized, redissolved in pH 2.2 sodium citrate buffer, and applied directly to the analyzer to determine the content of free aspartic acid as an index of cleavage. The preparation was then lyophilized and dissolved in suitable buffers prior to bioassay or further purification.

Sephadex Gel Filtration. The lyophilized 4-hr cleavage product (10–30 mg) was dissolved in 2.5 ml of 0.14 M ammonium acetate and applied to a 2.0 × 130 cm column of Sephadex G-50 (Superfine) (Pharmacia Fine Chemicals, Piscataway, N. J.). The column, maintained at 4°, was eluted with 0.14 M ammonium acetate buffer, pH 4.8. The effluent was monitored by reading optical density at 280 mμ. Aliquots were removed from the eluate tubes and their peptide content was evaluated by single-dimension high-voltage electrophoresis.

Ion-Exchange Chromatography. Ion-exchange chromatography was carried out using a 1.2 × 10-cm column of carboxymethylcellulose (Whatman CM-52; W. R. Balston, Ltd., London, England). A linear conductivity gradient was developed using 100 ml of 0.01 M ammonium acetate (pH 4.9, conductivity 0.6 mmho) and 100 ml of 0.15 M ammonium acetate (pH 5.9, conductivity 10 mmho). The column was run at 4°. Eluates were evaluated by measurement of optical density and by thin-layer chromatography.

Bioassays. Bioassays of hormone *in vivo* were carried out as described by Aurbach (1959b). The lyophilized hormone preparations were dissolved directly in 0.01 N acetic acid containing 2% cysteine·HCl and the solution was adjusted to pH 1.5 with sodium bicarbonate for assay. Aliquots (0.5 ml) of these solutions were injected subcutaneously into 80-g Sprague-Dawley rats immediately following parathyroidectomy. Blood was obtained for plasma calcium determination 5 hr later. Calcium levels were determined by the method of Hill (1965).

In vitro assays were performed using activation of renal-cortical adenylyl cyclase as described by Marcus and Aurbach (1969). The [^{32}P]ATP substrate was purchased from International Chemical and Nuclear, City of Industry, Calif. Radioactive products were counted on the Packard Tri-Carb scintillation counter using Bray's solution (Bray, 1960) as scintillator.

Both bioassay methods employed Medical Research Council (MRC) preparation 67/342 (potency 330 U/mg) (Robinson *et al.*, 1971) or a house standard calibrated against it.

Paper Electrophoresis and Chromatography. Peptide separation by two-dimensional chromatography–electrophoresis was carried out using 2–4 mg of peptide applied to Whatman No. 3MM paper. Descending chromatography was performed with a butanol–water–acetic acid (4:5:1) solvent system, with a running time of 15 hr at room temperature. High-voltage electrophoresis was carried out for 3 hr at 3500 V, 25°, in the Gilson Model D Electrophorator, using a pH 3.2 pyridine acetate buffer system. Peptides were detected by spraying the filter paper with 0.5% ninhydrin in ethanol, and were eluted from the paper for amino acid analysis by use of 6 N HCl.

Thin-Layer Chromatography. Thin-layer chromatography (tlc) was carried out using 20–40 μg of peptide on Brinkmann glass-backed cellulose plates of 90-μ thickness. The solvent system employed was butanol–pyridine–water–acetic acid (15:10:12:3). Plates were stained with ninhydrin. Preparative-scale thin-layer chromatography was performed using 100–200 μg of peptide spread along 2 cm at the origin. Guide spots, containing 20 μg of peptide, were developed alongside the bulk of the peptide and stained with ninhydrin, while the preparative load of peptide was shielded, to locate the peptides to be eluted. Peptides were scraped from the plates and eluted using 50% acetic acid.

Carboxypeptidase Digestion. Highly purified, endopeptidase-free carboxypeptidases A and B were prepared by treatment of the colloidal suspension of the respective enzymes (obtained from Worthington Biochemicals, Freehold, N. J.) with DFP (Potts, 1967). A 10 mg/ml solution of carboxypeptidase in 2 M ammonium bicarbonate, pH 8.0, was incubated at 20° for 2 hr following addition of a 60-fold molar excess of freshly opened, undiluted DFP (Sigma Chemicals, Inc., St. Louis, Mo.). The treated preparations were then

TABLE I: Hypercalcemic Effect of Parathyroid Hormone after Treatment with 0.03 N HCl for Varying Time Periods.[a]

Reaction Time (hr)	Injected Hormone (μg/Animal)	Plasma Calcium (mg%)
0	50	10.40
3	50	9.95
6	50	8.50
9	250	5.40
12	250	4.50
15	250	4.80
	0	4.75

[a] Plasma calcium measured 5 hr after injection of 0.5-ml aliquots of hormone into each parathyroidectomized rat. Calcium value for each hydrolysis time and control injection represents average of results achieved in the four animals of each group.

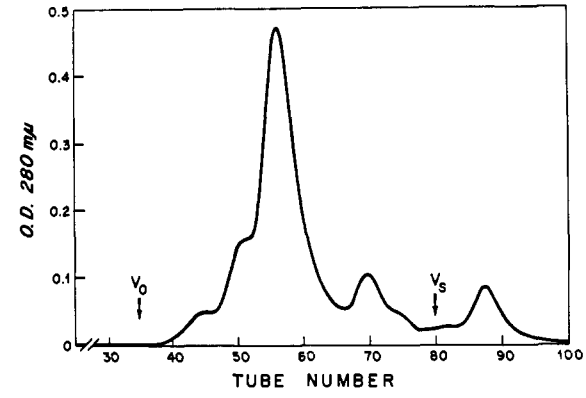

FIGURE 1: Elution pattern obtained by passage of dilute acid treated bovine parathyroid hormone over a column of Sephadex G-50 in 0.14 M ammonium acetate buffer, pH 4.9. The active fragment 1–29 eluted in the large peak, tubes 52–60. Fraction size was 4 ml.

frozen directly in small aliquots for storage. The carboxypeptidase preparations were shown to be free from endopeptidase contaminants by exhaustive digestion of several test peptides including bovine pancreatic ribonuclease and bovine ACTH.

Digestion of the active fragment peptide from BPTH was carried out by treating 0.1 mg of peptide with carboxypeptidase A or B at an enzyme:substrate ratio of 1:10 (M:M). Incubations were performed in 1 ml of 0.1 M ammonium bicarbonate buffer, pH 8.5, at 37° for time periods of 20 min and 1 hr.

Amino Acid Analyses. Acid hydrolysis was carried out in 5.7 N HCl at 110° for 24 hr in the presence of 1:2000 mercaptoethanol to prevent oxidation of methionine (Keutmann and Potts, 1969). Total enzymatic digestion, for determination of the content of glutamine, asparagine, and tryptophan, was carried out using papain and aminopeptidase M as previously described (Keutmann *et al.*, 1970).

Amino acid analyses were performed using the Beckman Model 121 amino acid analyzer, equipped for high sensitivity analyses (Hubbard, 1965). Elution programs included the 2-hr sodium citrate system (Hubbard and Kremen, 1965) and the lithium citrate system for evaluation of asparagine and glutamine (Benson *et al.*, 1967). Quantitation of amino acid yields employed digital integration using the Infotronics Model CRS-12AB integrator.

End-Group Analysis. End groups were determined by the Edman phenyl isothiocyanate procedure (Edman, 1960), modified as described by Niall and Potts (1970). Phenylthiohydantoins were identified by gas chromatography (Pisano and Bronzert, 1969) and thin-layer chromatography (Edman and Begg, 1967).

Results

Hydrolysis Time Study. Evaluation of optimal hydrolysis time was begun by treating 1.85-mg aliquots of hormone with dilute acid for varying lengths of time. Subcutaneous injections of each preparation were given at a single dose level to parathyroidectomized rats. The hypercalcemic responses, evaluated 5 hr after injection, are summarized in Table I. A marked decrease in potency was observed to take place between 6 and 9 hr of hydrolysis.

Formal, parallel-line bioassays at multiple dose levels (Aurbach, 1959b) were then carried out using samples from several repeat 6- and 9-hr hydrolyses, in order to confirm the rapid fall in potency after 6 hr. The average potency of the hormone preparations hydrolyzed for 6 hr was 18% when compared to that of untreated BPTH, while the 9-hr hydrolysates averaged 3.5%. Lengthening of hydrolysis time from 6 to 9 hr was thus accompanied by a fivefold drop in specific biological activity of the crude preparation.

Preparative 4-Hr Hydrolysis. The results of the preliminary studies indicated that hydrolysis times of 6 hr or less provided satisfactory recovery of biological activity. To minimize chemical heterogeneity caused by partial deamidation of asparagine and glutamine residues, a hydrolysis time of 4 hr was ultimately chosen for the preparative-scale experiments.

Direct amino acid analysis of an aliquot of the cleavage product following treatment with 0.03 N HCl for 4 hr revealed the presence of 3.2 moles of aspartic acid per mole of peptide. This indicated that 55% of the total aspartic acid content (6 moles per mole of BPTH) had been cleaved from the molecule. In addition, small quantities of free valine, alanine, and lysine were seen in the product.

Sephadex Gel Filtration. The cleavage products eluted from Sephadex G-50 in the pattern shown in Figure 1. Biologically active peptide of rapid electrophoretic mobility eluted in the centrally located peak of optical density (tubes 55–60). Assays of peptide from tube 57, near the center of the peak, showed a potency *in vitro* of 455 U/mg (95% confidence limits 385–555) and *in vivo* of 200 U/mg (95% confidence limits 100–350). Amino acid analysis indicated that this material originated from the amino-terminal portion of the molecule.

Material eluting in the earlier peaks represented larger peptides, including one of electrophoretic mobility identical to that of intact BPTH. Presence of these large fragments was anticipated by the hydrolysis conditions chosen; the reaction had not gone to completion, since not all aspartate residues (even those present originally as aspartic acid and not as asparagine) had been cleaved in 4 hr.

Tubes from the central peak, shown by electrophoresis to contain the active fragment peptide but free from larger fragments that might contain intact hormone, were pooled for further purification using a narrow cut from the peak.

Later peaks from the G-50 column consisted of multiple

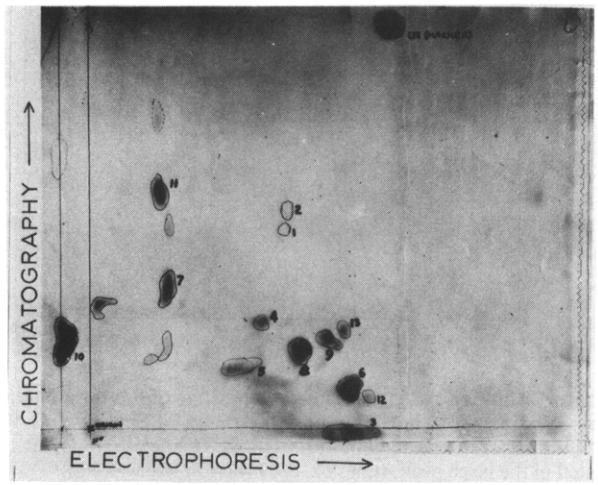

FIGURE 2: Two dimensional chromatography/electrophoresis of late eluting fractions (Tubes 61-85) from Sephadex G-50 gel filtration of dilute acid cleaved parathyroid hormone. Compositional analyses of the peptides (1–10) and free amino acids (11–13) are described in the text. The paper was sprayed with ninhydrin. Spots 1 and 2 stained yellow and are not visible in the photograph. Phenol red marker for chromatography is identified by the dotted circle.

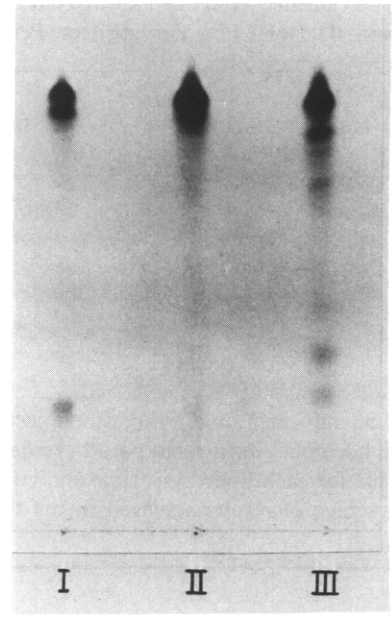

FIGURE 4: Thin-layer chromatography of peptide material from each of the three principal peaks from carboxymethylcellulose chromatography (Figure 3). The most rapidly moving component (R_F 0.65) represents the active fragment 1–29.

inactive, small peptide fragments and free amino acids. These fragments were separated by two-dimensional chromatography/electrophoresis as shown in Figure 2. The principal spots were eluted and the following compositions obtained: 1 (Ala_2,Ser,Ile,Tyr,Arg); 2 (Ala,Ser,Ile,Tyr,Arg); 3 (Ser_2Arg_2,-Lys_2,Glu_2,Pro,Gly); 4 (Glu_3,Leu_2,Val_2,Ser_2,Asp_2,His,Gly,Lys); 5 (Ala,Asp); 6 (Lys,Ala); 7 (Lys,Ala-Asp,Val); 8 (Lys_2,Val,-Ile,Leu,Ala,Pro,Glu); 9 (Lys_2,Val,Ile,Leu,Ala,Pro); 10 aspartic acid; 11 valine; 12 lysine; 13 alanine.

Ion-Exchange Chromatography. The pooled active material from gel filtration was subjected to ion-exchange chromatography on carboxymethylcellulose with an ammonium acetate buffer gradient; the elution pattern is shown in Figure 3. Three principal peaks were found, all of which contained biologically active peptide. The fractions from each peak were pooled and examined by thin-layer chromatography; each pool contained a principal peptide component of closely comparable chromatographic mobility, but differed considerably in homogeneity (Figure 4). Peak II, appearing in highest yield and in most homogeneous form, was studied the most extensively.

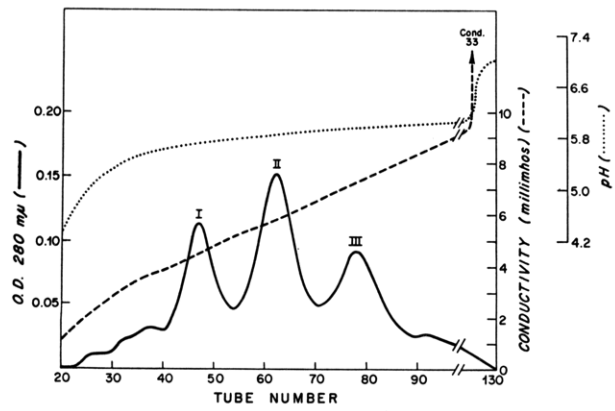

FIGURE 3: Elution pattern obtained by carboxymethylcellulose ion-exchange chromatography of biologically active fractions from Sephadex G-50 gel filtration (see Figure 1). Active 1–29 peptide was found in each of the three principal peaks. Fraction size was 2.5 ml.

Peak II Polypeptide (Active Fragment 1–29). The peak II material consisted of a single peptide with an R_F of 0.65 on thin-layer chromatography (Figure 4). The amino acid composition of the peak II peptide is shown in Table II. This composition is consistent with that of a 29-residue amino terminal fragment, generated by cleavage from the molecule of aspartic acid at position 30 (Figure 5).

Further analyses were made to confirm that the active peptide consisted of residues 1–29. Edman end-group analysis indicated a single amino acid end group—the phenylthiohydantoin of alanine, as predicted since alanine is the amino-terminal residue of BPTH. Results achieved after digestion with carboxypeptidase were also consistent with the conclusion that the isolated peptide consisted of residues 1–29. Glutamine and leucine were released in equal amounts, corresponding to the expected release of residues 29 and 28 of the parathyroid hormone molecule. Further digestion by carboxypeptidase A at pH 8.5 was not expected, because of the arginyllysyllysine sequence that should be encountered next, corresponding to the sequence found at positions 25 through 27 of the hormone (Figure 5).

Total enzymatic digestion was employed to evaluate the amide content of the peptide, of particular interest in these studies because of the cleavage of the side-chain amides of glutamine and asparagine known to take place under the acid conditions employed here (Tsung and Fraenkel-Conrat, 1965). Analysis of the enzymatic digest using the lithium citrate analyzer buffer system showed full recovery of the asparagine at position 10 (Figure 5).

Recovery of glutamine after total enzymatic digestion was low—one residue, rather than the expected two, was found. Full recovery of glutamine plus glutamic acid was obtained, as glutamic acid, after acid hydrolysis (5 residues, the expected total from 2 Gln and 3 Glu). The recovery of glutamic acid in the enzymatic digest did not exceed the expected 3 residu-

TABLE II: Amino Acid Composition of Active Fragment 1–29 (Carboxymethylcellulose Peak II Peptide).[a]

Amino Acid	Acid Hydrolysis	Enzymic Hydrolysis	Combined Results	Theory[b]
Aspartic acid	1.30	0.10	0	0
Asparagine		0.90	1	1
Threonine	0.0	0.0	0	0
Serine	3.10	3.05	3	3
Glutamic acid	4.90	3.10	3	3
Glutamine		0.90	1	2
Proline	0.0	0.0	0	0
Half-cystine	0.0	0.0	0	0
Glycine	1.20	1.20	1	1
Alanine	1.10	1.15	1	1
Valine	2.00	1.65	2	2
Methionine	1.55	1.60	2	2
Isoelcuine	0.85	0.90	1	1
Leucine	4.05	4.05	4	4
Tyrosine	0.0	0.05	0	0
Phenylalanine	0.95	0.90	1	1
Tryptophan		0.80	1	1
Lysine	2.95	3.15	3	3
Histidine	1.90	1.85	2	2
Arginine	2.15	2.05	2	2

[a] All values expressed as moles of amino acid per mole of peptide, rounded to the nearest 0.05 residue/mole and given best average fit to known composition, normalized as previously described (Keutmann *et al.*, 1971). [b] Based on sequence of amino-terminal 29 amino acids of BPTH I.

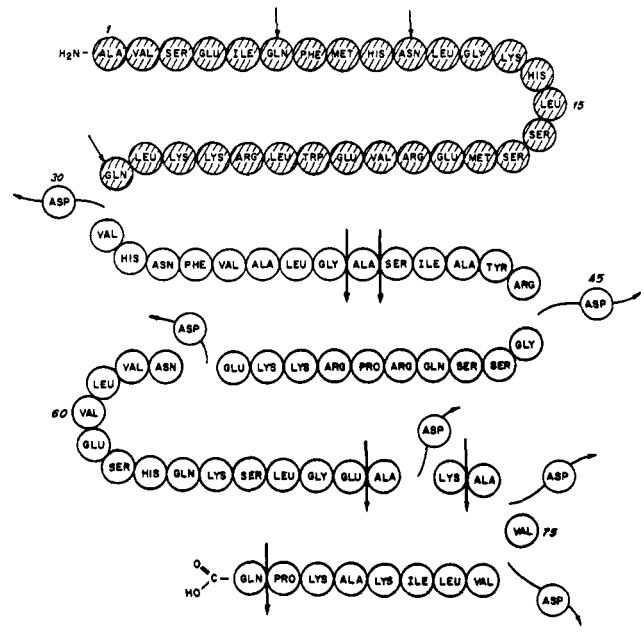

FIGURE 5: Amino acid sequence of bovine parathyroid hormone I, showing location of cleavages obtained by treatment with dilute acid. Aspartic acid residues were cleaved out of the molecule as the free amino acid; other cleavages, involving only a single peptide bond, are designated by arrows through the chain. Shaded residues indicate the active 1–29 fragment. Short arrows designate the glutamine and asparagine residues within the fragment which are subject to partial deamidation during dilute acid treatment.

so we cannot conclude that the lower recovery of glutamine reflects deamidation. The most likely explanation is that some of the glutamine underwent cyclization during enzymic cleavage. Since glutamine rather than glutamic acid was found on carboxypeptidase digestion we can at least conclude that the glutamine at position 29 did not undergo deamidation, but we do not know the state of the glutamine at position 6.

Assay of 1–29 Fragment from CMC Peak II. Four separate *in vitro* renal adenylyl cyclase assays at multiple doses against MRC standard 67/342 were performed with the peak II peptide. The specific activity was 650 U/mg (95% confidence limits 450–975). Dose–response curves were parallel to the intact 1–84 bovine hormone, consistent with a result shown earlier based on assays *in vitro* of the 1–34 peptide prepared by peptide synthesis (Potts *et al.*, 1971a).

The fragment 1–20, prepared by tryptic digestion of BPTH following reversible ε-amino lysine blockade with maleic anhydride (Niall *et al.*, 1970), was also tested in the same assay system and found to be devoid of detectable biological activity. The purified native 1–84 BPTH molecule has a potency in this system of 1500 U/mg (Keutmann *et al.*, 1971).

CMC Peak I. Peak I from carboxymethylcellulose contained peptide of specific activity closely comparable to that of peak II. Contamination with an inactive peptide of slow chromatographic mobility (R_F 0.18), identified after elution from tlc as fragment 46–71 of the BPTH sequence, was evident in this pool. The bulk of the peptide, however, comprised a double spot of similar R_F to the active material from peak II (Figure 4). This spot was eluted *in toto* from a preparative-scale thin-layer chromatogram and hydrolyzed. Because of the low recovery of peptide from the tlc eluates, the active fragment from peak I could not be subjected to definitive compositional studies as was performed with peak II. However, analysis after acid hydrolysis indicated that its composition was also consistent with a fragment derived from the 1–29 region of the sequence, *i.e.*, $Asp_1,Thr_0,Ser_3,Glu_5,Pro_0,Gly_1,Ala_1,Val_2,Met_2,Ile_1,Leu_4,Tyr_0,Phe_1,Lys_3,His_2,Arg_2$.

We have found that reliable enzymatic digestions cannot be performed on eluates from thin-layer plates; thus, the recovery of side-chain amides could not be assessed. Since peak I eluted earlier than peak II it is plausible to conclude that one or more of the asparagine or glutamine residues must have been deamidated, accounting for greater acidity of the peptide.

CMC Peak III. Inspection of Figure 4 indicates that peak II contained numerous contaminating peptides. The small quantity of usable material precluded further purification. Recovery of the major peptide spot corresponding to the active 1–29 fragment from peak I and II after preparative thin-layer chromatography provided sufficient material to permit a limited examination of the composition and biological activity of the peptide. Compositional analysis again indicated that this peak represented a peptide subfragment from the 1–29 sequence region of the hormone. The peptide was also shown in the adenylyl cyclase assay to have biological activity equivalent to that of CMC peaks I and II, but there was insufficient material to perform the additional assays required to obtain a definitive potency figure.

Overall recovery of active 1–29 peptide in all three CMC peaks was approximately 3 mg of peptide from 30 mg of BPTH, or about 30% on a molar basis.

Discussion

Although the vigorous conditions of the original method

(Collip, 1925) for extraction of parathyroid hormone had long been appreciated (Allardyce, 1931), definitive evidence for the existence of acid-cleaved fragments awaited the introduction of readily applicable bioassay methods to monitor purification. When useful assays became available (Davies *et al.*, 1954; Munson, 1955), several workers observed that, with progressive purification of the hormone, multiple active fractions were obtained without improvement in overall potency (Handler *et al.*, 1954; Aurbach *et al.*, 1958; Aurbach, 1961).

By use of countercurrent distribution, Rasmussen (1960) isolated three fragments, reported to be active and to consist of peptides 33, 47, and 51 residues in length, from parathyroid extracts prepared by hot acid. The predominating size was dependent upon the length of exposure to acid in the original extraction. The specific activity of these fragments was reported to be approximately one-third that of the native uncleaved molecule isolated from phenol extracts. In 1962 Rasmussen and Craig recovered a fragment, similar to the 47-residue peptide described earlier, after treatment of native BPTH with 2 N HCl for 5 min at 70°.

Using similar conditions, Potts and Aurbach (1965) reported recovery of a fragment containing significant biological and immunological activity. Tashjian *et al.* (1965) also observed that this treatment resulted in retention of biological activity, but loss of immunological activity by complement fixation.

The tendency in acid hydrolysis for peptides to be cleaved initially at their aspartic acid residues had been recognized since 1950 (Partridge and Davis), but this method was not widely explored as a specific cleavage technique until the kinetics and optimal reaction conditions were described in detail by Schultz *et al.* (1962) and Tsung and Fraenkel-Conrat (1965).

Their studies showed that hydrolysis in 0.03 M hydrochloric acid at 110° resulted in concurrent cleavages of the peptide bond at each side of the aspartic acid residues, at a rate 100-fold greater than the cleavage rate of peptide linkages involving any other residue. The half-time of this aspartate cleavage was 5.5 hr (Tsung and Fraenkel-Conrat, 1965).

When these conditions were applied to BPTH, detectable biological activity was retained after up to 12 hr treatment with 0.03 N HCl at 110°, at which time 75% of the aspartate had been cleaved. The time-course studies, described in the Results section, suggested that 6-hr cleavage resulted in optimal yield of active polypeptide.

We reported earlier (Potts *et al.*, 1968) that an active product isolated from a 6-hr hydrolysate approximated 35 residues in length and, by use of the partial sequence information available at that time, concluded that the peptide came from the amino-terminal end of the BPTH molecule. The sequence studies also suggested, however, that several aspartic acid and asparagine residues were present in the region of the molecule between residues 30 and 45. Potentially, cleavage in dilute acid could therefore result in a number of peptides closely similar in size and charge, difficult to separate by conventional methods.

In carrying out the definitive active-fragment isolation reported here, the reaction time was shortened further, to 4 hr, in order to minimize conversion of asparagine residues to aspartic acid which could give rise to both heterogeneity and extra cleavage sites within the active peptide. In so doing, the overall yield of active peptide (1–29) was reduced as well; typical recovery in a 4-hr hydrolysis was about 30% on a molar basis.

The concurrent completion of amino-terminal amino acid sequence studies (Niall *et al.*, 1970) permitted more precise evaluation of the cleavage products. Thus, aspartic$_{30}$ and aspartic$_{45}$ constituted the expected sites of cleavage in the amino-terminal region. No fragment shorter than 1–29 was expected provided deamidation at asparagine$_{10}$ was minimal as hoped.

Figure 5 summarizes all of the cleavages obtained within the BPTH molecule. The active peptide was shown to comprise the 1–29 amino terminal fragment generated by cleavage at aspartic$_{30}$. No fragments arising from within the 1–29 peptide could be detected.

Based on compositions of the peptides eluted from paper (Figure 2), combined with sequence studies of the entire BPTH molecule, most of the cleavages were found to have occurred in the aspartic-rich carboxyl-terminal region. Of special interest were several cleavages, in high yield, at alanine residues (positions 39, 70, and 73). Such cleavage sites were originally described by Tsung and Fraenkel-Conrat (1965). Unlike aspartic acid, which is released outright as the free amino acid, alanine was found to be cleaved at only a single side. Thus, for example, substantial amounts of both peptide 39–44 (N-terminal cleavage of Ala$_{39}$) and peptide 40–44 (C-terminal cleavage of Ala$_{39}$) were obtained (Figure 2, peptides 1 and 2, respectively).

Consistent with the complete removal of aspartic acid during cleavage, no peptide was found with aspartic acid as its terminal residue; a single exception was the dipeptide Ala$_{70}$–Asp$_{71}$ (Figure 2, peptide 5), a combination possibly resulting from the unusual cleavage properties of alanine residues. An unusual cleavage was the removal of the carboxyl-terminal glutamine, residue 84. Perhaps the presence of the free α-carboxyl function contributed to the lability of the prolyl–glutamine peptide bond.

The concurrent loss of side-chain amides presented the most complex aspect to the evaluation of the active fragment. In analysis of the results, it would be of particular interest to account for the distribution of the biologically active subfragment from the 1–29 sequence region into the 3 peaks of increasing apparent basicity in the order of their elution from CMC. It seems reasonable to speculate that deamidation of glutamine and asparagine is most marked in peak I and least in peak III, but the poor recoveries of pure peptide in peaks I and III and the uncertainty about the state of one of the glutamine residues in peak II will not permit any definite conclusions. It would appear likely, however, that since the peptides from all three CMC peaks were equivalently active, the BPTH molecule can tolerate the loss of at least some side-chain amides without complete loss of biological activity.

The specific activity of the purified 1–29 peptide by *in vitro* adenylyl cyclase assay was 650 U/mg. Because of the small quantity of product and the high doses required, sufficient *in vivo* rat bioassays of the CMC-purified fragment could not be carried out to obtain a formal activity figure. Potency values *in vivo* and *in vitro* could be compared, however, using the G-50 preparation which was, like the CMC peptide, entirely free from unreacted native hormone. The higher potency obtained by *in vitro* assay (455 U/mg) compared to that obtained *in vivo* (220 U/mg) was a similar observation to that noted for the 1–34 fragment prepared by peptide synthesis (Potts *et al.*, 1971a). The lower potency *in vivo* suggests that the middle and carboxyl terminal portions of the molecule may serve to protect the molecule against rapid degradation during transport in the circulation.

The dilute-acid cleavage studies reported here are of interest in providing some detailed information about the specificity

and pattern of cleavage of an 84-amino acid polypeptide rich in aspartic acid, asparagine, glutamic acid, and glutamine. While the studies have led to definitive isolation of the 29-residue active fragment, considerable complexity was evident in the cleavage process with random attack on aspartic acid (peptides corresponding to cleavage at every aspartate residue were found when only about 50% of the aspartates had been cleaved overall) along with the additional possibility of random deamidation of the glutamine and asparagine residues. This results in the generation of many peptides separable by charge during isolation, and could explain the great difficulties encountered earlier in attempts to isolate active peptides from hot acid extracts of the parathyroids. It is, for example, not possible to conclude in retrospect which peptides were present in the material described by Rasmussen and Craig (1962). None of their reported analyses correspond closely to the 1–29 subfragment described here.

The isolation of the biologically active amino-terminal fragment has already proven of great interest in our understanding of the structural features essential for receptor binding and biological activity. This 29-residue fragment constitutes the shortest sequence, derived either from natural sources or prepared by synthesis, that has been shown to have hormonal biological activity.

Shortening of the active region at the amino terminus is not tolerated, as shown both by treatment of native BPTH with exopeptidases (Potts *et al.*, 1968) and by studies of synthetic fragments (Potts *et al.*, 1971b; Tregear *et al.*, 1972). It is not known to what extent further shortening at the carboxyl terminus is tolerated. A minimum size limit is established, however, by the finding that the fragment consisting of residues 1–20 is devoid of biological activity.

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