Acknowledgment

We thank Dr. Ray Iverson and Dr. H. E. Lehman for the courtesies extended us at the University of Miami, Miami, Fla., and Bermuda Biological Station, St. George's West, Bermuda, respectively.

References

Bellamy, A. R., and Ralph, R. K. (1968), *Methods Enzymol. 12B*, 156.

Britten, R. J. (1970), *in* Problems in Biology: RNA in Development, Hanly, E. W., Ed., Salt Lake City, Utah, University of Utah Press, pp 187–216.

Britten, R. J., and Kohne, D. E. (1965), *Carnegie Inst. Wash. Yearb. 64*, 78.

Dawid, I. B., Brown, D. D., and Reeder, R. H. (1970), *J. Mol. Biol. 51*, 341.

Emerson, C. P., and Humphreys, T. (1970), *Develop. Biol. 23*, 86.

Felsenfeld, G., and Hirschman, S. Z. (1965), *J. Mol. Biol. 13*, 407.

Felsenfeld, G., and Sandeen, G. (1962), *J. Mol. Biol. 5*, 587.

Gillespie, C., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.

Glišin, V. R., and Gilšin, M. V. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1548.

Hirschman, S. Z., and Felsenfeld, G. (1966), *J. Mol. Biol. 16*, 347.

Hirschman, S. Z., Gellert, M., Falkow, S., and Felsenfeld, G. (1967), *J. Mol. Biol. 28*, 469.

Hultin, T. (1961), *Exp. Cell Res. 25*, 405.

Inman, R., and Bertani, G. (1969), *J. Mol. Biol. 44*, 533.

Inman, R. B. (1967), *J. Mol. Biol. 28*, 103.

Kirby, K. S., Fox-Carter, E., and Guest, M. (1967), *Biochem. J. 104*, 258.

Loening, U. E. (1970), *in* Organization and Control in Prokaryotic and Eukaryotic Cells, Charles, H. P., and Knight, B. C. J. G., Ed., London, Cambridge University Press, pp 77–106.

Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.

Mirsky, A. E., and Ris, H. (1951), *J. Gen. Physiol. 34*, 451.

Nygaard, A. P., and Hall, B. D. (1963), *Biochem. Biophys. Res. Commun. 12*, 98.

Patterson, J. B., and Stafford, D. W. (1970), *Biochemistry 9*, 1278.

Perry, R. P., Cheng, T., Freed, J. J., Greenberg, J. R., Kelley, D. E., and Tartof, K. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 609.

Schildkraut, C., and Lifson, S. (1965), *Biopolymers 3*, 195.

Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.

Subirana, J. A., and Doty, P. (1966), *Biopolymers 4*, 171.

Sy, J., and McCarty, K. S. (1968), *Biochim. Biophys. Acta 166*, 571.

Wetmur, J. G. (1967), Ph.D. Thesis, California Institute of Technology.

Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31*, 349.

Isolation and Characterization of the Bovine Parathyroid Isohormones*

Henry T. Keutmann,† Gerald D. Aurbach, Bess F. Dawson, Hugh D. Niall, Leonard J. Deftos, and John T. Potts, Jr.

ABSTRACT: Several different molecular forms of bovine parathyroid hormone (BPTH) have been isolated in high yield from extracts of pooled bovine parathyroid glands. Difficulties were previously encountered in the final stages of purification due to the persistence in the purest hormone preparations of several nonhormonal peptides similar in size and charge to the parathyroid polypeptides. These problems have been overcome by the introduction of 8 M urea into the buffers used in the final chromatography step on carboxymethylcellulose. In addition to the predominant form of the hormone (BPTH-I), two other biologically active hormonal peptides (BPTH-II and -III) were also isolated. These three active polypeptides, each containing 84 amino acid residues, all possess amino-terminal alanine and are devoid of cystine. The amino acid compositions of BPTH-I and -II differ only in the presence in BPTH-II of a single threonine residue (BPTH-I lacks threonine) and one less valine residue. BPTH-III also contains threonine; this form of the hormone is the most basic, eluting last from carboxymethylcellulose.

The purification procedure has proven satisfactory for preparation of sufficient BPTH-I to permit the complete structural analysis of the molecule.

The isolation of parathyroid hormone in completely pure, homogeneous form has been the object of extensive investigations over the 40-year period since the first active extracts from bovine parathyroid glands were reported by Collip (1925). These early procedures involved use of hot hydrochloric acid; subsequently it was appreciated that this caused considerable hydrolysis of the polypeptide during the extrac-

* From the Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Metabolic Diseases Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received February 9, 1971.* This work was supported in part by Grants AM-04501 and AM 11794 of the National Institute of Arthritis and Metabolic Diseases.

† To whom to address correspondence.

tion (Aurbach, 1959a). Cold hydrochloric acid (Munson, 1959) and hot 80% acetic acid (Rasmussen, 1957) both represented a considerable improvement but low yields and instability of the hormone were frequently encountered. Recognition of these problems and the introduction of phenol as an extraction medium (Aurbach, 1959a) which provided consistently high yields of relatively stable hormone was a great advance. Later use of urea, cysteine, and cold hydrochloric acid was introduced as an efficient extraction method (Rasmussen *et al.*, 1964).

Further purification of the phenol extracts by either countercurrent distribution (Rasmussen and Craig, 1961) or gel filtration (Rasmussen and Craig, 1962; Aurbach and Potts, 1964) subsequently provided hormone preparations of a purity sufficient for many applications. Persistence in these preparations, however, of a significant quantity of nonhormonal contaminants, as well as of a variety of different polypeptides reported to contain biological activity (Rasmussen *et al.*, 1964; Hawker *et al.*, 1966), prevented the definitive chemical characterization of the hormone.

In 1965 Potts and Aurbach reported, by use of ion-exchange chromatography on carboxymethylcellulose (CMC),[1] isolation of a parathyroid polypeptide thought to be in relatively pure form by the criteria of fluorodinitrobenzene end-group analysis, immunoelectrophoresis, and ultrafiltration. However, disc gel electrophoresis still indicated the presence of several polypeptide bands. Two of the bands were isolated by preparative disc gel electrophoresis (Potts *et al.*, 1966) and shown to be variants of parathyroid hormone that were both biologically and immunologically active and differed in amino acid composition by only a few residues. Not all of the bands could be isolated and it was difficult to obtain sufficient quantities, even by preparative disc gel electrophoresis, to permit further characterization.

During the course of sequence analysis using the phenyl isothiocyanate procedure it was appreciated that even the best preparations of hormone purified by CMC chromatography contained at least two nonhormonal contaminants. That these were entirely different polypeptides and not parathyroid isohormones was established by ten-step Edman degradations which showed minor sequences, contributed by the contaminants, differing at each amino acid position from the parathyroid hormone molecule.

Recently we have investigated the nature of these contaminants and their relation to parathyroid hormone, and undertaken several newer approaches to isolation of the hormone. We considered the possibility that the pattern of coelution of contaminants with hormone from CMC, despite extensive manipulation of gradient elution conditions, indicated a physical–chemical interaction between the different polypeptides.

The result, described in this report, has been the successful separation of the several molecular variants of bovine parathyroid hormone (BPTH) and the isolation of two of them, including the predominant form (BPTH-I) free of nonhormonal contaminants in high yield satisfactory for compositional and structural analysis.

This was accomplished by the use of CMC chromatography in the presence of 8 M urea. This report outlines the methods used in purification and the results of detailed chemical and biological analyses of the purified parathyroid polypeptides.

[1] Abbreviations used are: CMC, carboxymethylcellulose; BPTH, bovine parathyroid hormone; PPTH, porcine parathyroid hormone; NBS, *N*-bromosuccinimide; MRC, Medical Research Council.

Materials and Methods

Hormone Preparations. Partially purified parathyroid hormone (trichloroacetic acid precipitate) (Aurbach, 1959a) obtained from Wilson Laboratories, Chicago, Ill., was used as the starting material for most of the purification work described. For the study of extraction efficiencies during earlier purification stages, acetone-dried parathyroid glands obtained from Wilson were employed. These glands were extracted with 90% (w/v) phenol as described by Aurbach (1959a). Following precipitation of the active extract with acetone and ether, the precipitate was redissolved in 80% acetic acid, additional impurities were removed by precipitation with sodium chloride and the crude PTH precipitated with 3% trichloroacetic acid (Aurbach, 1959a). After resuspension in 0.02 N hydrochloric acid, residual trichloroacetic acid was removed from the preparation by ether extraction followed by treatment with freshly washed IRA-400 acetate resin (Rohm and Haas, Philadelphia, Pa.) (1–2 g of resin/100 ml of aqueous phase, separated afterward by filtration).

Column Chromatography. Gel filtration on Sephadex G-100 (Pharmacia, fine grade) was carried out in 0.14 M ammonium acetate (pH 4.9) at 4° (Aurbach and Potts, 1964). Chromatography on Sephadex G-25 (Pharmacia, fine grade) was performed in 0.01 M acetic acid at 4° (Hawker *et al.*, 1966).

Ion-exchange chromatography on carboxymethylcellulose (Whatman CM-52) was carried out essentially by the method of Potts and Aurbach (1965). The linear gradient was developed using 0.01 M ammonium acetate (pH 5.15, conductivity 0.7 mmho) and 0.33 M ammonium acetate (pH 6.85, conductivity 16 mmhos). For a 2 × 40 cm column, 750 ml of each buffer was used.

CMC chromatography using buffers equilibrated in 8 M urea was carried out over a smaller range of conductivity as described in detail in the Results section.

Desalting of samples, where necessary, was performed using Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) equilibrated with 0.1 M acetic acid. Column effluents were monitored by reading optical density at 280 and 250 mμ with a Beckman DBG spectrophotometer. Buffer conductivity was measured at room temperature on a Radiometer CDM-2e conductivity meter calibrated to read in the range 0–50 mmhos.

In experiments employing hydroxylapatite, the preparation available from Calbiochem was equilibrated with 0.001 M sodium phosphate buffer and centrifuged. BPTH, also dissolved in phosphate buffer (1 mg/ml), was added to the hydroxylapatite (1 g of hydroxylapatite/mg of BPTH). After standing in a 1.5 × 10 cm test tube for 1.5–2 hr, BPTH was eluted off batchwise by increasing concentrations of phosphate buffer (Tiselius *et al.*, 1956).

Assay Procedures. BIOASSAYS. *In vivo* assays were performed based on calcium mobilization using 80-g parathyroidectomized Sprague–Dawley rats as previously described (Aurbach, 1959b). Rats were maintained on a low-calcium diet for 4 days prior to the assay and injected subcutaneously immediately after parathyroidectomy. Blood was obtained 5 hr later and serum calcium values determined by the fluorometric method of Hill (1965). The standard, potency 330 U/mg, was Medical Research Council (MRC) preparation 67/342 (National Institute for Medical Research London, England).

In vitro bioassays employed tests of renal cortical cell adenylylcyclase stimulation. These assays were carried out as previously described by Marcus and Aurbach (1969). [^{32}P]ATP and [^{3}H]AMP were purchased from International Chemical and Nuclear Corp. and from Schwarz BioResearch,

respectively. Tritiated and ^{32}P-labeled compounds were counted by liquid scintillation counting (Packard Tri-Carb) using Bray's solution (Bray, 1960) as scintillator. The standard used was the same MRC preparation used for the *in vivo* assays, or a house standard of 1300 U/mg calibrated against the MRC standard. All *in vitro* assays employed three concentrations of standard and three or four concentrations of unknown within the linear portion of the dose–response curve. *In vitro* and *in vivo* assays were evaluated according to standard methods for parallel-line assays (Gaddum, 1953).

RADIOIMMUNOASSAYS. Radioimmunoassay for parathyroid hormone was carried out by the original method of Berson *et al.* (1963) using ^{125}I-labeled purified parathyroid hormone as tracer, guinea pig 1 antiserum (O'Riordan *et al.*, 1969; Deftos and Potts, 1969) and phase separation by chromato-electrophoresis, charcoal, or double antibody. ^{125}I hormone samples were counted on a Packard Model 3001 Auto-Gamma spectrometer.

Chemical Analyses. EDMAN DEGRADATIONS. Homogeneity of the various BPTH preparations was examined using the manual phenyl isothiocyanate end-group method. These were done using a modification (Niall and Potts, 1970) of the three-stage procedure of Edman (Edman, 1960; Blomback *et al.*, 1966). Phenylthiohydantoins were evaluated quantitatively by gas–liquid chromatography using the Beckman GC-45 gas chromatograph (Pisano and Bronzert, 1969; Niall and Potts, 1970) and qualitatively by thin-layer chromatography in several solvent systems (Edman and Begg, 1967).

AMINO ACID ANALYSES. Acid hydrolysis was carried out in 5.7 N HCl at 110° in an evacuated desiccator as previously described (Keutmann and Potts, 1969); hydrolysis time was routinely 24 hr, except for the definitive compositional studies which were carried out for 24, 48, 72, and 96 hr. All hydrolyses were done in the presence of 1:2000 (v/v) mercaptoethanol to protect methionine residues (Keutmann and Potts, 1969).

Total enzymatic digestion was performed using papain (enzyme:substrate ratio 1:50, pH 5.4, 2 hr, 37°) followed by aminopeptidase M (Rohm and Haas GMBH, Darmstadt, Germany) (enzyme:substrate ratio 1.5:1, pH 8.2, 3 hr, 37°) in order to evaluate content of the labile residues glutamine, asparagine, and tryptophan (Keutmann *et al.*, 1970).

Amino acid analyses were carried out using the Beckman Model 121 automatic amino acid analyzer equipped with the high-sensitivity range card (Hubbard, 1965) and Infotronics Model CRS-12AB digital integrator. Both 2-hr and 4-hr elution schedules with sodium–citrate buffers were used (Hubbard and Kremen, 1965). Glutamine and asparagine were analyzed using the lithium–citrate system as described by Benson *et al.* (1967). Values for serine and threonine were corrected for loss by extrapolation to zero time.

Normalization of amino acids into moles per mole of BPTH was derived from mole fractions by best fit based on recovery of all residues except Thr, Ser, Met, and Tyr, using a computer program developed for use on the SDS Sigma-7 System (Harvard Computer Center, Cambridge, Mass.) (H. T. Keutmann, unpublished data).

PERFORMIC ACID OXIDATION. Performic acid was prepared by adding hydrogen peroxide to formic acid (2:18, v/v) and allowing the mixture to stand at room temperature for 1 hr. The solution was then cooled to 0° and BPTH (1 mg in 1 ml) added. After 2 hr at 0°, five volumes of water was added and the preparation lyophilized.

TITRATION WITH *N*-BROMOSUCCINIMIDE. BPTH was titrated for tryptophan content using *N*-bromosuccinimide (NBS) (Eastman; recrystallized) (Spande and Witkop, 1967). BPTH (2 mg) was added to 2 ml of a 1-mg/ml solution of NBS in 8 M urea, pH 4, 20°, and the change in optical density at 280 mμ read using untreated BPTH as a reference.

EXTINCTION COEFFICIENT. Several samples of purified BPTH were dissolved in 0.1 N acetic acid to a concentration of 1 mg/ml. Optical density was read at 280 mμ on the Beckman DBG spectrophotometer in quartz cuvets with a 1-cm light path; correction was made for any light scattering by subtracting the optical density at 320 mμ. Precisely measured aliquots were then taken from the solutions for acid hydrolysis and amino acid analysis.

IODINE DETERMINATIONS. Determination of total iodine content was carried out on 50 μg of dried, purified BPTH by the method of Benotti and Benotti (1963) (Boston Medical Laboratory, Waltham, Mass.)

DISC GEL ELECTROPHORESIS was carried out at pH 3.6, 25° using a 7% (w/v) acrylamide monomer in the presence of 6 M urea (Potts *et al.*, 1967). Gels were stained using Amido Black (1% in 7% acetic acid).

TABLE I: Recovery of Immunoreactive Parathyroid Hormone from Successive Steps of Purification Procedure.[a]

Step	Efficiency (%)	Cumulative Yield (%)
Phenol extract		(100)
Acetone–ether precipitate	97	97
NaCl supernatant	93	90
Trichloroacetic acid precipitate	88	79
Sephadex G-100	95	75
CMC	81	61

[a] Starting material: phenol extract of 100 g of acetone-dried bovine parathyroid glands.

Results

Extraction of BPTH from Glands. Acetone-dried bovine parathyroid glands (100 g) were extracted by the method of Aurbach (1959a), precipitated with trichloroacetic acid, and carried through gel filtration on Sephadex G-100 (Aurbach and Potts, 1964) and ion-exchange chromatography on CMC (Potts and Aurbach, 1965). In order to evaluate the over-all efficiency of the purification process, yields of hormone recovered at each step were evaluated by radioimmunoassay (Table I). For this purpose repeated quantitative assays were performed at multiple dilutions on an exactly measured aliquot of each fraction that contained hormone during solvent and salt fractionation or an identical aliquot of every tube from the region of elution volume that contained hormone during chromatography on Sephadex or CMC.

At the trichloroacetic acid stage, 280 mg of partially purified hormone had been recovered from the original 100 g of tissue, representing a 70% recovery of immunoassayable hormone. Overall yield after CMC chromatography was 61%. More importantly, the efficiency of recovery at each step was usually greater than 90%; at the CMC step efficiency was 81%.

Gel Filtration on G-100. Aliquots as large as 2 g of trichloroacetic acid precipitated BPTH (trichloroacetic acid–BPTH)

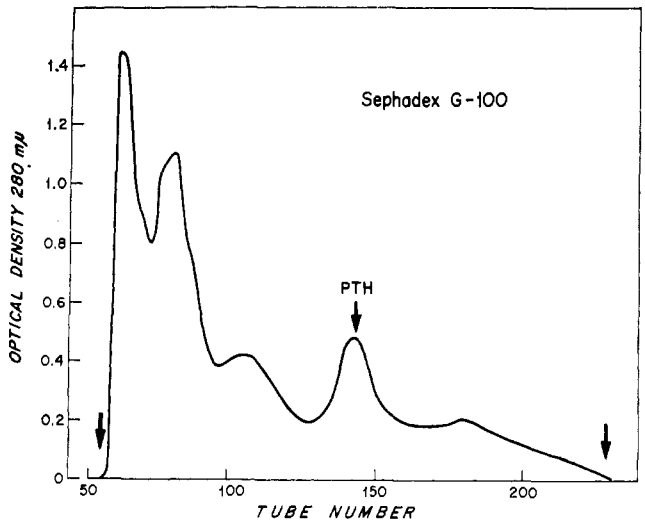

FIGURE 1: Elution pattern obtained from passage of 1 g of trichloroacetic acid–BPTH over a 5 × 130 cm column of Sephadex G-100, using 0.14 M ammonium acetate buffer (pH 4.9). Fraction size was 10 ml.

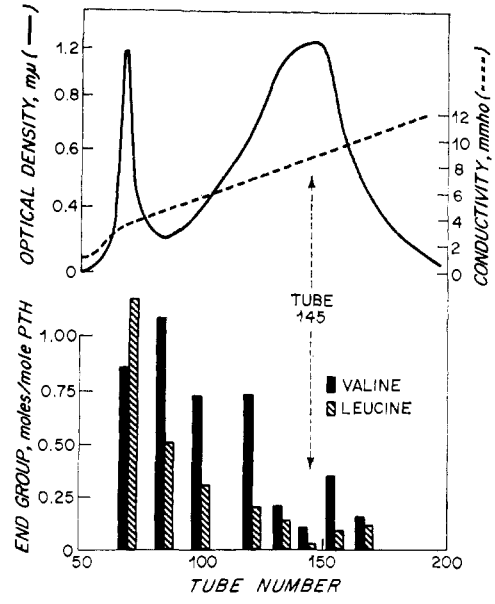

FIGURE 2: Elution pattern of bovine parathyroid hormone from a carboxymethylcellulose column developed with a linear gradient of ammonium acetate buffer as described in the Methods section. Vertical bars represent content of nonhormonal contaminants, as measured by valine and leucine phenylthiohydantoin end groups, in selected aliquots across the peak.

were subjected to gel filtration on a 5.0 × 130 cm column of Sephadex G-100 (fine grade) using 0.14 M ammonium acetate buffer (pH 4.9). A typical elution pattern is shown in Figure 1. BPTH, accounting for 95% of the applied immunological activity (Table I), eluted at a K_d of 0.50; this result was consistent with those reported earlier (Aurbach and Potts, 1964) on the elution of BPTH from G-100 columns.

Since BPTH has been observed to aggregate under certain conditions, especially lyophilization in high salt (Potts and Aurbach, 1965), material from the void volume was incubated in 8 M urea for 2 hr at room temperature and rerun on Sephadex G-100. No additional hormone was found to elute as a result of this treatment.

Amino-terminal end-group analyses by the Edman technique on aliquots from the hormone peak (tubes 130–160, Figure 1) disclosed that, in addition to the expected alanine, phenylthiohydantoin derivatives of valine and leucine were also present, representing contaminating peptides. When evaluated in successive aliquots, these contaminants were found to be broadly distributed all across the peak of hormone; the contaminants represented 20–30% of the polypeptide.

Chromatographic Procedures. Figure 2 illustrates the elution profile obtained by passage of 500 mg of G-100 BPTH over a 2 × 40 cm CMC column (Potts and Aurbach, 1965). The bar graphs indicate the distribution of contaminants as found by end group. BPTH eluted at a conductivity of 8–9 mmhos. Recovery of the applied immunoassayable activity was 80%. Evidence interpreted as indicative of the presence of a larger polypeptide, a "prohormone," in parathyroid gland extracts has recently been presented (Arnaud *et al.*, 1971). The present recovery experiments suggest that such a molecule, if present, must either be present in small amounts, or must react much more weakly in our immunoassay system than the 84-amino acid hormonal polypeptide which we have isolated.

Tube 145 (specific activity 1800 U/mg) from the center of the peak was 91% pure by the end-group determination (Figure 2). Contamination of the hormone, however, increased rapidly in tubes on either side of the center; thus, despite the 80% recovery of immunoassayable activity, the yield even of 90% pure hormone was small. These results indicated that the CMC method, as such, was not a practical final step in the purification of workable amounts of hormone. In numerous further trials, even with a more narrow gradient, it was not possible to obtain hormone free of contaminants. It was not known if the three amino-terminal end groups detected across the elution volumes of polypeptide (Figure 2) reflected the presence only of hormone (alanine end group) and two discrete nonhormonal polypeptides (leucine and valine end groups, respectively) or of a larger number of peptides. This was not pursued further because the important issue was to obtain hormone free of contaminants.

Several approaches to the further purification of the CMC hormone were then undertaken.

The tendency of BPTH to adsorb to Sephadex gels at low ionic strength is well known (Rasmussen and Craig, 1962; Aurbach and Potts, 1964), and was proposed by Hawker *et al.* (1966) as a method for further purification of G-100 hormone. Therefore, 100 mg of CMC–BPTH was applied to a 2 × 130 cm Sephadex G-25 column and eluted with 0.01 N acetic acid. The BPTH eluted sharply near the salt volume (K_d = 0.9) but we did not find any significant improvement in purity when evaluated by end-group analysis.

The adsorbent properties of BPTH were further investigated as a means of purification by use of solid–liquid chromatography on hydroxylapatite. Five milligrams of PTH adsorbed fully to 5 g of hydroxylapatite; stepwise elution with sodium phosphate buffer (pH 6.5) resulted in recovery of about 40% of the hormone at a molarity of 0.075 or higher. Although some improvement was seen in purity, the low yields, poor solubility of BPTH in this pH range, and tendency of the apatite to break down rapidly to smaller soluble (and difficult to remove) forms of calcium phosphate all mitigated against this as a practical method.

Fractionation of Hormone on CMC with Addition of 8 M Urea. The possibility that noncovalent association might

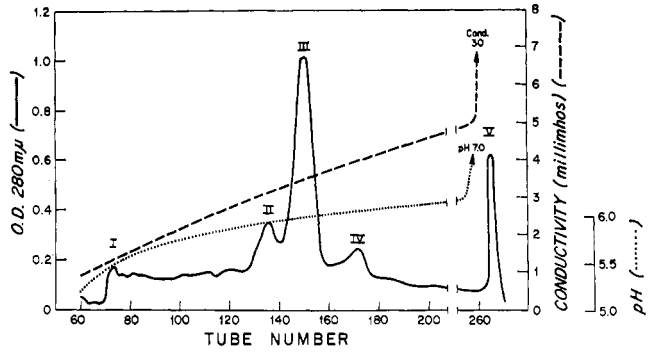

FIGURE 3: Elution pattern of purification of 150 mg of G-100 BPTH on a 2 × 40 cm carboxymethylcellulose column using a linear gradient of ammonium acetate in 8 M urea. Details of the procedure are found in the Results section. Peak III, eluting at a conductivity of 3–4 mmhos, contained the predominant form of the hormone (BPTH-I). The minor variants were found in peak II (BPTH-II) and peak IV (BPTH-III). Fraction size was 6 ml.

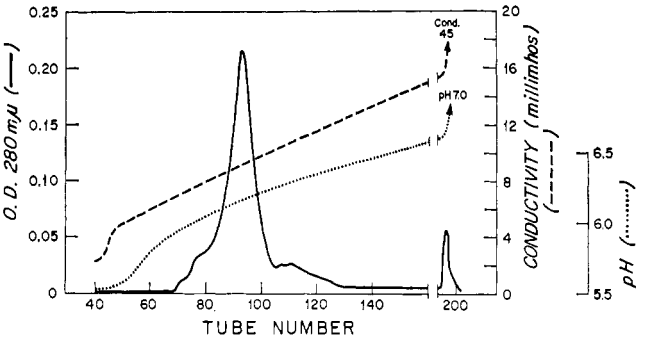

FIGURE 4: Elution of 30 mg of urea–CMC-purified BPTH-I (peak III, Figure 3) from a 2 × 40 cm carboxymethylcellulose column using urea-free ammonium acetate buffer gradient as described in the Results section. Urea, present in the starting material, passed through the column at the outset of the gradient. BPTH, freed from urea, eluted at a conductivity of 8–9 mmhos. Fraction size was 5 ml.

exist between the hormone and the contaminants that co-eluted with hormone from carboxymethylcellulose prompted us to evaluate CMC chromatography with 8 M urea added to the buffers.

All buffers were prepared by adding the appropriate quantities of solid ammonium acetate to a solution of 8 M urea; the urea was deionized beforehand by passage over a 3 × 50 cm column of Rexyn I-300 (Fisher Scientific) to remove cyanate or other ions. A 2.0 × 40 cm CMC column was then equilibrated at 20° with 0.01 M ammonium acetate–8 M urea buffer (pH 4.9, conductivity 0.6 mmho). Lyophilized G-100 BPTH (150 mg) was dissolved in a sufficient volume of the same buffer to ensure a conductivity of 0.65 mmho or less, and was allowed to run onto the column. A linear gradient (Varigrad, Büchler Instrument Corp.) was developed using 750 ml of the 0.01 M starting buffer (pH 4.9, conductivity 0.6 mmho) and 750 ml of 0.33 M ammonium acetate–8 M urea (pH 6.85, conductivity 16 mmhos). It was found that the hormonal peptide eluted much earlier in the linear gradient than when the same gradient was used without urea. Hence the gradient was narrowed and for the second buffer we employed 750 ml of 0.1 M ammonium acetate (pH 5.85, conductivity 6.0 mmhos). At the conclusion of the gradient, 1.0 M ammonium acetate in 8 M urea (pH 6.9, conductivity 35 mmhos) was introduced to clear any remaining protein from the column. The column was run at room temperature throughout, using a gravity flow rate of 30–60 ml/hr.

The elution pattern (Figure 3) was different from that obtained from CMC in the absence of urea. The majority of the polypeptide eluted in a central peak (peak III) at a lower conductivity (3–4 mmhos) than the range observed for urea-free CMC columns (8–9 mmhos). End-group determinations on aliquots of salt and urea-free protein from this peak (III) showed only alanine, indicating purity close to 100%. Preliminary tests by the *in vitro* bioassay indicated that two other peaks (II and IV, Figure 3) also contained considerable hormonal activity.

The large volume of eluate constituting the central peak (III) necessitated the use of ion exchange, rather than gel filtration, as a means of removing the urea from the peptide. The eluate pool of peak III was thus diluted with water to reduce the conductivity to 0.8 mmho or less, then passed over a second 2 × 40 cm CMC column equilibrated with 0.01 M ammonium acetate (pH 4.9, conductivity 0.7 mmho) devoid of urea. The column was then eluted with a linear gradient of ammonium acetate from 0.01 M (pH 4.9, conductivity 0.7 mmho; volume 750 ml) to 0.33 M (pH 6.8, conductivity 16 mmhos; volume 750 ml). The urea passed freely through the column at the outset, and the peptide eluted at a conductivity of 8–10 mmhos, the characteristic elution position occupied by BPTH from CMC columns in the absence of urea (Figure 4).

Aliquots across the peak (tubes 80–103) were examined by Edman end-group analysis. These fractions yielded essentially undetectable (less that 1%) quantities of valine and leucine phenylthiohydantoins (Figure 5). This material had a specific activity of 1800 MRC U/mg by the *in vivo* rat bioassay, and 1500 MRC U/mg by the *in vitro* adenylylcyclase assay.

Duplicate samples of the pooled, purified material were hydrolyzed for 24, 48, 72, and 96 hr and analyzed for amino acid composition. The results are presented in Table II. All amino acids were recovered with good stoichiometry except for threonine, of which 0.15–0.25 residue was consistently observed, and valine which analyzed consistently at 7.5 resi-

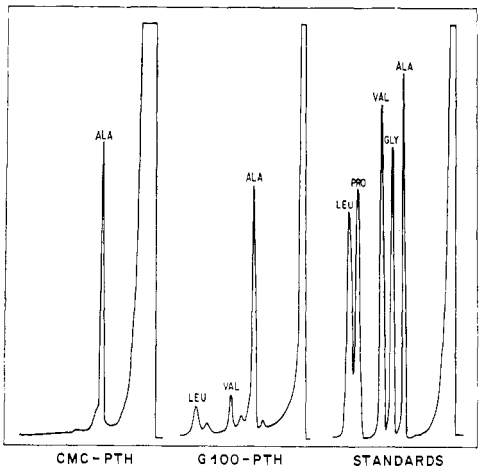

FIGURE 5: Gas–liquid chromatography, over a DC-560 column at 180°, of phenylthiohydantoins from Edman amino-terminal end-group determinations of G-100 BPTH before and after purification on carboxymethylcellulose in 8 M urea. Alanine end group was derived from the BPTH; valine and leucine end groups represented 25–30% contamination by nonhormonal polypeptides, eliminated by the urea–CMC chromatography.

TABLE II: Amino Acid Analysis of Bovine Parathyroid Hormone.[a]

Amino Acid	Total Enzymic Digestion	Timed Acid Hydrolysis (5.7 N HCl, 110°) 24 hr	48 hr	72 hr	96 hr	Residues/ Mole (Combined Results)	BPTH[b]-I	BPTH[b]-II
Asp	6.05	8.80	8.95	8.85	8.90	6	6	6
Asn	3.10					3	3	3
Thr	0.20	0.25	0.20	0.20	0.15	0.3	0	1
Ser	7.55	7.30	6.79	6.23	5.80	8	8	8
Glu	5.95	11.35	11.05	10.85	11.05	6	6	6
Gln	4.63					5	5	5
Pro	1.85	2.00	2.00	2.05	1.85	2	2	2
1/2-Cys[c]	0	0	0	0	0	0	0	0
Gly	4.05	4.05	4.00	4.05	4.05	4	4	4
Ala	7.30	6.95	7.00	7.00	7.05	7	7	7
Val	6.38	7.40	7.50	7.45	7.55	7.5	8	7
Met[c]	1.75	1.60	1.55	1.50	1.40	2	2	2
Ile	3.05	2.85	2.90	2.90	2.85	3	3	3
Leu	8.50	8.25	8.15	8.35	7.80	8	8	8
Tyr	1.20	0.85	0.80	0.80	0.80	1	1	1
Phe	1.85	1.90	1.95	1.80	1.95	2	2	2
Trp[d]	0.90	0.58	0.32	0.28	0.18	1	1	1
Lys	9.00	8.85	8.95	9.00	9.00	9	9	9
His	3.10	3.65	3.90	3.90	3.85	4	4	4
Arg	5.20	5.10	5.00	5.05	5.00	5	5	5
					Total	84	84	84

[a] Timed acid hydrolyses done on duplicate aliquots of BPTH pooled from a single-step CMC–urea chromatography (Figure 3). All values expressed as moles of amino acid per mole of BPTH and normalized as described in text. [b] Compositions from analyses of BPTH-I and -II purified by rechromatography on urea–CMC (Figure 6). [c] Analysis of performic acid oxidized preparation showed no cysteic acid and 2 moles of methionine sulfone per mole of BPTH. [d] 1.08 moles of tryptophan per mole of BPTH by titration with *N*-bromosuccinimide.

dues. In addition, the hormone still showed several bands on disc gel electrophoresis. These lines of evidence prompted closer evaluation of the adjacent smaller but highly active peaks (II and IV) from the original urea–CMC column run.

Isolation and Final Purification of BPTH-I and BPTH-II. Peak II (Figure 3), eluting from the urea–CMC column at a conductivity of 2.5–3.5 mmhos with a biological potency similar to that of peak III, resembled peak III closely in amino acid composition except for presence of larger amounts of threonine. To further eliminate any cross-contamination between peaks III and II, the peak II material was rechromatographed on CMC in the presence of 8 M urea, using a 1.2 × 10 cm column with 100 ml of the same starting and finishing buffers as previously employed. A comparable (10 mg) aliquot of peak III material was similarly rechromatographed. The two elution patterns are shown in Figure 6; the difference in elution positions of the two preparations is again seen. The two products were passed over a second 1.2 × 10 cm CMC column to remove the urea.

The rechromatographed peak III BPTH showed a single sharp band on disc gel electrophoresis, corresponding to the fastest of the multiple bands previously present (Figure 7). The peak II preparation showed a predominant, slightly slower band; although a second faint slow band persisted, it was completely free from the band corresponding to peak III (Figure 7). Both preparations yielded a single alanine phenylthiohydantoin end group. The amino acid composition after acid hydrolysis of each preparation was identical except for presence in the peak II material of a single residue of threonine and one less (7 instead of 8) residue of valine (Table II).

It was concluded that two forms of BPTH had been separated on urea–CMC chromatography. The predominant peak III material was designated BPTH-I, while the threonine-containing variant present in much lower amount was called BPTH-II. The pooled urea–CMC material (Figure 4) subjected to timed acid hydrolysis (Table II) thus contained predominantly BPTH-I with a 25% contamination by BPTH-II, explaining the persistence of submolar quantities of threonine and the 7.5 moles/mole of valine in the hydrolyses.

Bioassay of BPTH-I and-II by *in vitro* adenylylcyclase bioassay gave identical specific activities of 1500 units/mg. The column procedures described here were carried out in the absence of reducing agent, a departure from our earlier purification routines which were performed in the presence of 1:2000 (v/v) mercaptoethanol. We have found that the inevitable accumulation of mercaptoethanol disulfides (resulting from exposure of the reducing agent to alkaline pH) creates spurious optical density peaks interfering with evaluation of the elution profiles. The BPTH obtained from a single cycle of

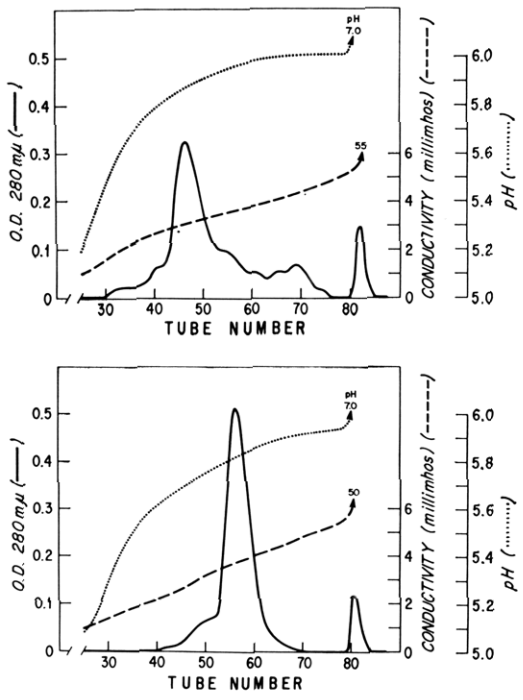

FIGURE 6: Purification of BPTH-II (top) and BPTH-I (bottom) by rechromatography on carboxymethylcellulose in presence of 8 M urea. Details of the procedure are described in the Results section. Pure BPTH-II again elutes earlier than BPTH-I from the column (see Figure 3). Fraction size was 3 ml.

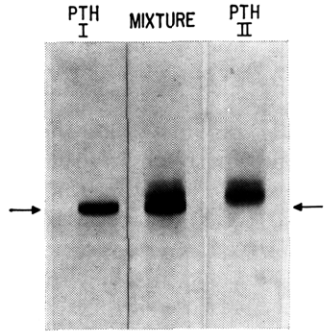

FIGURE 7: Disc gel electrophoresis of BPTH-I and BPTH-II following rechromatography on urea–CMC (Figure 6). BPTH-I (arrow) migrates more rapidly than a corresponding amount of BPTH-II.

urea–CMC purification in the absence of reducing agent (Figures 3 and 4) showed methionine losses, if any, no greater than those previously observed after purification in the presence of mercaptoethanol. However, in some instances rechromatography of BPTH-I or BPTH-II through urea–CMC (Figure 6) did result in the conversion of some methionine to the sulfoxide form with accompanying decrease in biological activity. Full activity could be restored by reduction in 0.12 M cysteine at 80° for 3 hr (Tashjian *et al.*, 1964).

Recovery of BPTH-III from Urea–CMC Column. Peak IV from the urea–CMC column eluate (Figure 3) had a specific activity of 800 U/mg by adenylylcyclase bioassay. When subjected to rechromatography on urea–CMC, this material, designated BPTH-III, again eluted at a higher conductivity than BPTH-I. On disc gel electrophoresis, this peptide migrated more rapidly than either BPTH-I or BPTH-II. By Edman end-group analysis, a single alanine phenylthiohydantoin was detected. It is clear that this peak represents a physicochemically distinct form of BPTH since the biological activity of the preparations is not attributable to contamination by BPTH-I or -II. Its amino acid composition closely resembles BPTH-II; threonine is present. We considered the possibility that BPTH-III eluted differently because of alterations in labile functional groups of one or more amino acids (methionine or amidated amino acids) during fractionation. Analysis by total enzymatic digestion showed no difference in amide content or methionine oxidation state between BPTH-III and the other forms.

Peaks I and V were devoid of biological activity, and their amino acid analyses varied considerably from those of any of the three BPTH variants.

The total yield of active hormone (BPTH-I, -II, and -III), calculated on a weight basis, from the urea–CMC column was 70%, only slightly less than the recovery (based on immunoassay) of hormone achieved after chromatography on CMC without urea (Table I).

Characterization of BPTH-I. Purified parathyroid hormone I contained 84 amino acids (Table II) with a calculated molecular weight of 9563. Tryptophan (1.08 moles/mole) was found after titration with *N*-bromosuccinimide, in agreement with the result obtained after total enzymatic digestion (Table II). A shift in tyrosine absorbance at pH 11 (Potts *et al.*, 1966) was again demonstrated. The molar extinction coefficient at 280 mμ of this homogeneous preparation was 6600 and the extinction for a 1-mg/ml solution was 0.69; these values are in close agreement with that previously reported and are consistent with the presence in the molecule of 1 mole of tryptophan and 1 mole of tyrosine. Analysis for iodine was negative.

Parathyroid hormone I prepared as described above was employed in the complete structural analysis of the molecule (Niall *et al.*, 1970).

Discussion

Previous work with the purification of parathyroid hormone led to the preparation of highly purified hormone and the detection of slightly different forms of biologically active hormone (Potts *et al.*, 1966), but nonhormonal contaminants persisted in hormone preparations despite the use of high-resolution ion-exchange chromatography.

Neither preparative disc gel electrophoresis nor other methods of fractionation proved entirely satisfactory, either because of the difficulty in their use for large-scale purification or because the nonhormonal contaminants could not be completely eliminated.

However, the use, described here, of carboxymethylcellulose ion-exchange columns equilibrated with urea as the final step in purification has been successful in completely removing the nonhormonal contaminants. In addition, this procedure resolved the hormonal material into three biologically active components, found to differ by only minor changes in amino acid composition.

Other workers have found urea + CMC systems useful in separation of various components of insulin (Dillon and Rowans, 1967) and proinsulin (Steiner *et al.*, 1968). The reason for the success of the urea method is not certain. The use of urea might result in disruption of noncovalent interactions between contaminants and the hormone. An alternative possibility is suggested by the observation that the presence of urea alters the elution position of parathyroid hormone from CMC. This could reflect disruption by the urea of the conformation of the molecule, thus altering its effective

charge; this effect could be taking place to a different degree with the contaminating polypeptides, thus altering both their ionic interaction with BPTH and their own elution behavior on the CMC column.

The yield of BPTH from urea–CMC chromatography was comparable to that previously observed with CMC columns run in the absence of urea. Among the three components, the bulk of the material (75%) comprised BPTH-I. BPTH-II (15%) and BPTH-III (10%) were consistently present in much smaller quantities. The occurrence of three forms of bovine hormone could reflect presence of different strains of cattle in the pooled tissue used for preparation of extracts. On the other hand, more than one isohormone could arise from a single gland, obtained from a heterozygous animal.

In using the urea system, certain precautions should be taken in order to minimize potential damage to the hormone from urea breakdown products, particularly cyanates which can react with the free amino group. Presence of cyanates or other ions can be detected by an increase in conductivity of urea solutions after standing for 3–4 days at room temperature. The hormone should thus be exposed to the freshly deionized urea-containing buffer immediately before chromatography, and should be passed over the second CMC column promptly after chromatographic resolutions to remove the urea. When prompt removal is not practical, pools containing urea can be stored frozen at −20° for at least several weeks without obvious loss of activity. Solutions containing 8 M urea cannot be employed at 4°; there does not seem to be significant loss of biological activity when chromatography is conducted at 20°. It is most convenient to employ separate columns (urea and urea free) for the two CMC steps.

Parathyroid hormone I was found on detailed compositional analysis to consist of 84 amino acids. The presence of a single tyrosine and tryptophan residue and two methionine residues, and the complete absence of cysteine or threonine were of special interest. BPTH-II was identical in composition to BPTH-I except for the presence of a single threonine residue and one less valine residue (7 instead of 8). Thus, BPTH-II would appear to be, at least in part, the threonine-rich form of the hormone detected previously by Potts *et al.* (1966). Current evidence from studies on human parathyroid hormone (O'Riordan *et al.*, 1971) suggests that the human molecule may also be relatively threonine rich. On the other hand, porcine parathyroid hormone (PPTH), recently purified and characterized (Woodhead *et al.*, 1971), contains, like BPTH-I, no threonine.

Comparison of the amino acid contents of BPTH-I and porcine PTH reveals several other interesting compositional differences. Although both are 84-amino acid polypeptides, the porcine molecule contains amino-terminal serine, lacks a tyrosine residue, and contains only a single methionine residue. Oxidation of the single methionine to the sulfoxide results in loss of biological activity, as with the bovine hormone.

The precise nature of BPTH-III is currently under further evaluation. Its amino-terminal residue is alanine. The composition closely resembles BPTH-I and -II; at least 1 mole/mole of threonine is present. If there is a compositional difference between BPTH-III and BPTH-II, detailed analysis of amino acid content based on hydrolyses performed for varying periods of time, as done with BPTH-I, may be required to detect such a difference. Alternatively, there may be a sequence change without a difference in overall amino acid composition. For example, the porcine molecule and BPTH-I have identical contents of serine and alanine but the porcine contains a serine–alanine substitution (at the amino terminus). It will therefore be necessary to do complete sequence analysis o each form of the bovine hormone.

Isolation on a large scale of a homogeneous preparation of bovine parathyroid hormone I, free from both nonhormonal contaminants and other variants of the hormone, has already made possible several important advances. The complete structural analysis has been carried out (Niall *et al.*, 1970), and cleavage of the pure native polypeptide by hydrolysis in dilute acid has led to isolation of a biologically active fragment consisting of residues 1–29 (H. T. Keutmann *et al.*, in preparation). These structural studies have led to the preparation by peptide synthesis of a 34-residue amino-terminal fragment possessing the full range of biological activity of the native hormone on both kidney and bone, *in vivo* and *in vitro* (Potts *et al.*, 1971).

All forms of the bovine hormone, as well as porcine and human hormone, are active in rats; bovine hormone has been shown to be active in man. These cross-species tests of biological activity of bovine, porcine, and human parathyroid hormone suggest a common core of structural requirements for biological activity in mammals among naturally occurring forms of the hormone.

Structural evaluation of the other variants of the bovine hormone, BPTH-II and -III, are currently in progress. The differences found in the amino acid sequence of these molecules and of the porcine hormone should serve as guides in studies, currently underway, to define more fully the structural basis for biological potency and immunological activity of parathyroid hormone through synthesis and detailed testing of peptide fragments and analogs of these sequences.

References

Arnaud, C. D., Sizemore, G. W., Oldham, S. B., Fischer, J. A., Tsao, H. S., and Littledike, E. T. (1971), *Amer. J. Med. 50*, 630.

Aurbach, G. D. (1959a), *J. Biol. Chem. 234*, 3179.

Aurbach, G. D. (1959b), *Endocrinology 64*, 296.

Aurbach, G. D., and Potts, J. T., Jr. (1964), *Endocrinology 75*, 290.

Benotti, J., and Benotti, N. (1963), *Clin. Chem. 9*, 408.

Benson, J. V., Jr., Gordon, M. J., and Patterson, J. A. (1967), *Anal. Biochem. 18*, 228.

Berson, S. A., Yalow, R. S., Aurbach, G. D., and Potts, J. T., Jr. (1963), *Proc. Nat. Acad Sci. U. S. 49*, 613.

Blomback, B., Blomback, M., Edman, P., and Hessel, B. (1966), *Biochim. Biophys. Acta 115*, 371.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Collip, J. B. (1925), *J. Biol. Chem. 63*, 395.

Deftos, L. J., and Potts, J. T., Jr. (1969), *Brit. J. Hosp. Med.*, 1813.

Dillon, W. W., and Romans, R. G. (1967), *Can. J. Biochem. 45*, 221.

Edman, P. (1960), *Ann. N. Y. Acad. Sci. 88*, 602.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.

Gaddum, J. H. (1953), *J. Pharm. Pharmacol. 5*, 345.

Hawker, C. D., Glass, J. D., and Rasmussen, H. (1966), *Biochemistry 5*, 344.

Hill, J. B. (1965), *Clin. Chem. 11*, 122.

Hubbard, R. W. (1965), *Biochem. Biophys. Res. Commun. 19*, 679.

Hubbard, R. W., and Kremen, D. M. (1965), *Anal. Biochem. 12*, 593.

Keutmann, H. T., Parsons, J. A., Potts, J. T., Jr., and Schlueter, R. J. (1970), *J. Biol. Chem. 245*, 1491.

Keutmann, H. T., and Potts, J. T., Jr. (1969), *Anal. Biochem. 29*, 175.
Marcus, R., and Aurbach, G. D. (1969), *Endocrinology 85*, 801.
Munson, P. L. (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18*, 291.
Niall, H. D., Keutmann, H. T., Sauer, R., Hogan, M., Dawson, B., Aurbach, G., and Potts, J. T., Jr. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351*, 1586.
Niall, H. D., and Potts, J. T., Jr. (1970), Peptides: Chemistry and Biochemistry, Proceedings of the First Biochemistry Symposium, New York, N. Y., Marcel Dekker, p 215.
O'Riordan, J. L. H., Aurbach, G. D. and Potts, J. T., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 692.
O'Riordan, J. L. H., Potts, J. T., Jr., and Aurbach, G. D. (1971), *Endocrinology* (in press).
Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 24*, 5597.
Potts, J. T., Jr., and Aurbach, G. D. (1965), *in* The Parathyroid Glands: Ultrastructure, Secretion and Function, Gaillard, P. J., Talmage, R. V., and Budy, A. M., Ed., Chicago, Ill., University of Chicago Press, p 53.
Potts, J. T., Jr., Aurbach, G. D., and Sherwood, L. M. (1966), *Recent Progr. Hormone Res. 22*, 101.
Potts, J. T., Jr., Reisfeld, R. A., Hirsch, P. F., Wasthed, A. B., Voelkel, E. F., and Munson, P. L. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 328.
Potts, J. T., Jr., Tregear, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, L. J., Dawson, B. F., Hogan, M. L., and Aurbach, G. D. (1971), *Proc. Nat. Acad. Sci. U. S. 58*, 63.
Rasmussen, H. (1957), *J. Biol. Chem. 229*, 781.
Rasmussen, H., and Craig, L. C. (1961), *J. Biol. Chem. 236*, 759.
Rasmussen, H., and Craig, L. C. (1962), *Biochim. Biophys. Acta 56*, 332.
Rasmussen, H., Sze, Y. L., and Young, R. (1964), *J. Biol. Chem. 239*, 2852.
Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.
Steiner, D. F., Hallund, O., Rubenstein, A., Cho, S., and Bayliss, C. (1968), *Diabetes 17*, 725.
Tashjian, A. H., Ontjes, D. A., and Munson, P. L. (1964), *Biochemistry 3*, 1175.
Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.
Woodhead, J. S., O'Riordan, J. L. H., Keutmann, H. T., Stoltz, M. L., Dawson, B. F., Niall, H. D., Robinson, C. J., and Potts, Jr., J. T. (1971), *Biochemistry 10*, 2787.

Isolation and Chemical Properties of Porcine Parathyroid Hormone*

J. S. Woodhead, J. L. H. O'Riordan,† Henry T. Keutmann, M. L. Stoltz, Bess F. Dawson, Hugh D. Niall, Christopher J. Robinson, and John T. Potts, Jr.

ABSTRACT: Porcine parathyroid hormone was isolated from phenolic extracts of pig parathyroid glands. By solvent and salt fractionation, gel filtration, and ion-exchange chromatography, a preparation that was homogeneous by several criteria was obtained; it contained a single amino-terminal residue.

While the physical properties of the porcine hormone are similar to those of bovine parathyroid hormone, the hormones differ in amino acid composition. The composition of porcine parathyroid hormone is Asp_5,Asn_3,Ser_8,Glu_6,Gln_5,Pro_2,Gly_5,-Ala_6,Val_9,Met_1,Ile_3,Leu_{10},Phe_1,Lys_9,His_5,Arg_5,Trp_1. The calculated molecular weight is 9423.

Prominent among the differences in amino acid composition between the hormone from the two species is the presence in the porcine hormone of serine instead of valine at the amino terminus and the lack of tyrosine and one methionine residue. Despite the lack of tyrosine, the peptide can be labeled with ^{125}I by standard techniques used in radioimmunoassays. Oxidation of the methionine destroys biological activity. The sequence of the first six residues by Edman degradation was found to be Ser-Val-Ser-Glu-Ile-Gln; residues 2–6 were thus identical with those found in bovine parathyroid hormone.

The most extensively studied forms of parathyroid hormone are those of bovine origin. These are single-chain peptides with 84 amino acid residues (Keutmann *et al.*, 1971). The complete covalent structure of the major form of bovine hormone has recently been determined (Niall *et al.*, 1970; Brewer and Ronan, 1970). Human parathyroid hormone has only been isolated in microgram quantities (O'Riordan *et al.*, 1971). Its charge and size properties are similar to those of bovine parathyroid hormone (BPTH)[1] but chemical and immunological differences from bovine parathyroid hormone have been shown (O'Riordan *et al.*, 1969).

* From the Department of Medicine, The Middlesex Hospital, London W-1, England (J. S. W., J. L. H. O'R., and M. L. S.), Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114 (H. T. K., B. F. D., H. D. N., and J. T. P., Jr.), and the National Institute for Medical Research, Mill Hill, London N.W.-7, England (C. J. R.). *Received February 9, 1971.* This work was supported in part by Grants AM-04501 and AM 11794, National Institute of Arthritis and Metabolic Diseases and by a grant to J. L. H. O'R. from the Medical Research Council.

† To whom to address correspondence.

[1] Abbreviations used are: PPTH, porcine parathyroid hormone; BPTH, bovine parathyroid hormone; CMC, carboxymethylcellulose; MCR, Medical Research Council.