Isolation of Human Parathyroid Hormone†

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ABSTRACT: A pure preparation of human parathyroid hormone has been isolated using as starting material 500 g of pooled gland tissue removed at surgery. Following initial extraction of the acetone-dried parathyroid tissue by means of phenol, a trichloroacetic acid precipitate was obtained which contained 4% hormone by radioimmunoassay. Further purification of the initial CCl_3COOH precipitate was achieved by polyacrylamide gel filtration followed by a final step of ion-exchange chromatography. Repeat extractions of the original tissue residue provided an additional yield of hormone, although the hormone content of most of these CCl_3COOH preparations was lower. Additional ion-exchange chromatographic steps were needed for final purification of hormone derived from these repeat tissue extractions. The purified human hormone preparations from the various extractions were, however, found to be identical chemically and immunologically. An overall yield of 3.2 mg of purified hormone suitable for structural studies was obtained, sufficient for sequence analysis of the biologically active amino-terminal portion of the molecule. The biological activity by *in vitro* renal adenylyl cyclase assay is 350 units/mg, considerably lower than that of the hormone from the bovine species. The amino acid composition of human parathyroid hormone bears considerable resemblance to that of bovine and of porcine hormone. There are, however, a number of compositional differences which have not been accounted for in the sequence of the amino-terminal region. This indicates that when structural studies of the carboxyl-terminal portion are undertaken, they will reveal several sequence positions which are unique to the human hormone.

Evidence from several laboratories suggests that the state of parathyroid hormone in the circulation, especially in certain disease states, may be very complex (Habener *et al.*, 1971; Canterbury and Reiss, 1972; Segre *et al.*, 1972; Goldsmith *et al.*, 1973; Silverman and Yalow, 1973). Application of the radioimmunossay to measurement of parathyroid hormone in the peripheral circulation has demonstrated the presence of multiple fragments in addition to the intact hormone. The possible contribution of large precursor forms (Cohn *et al.*, 1972; Habener *et al.*, 1972) to the spectrum of immunoreactive hormone may further complicate this picture.

Attempts to clarify the nature of circulating human hormone and to ascertain the significance of the various hormonal fragments have thus far relied principally on radioimmunoassay systems and structure–function studies based on the bovine and porcine hormones. These problems have emphasized the need for knowledge of the chemical structure of human parathyroid hormone (HPTH).[1]

The structural analyses previously carried out on the hormone from bovine (Brewer and Ronan, 1970; Niall *et al.*, 1970) and porcine (O'Riordan *et al.*, 1971a) species benefitted from the availability of gland tissue obtained from slaughterhouse sources. The study of human parathyroid hormone (Arnaud *et al.*, 1970; O'Riordan *et al.*, 1971b) has been limited by the extremely small quantities of available starting material: parathyroid tissue removed at surgery. Using this source, O'Riordan *et al.* (1971b) isolated 0.5 mg of purified hormone, sufficient for preliminary immunological and chemical characterization. It was observed, however, that throughout the isolation procedure the recoveries of hormone were considerably lower than those obtained during isolation of bovine or porcine hormone. These problems have stressed the need not only for efficient extraction and purification procedures, but also for the application of precise and economical methods for monitoring recoveries and purity throughout the isolation.

Recently, accumulation of sufficient quantities of human parathyroid tissue has permitted the resumption of efforts to characterize the human hormone, and, as a result, sequence analysis of the biologically active amino-terminal region has been reported by two groups of investigators (Brewer *et al.*, 1972; Niall *et al.*, 1974). To be described here is our procedure for the extraction and purification of hormone from 500 g of human gland tissue, using several modifications designed to improve recoveries. In the course of the work, compositional analyses of the hormone and an assessment of its biological activity have also been carried out.

The purified hormone obtained was sufficient for sequence determination of the amino-terminal 37 residues of the molecule (Niall *et al.*, 1974). Since the structural analysis of the entire carboxyl-terminal portion remains to be done, the information described in this report should be useful in planning and carrying out future hormone purifications when sufficient tissue again becomes available.

Materials and Methods

Parathyroid Tissue. The human parathyroid tissue used consisted predominantly of adenomas but also included hyperplastic tissue from patients with primary and secondary

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

hyperparathyroidism. This material was obtained through the cooperation of a large number of medical centers in the United Kingdom, United States, and several European countries. The tissue was frozen on dry ice immediately upon surgical removal, and stored at −20° or below until used.

Extraction Procedure. The procedure followed for extraction of a crude hormone preparation from the pooled gland tissue is outlined in Figure 1. The tissue was first homogenized in acetone at −20° and filtered. The residue was defatted by homogenization in hexane, followed by filtration and washing with acetone, all at −20°. The resulting powder (AP, Figure 1) was kept overnight in an evacuated desiccator, and processed into a trichloroacetic acid precipitate (CCl_3COOH–HPTH) after extraction with phenol, by means of a procedure derived from that described originally by Aurbach (1959).

The acetone powder was suspended in 1 l. of 90% phenol containing 0.5% 2-mercaptoethanol. The suspension was diluted out with 5 l. of 20% acetic acid in acetone, 13 ml of 4 M sodium chloride was added and, after standing for 1.5 hr, the suspension was filtered in the presence of Celite.

The residue was saved and ultimately subjected to repeat extractions, as outlined in the Results section and in Figure 1. The filtrate was treated by gradual addition of ether and collection of the resulting precipitate (AE, Figure 1). The ether powder was resuspended in glacial acetic acid in the presence of cysteine-HCl, diluted with water, and centrifuged. The precipitate was saved for reprocessing later while the supernatant was made to 6% (w/v) with sodium chloride.

The precipitate formed upon addition of the salt was separated by centrifugation, resuspended, and again treated with sodium chloride. The combined salt supernatants (AS, Figure 1) were made to 4% in trichloroacetic acid and the resulting suspension was centrifuged. The CCl_3COOH precipitate (AT, Figure 1) was resuspended in acetic acid and the CCl_3COOH removed by addition of IRA-400 acetate resin (Rohm and Haas, Darmstadt, Germany). When the solution had completely cleared, it was passed through a 1.5 × 20 cm column of IRA-400 and the eluate was lyophilized.

Measured aliquots were removed at successive stages of the extraction (AE, AS, AT; Figure 1) for radioimmunoassay, and a weighed aliquot of the acetone powder starting material (AP, Figure 1) was also retained for assays to assess overall recovery.

A 20-mg aliquot of CCl_3COOH precipitate from the initial extraction (AT-1) was also retained for calibration against the purified hormone, for use as a future radioimmunoassay standard for HPTH.

Gel Filtration. A 2.5 × 130 cm column of Bio-Gel P-100 (Bio-Rad, Riverside, Calif.) was equilibrated with 0.14 M ammonium acetate (pH 4.9) at 4°. The column was presaturated by application of 500 mg of a CCl_3COOH precipitate of bovine parathyroid hormone (BPTH) containing about 50 mg of hormone. The BPTH was eluted with the same buffer and then 2 l. (four column volumes) of buffer was allowed to pass through the column before application of any HPTH.

A flow rate of 20 ml/hr was used in all runs. Optical density of eluate tubes was read at 250 and 280 mμ using the Beckman DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif.). Elution of hormone was defined by radioimmunoassay, and the appropriate tubes pooled and lyophilized twice.

Ion-exchange chromatography was carried out using a 0.9 × 10 cm column of carboxymethylcellulose (CMC) (Whatman CM-52; Reeve-Angel, Inc., Clifton, N. J.). Prior to use for the human hormone, the CMC column was presaturated by

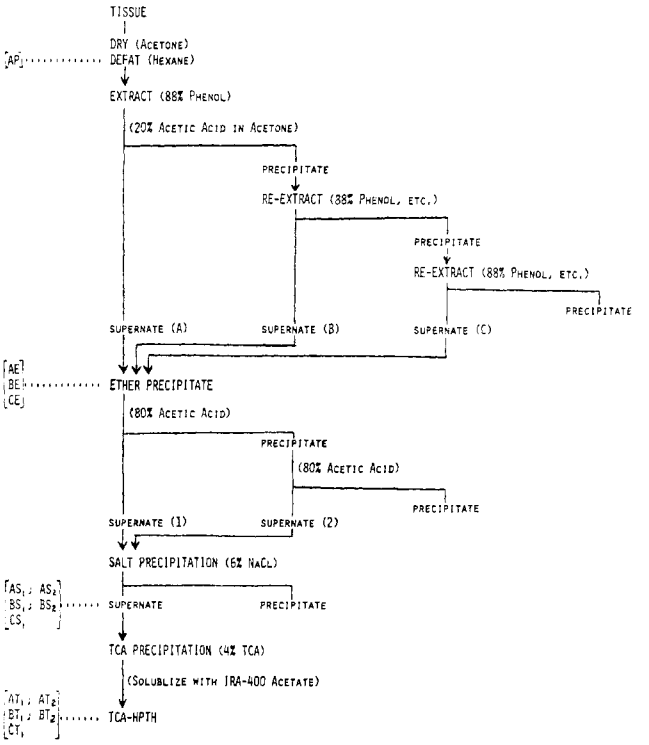

FIGURE 1: Outline of procedure for extraction of parathyroid hormone from pooled human gland tissue. Code designations for various reextracts of the phenol residue and ether powder (described in the text) appear along left margin.

chromatography of 20 mg of partially purified (Sephadex grade) bovine parathyroid hormone. After elution of the BPTH with high ionic strength buffer, the column was exhaustively reequilibrated with the starting buffer, 0.01 M ammonium acetate (pH 4.9, conductivity 0.6 mmho). The lyophilized human hormone pool from P-100 chromatography was applied in the same buffer, and a linear gradient of increasing conductivity established at 4° by means of a Varigrad gradient maker (Buchler Instruments, Fort Lee, N. J.), using 0.2 M ammonium acetate (pH 6.0, conductivity 12.5 mmhos) as second buffer. The most satisfactory linear gradient was achieved by use of two chambers (each 90 ml) of starting buffer and a third chamber (also 90 ml) of second buffer. Following completion of the gradient, 1 M ammonium acetate (pH 6.5, conductivity 30 mmhos) was allowed to run through the column. All conductivity measurements were taken at 20° using the Radiometer (Copenhagen) Model CDM-2e conductivity meter.

For CMC chromatography employing buffers in 8 M urea, the buffer constituents were added to the freshly deionized urea immediately before chromatography, which was carried out at room temperature. Column dimensions and buffer parameters were otherwise the same as described above.

Radioimmunoassays. Recoveries of hormone at the initial stages of extraction were assessed by use of the immunoradiometric assay as described by Addison *et al.* (1971) using ^{125}I-labeled antibodies extracted from guinea pig 199, an anti-bovine antiserum (O'Riordan *et al.*, 1972). The reference preparation was a CCl_3COOH precipitate prepared earlier (O'Riordan *et al.*, 1971b), designated HT-67. Radioiodine was purchased from Amersham Ltd. (Amersham, England).

The hormone preparations AE, AS, and AT (Figure 1) were diluted and added directly to the assay. Aliquots of the acetone powder starting material (AP, Figure 1), in amounts of 10–30

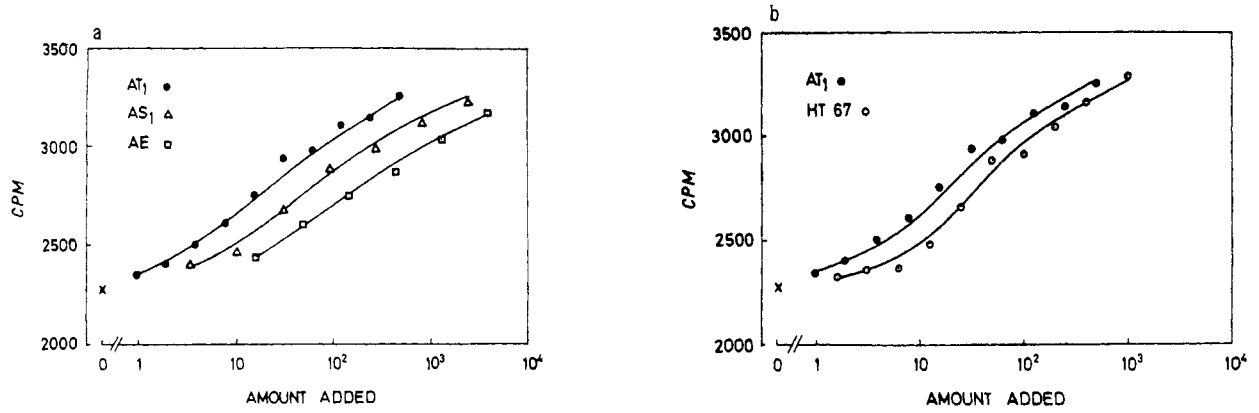

FIGURE 2: (a) Comparison of response curves for human parathyroid hormone aliquotted from successive stages of initial extraction, in an immunoradiometric assay using ^{125}I-labeled GP-199 antiserum (Addison *et al.*, 1971): AE, ether powder; AS_1, salt supernatant; AT_1, CCl_3-COOH precipitate. Ordinate represents counts of hormone-bound, labeled antibody remaining in supernatant after precipitation of unbound antibody with solid-phase immunadsorbant. Abscissa represents increasing volume aliquots of the respective extracts added into the assay. (b) Comparison of immunoassay response curves for CCl_3COOH–HPTH preparation AT-1 from current purification and CCl_3COOH–HPTH preparation HT-67 previously prepared by O'Riordan *et al.* (1971b). Assay conditions and coordinates are as described for part a.

mg, were extracted either with 20% acetic acid in 8 M urea or with 90% phenol (2 ml, 18 hr, room temperature for either method) prior to immunoassay.

Recovery studies during the steps of purification subsequent to the CCl_3COOH stage were carried out using a modification (Segre *et al.*, 1972) of the standard radioimmunoassay as developed for bovine parathyroid hormone by Berson *et al.* (1963). Guinea pig 1 antiserum, also an anti-bovine preparation (O'Riordan *et al.*, 1969) was used at a dilution of 1 : 300,000. The same CCl_3COOH–HPTH standard (HT-67) was used as a reference in these assays. ^{125}I for labeling of purified BPTH tracer was obtained from New England Nuclear (Wilmington, Mass.).

Scanning of the column profiles for location of eluted hormone was performed using a shortened assay procedure. Guinea pig 1 antiserum was used at a dilution of 1 : 50,000, enabling incubations to be carried out overnight instead of the usual 3 days. In these assays 20-μl aliquots from selected tubes were assayed in three successive 50-fold dilutions.

Edman Degradations. Chemical purity of the hormone following P-100 and CMC chromatography was evaluated using the phenyl isothiocyanate procedure of Edman (1960). Most degradations were carried out by the automated procedure (Edman and Begg, 1967); aliquots containing 3–5 nmol of peptide were subjected to several steps of degradation in the Beckman Sequencer, Model 201 (Beckman Instruments, Palo Alto, Calif.) using [^{35}S]phenyl isothiocyanate (Amersham-Searle, Arlington Heights, Ill.) to improve sensitivity of detection (Jacobs *et al.*, 1973). Manual degradations were carried out using previously described techniques (Niall and Potts, 1970). Phenylthiohydantoins were identified by thin-layer chromatography accompanied by autoradiography (Edman and Begg, 1967; Jacobs *et al.*, 1973) and by gas–liquid chromatography (Pisano and Bronzert, 1969).

Amino Acid Analyses. Amino acid analysis was carried out both to provide compositional information and to measure protein content of the purified preparations for use in standardizing the radioimmunoassays and biological assays. All hydrolyses were performed using 5.7 N HCl containing 1 : 2000 (v/v) mercaptoethanol (Keutmann and Potts, 1969). Analyses were carried out using the Beckman Model 121 automatic amino acid analyzer. Amino acids were normalized from mole fractions into moles per mole by best fit based upon recovery of all stable residues.

Bioassays. The *in vitro* potency of the CMC-purified human hormone was assessed using the rat renal-cortical adenylyl cyclase assay system as described by Marcus and Aurbach (1969). [^{32}P]ATP and [^{3}H]cAMP were purchased from New England Nuclear and Schwarz/Mann, respectively. Medical Research Council (MRC) preparation 72/286 (National Institute for Medical Research, Mill Hill, London, England), a highly purified bovine hormone preparation, was used as reference standard.

TABLE I: Recovery of Immunoassayable Hormone from Extraction of 520 g of Pooled Human Parathyroid Gland Tissue.

Preparation	Dry Weight	Hormone Content
Acetone powder (AP)	96 g	85 mg
CCl_3COOH precipitates		
AT-1	394 mg	16.0 mg
AT-2	160	2.0
BT-1	218	4.5
BT-2	38	0.3
CT-1	124	1.0

Results

Preparation of CCl_3COOH–HPTH. Following drying and defatting, 96 g of acetone powder was obtained from 520 g of pooled tissue. The initial extraction of this preparation yielded 394 mg of CCl_3COOH precipitate (AT-1, Table I) containing 16 mg of hormone by radioimmunoassay.

Immunoassay of the acetone powder itself after small-scale extraction by either urea–acetic acid or phenol showed, however, an immunoreactive hormone content of 80–90 mg (AP, Table I).

Therefore, it was felt that additional quantities of hormone might be obtained by a series of reextractions of the residues from the initial extraction procedure. The tissue residue from the original phenol step was thus extracted and processed a second and third time (Figure 1), yielding an additional 5.5 mg of immunoreactive hormone (BT-1, CT-1; Table I). In addition to these reextractions of the phenol residue, the ether–powder residues from the first two extractions (AE, BE;

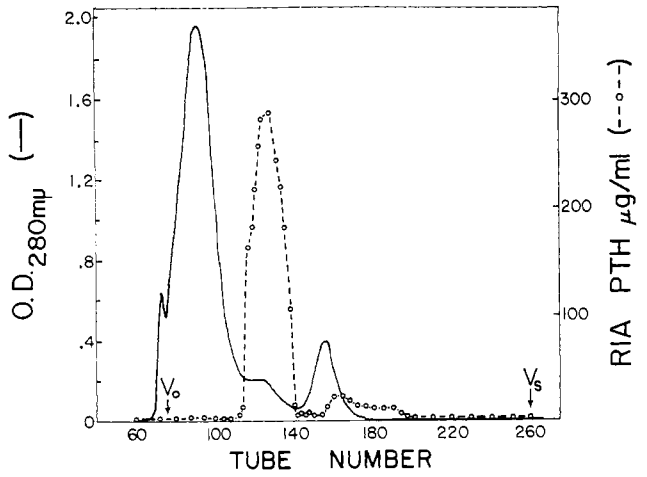

FIGURE 3: Elution profile obtained by passage of 273 mg of CCl_3-COOH–HPTH (AT-1) through a 140 × 2.5 cm column of Bio-Gel P-100 using 0.14 M ammonium acetate buffer (pH 4.9). Fraction size was 2.2 ml. Parathyroid hormone, detected by radioimmunoassay, eluted between tubes 118 and 140.

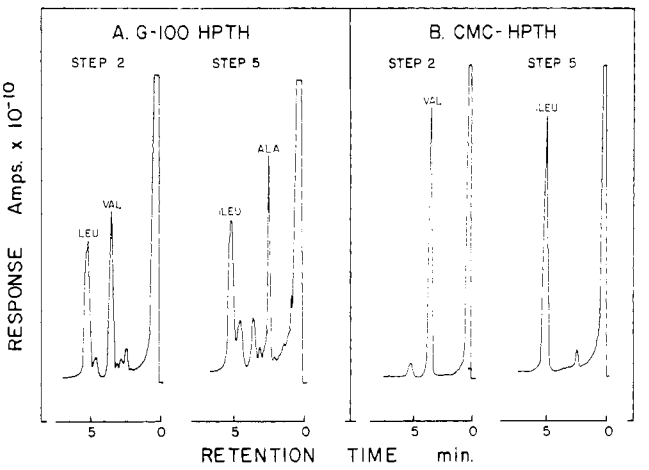

FIGURE 4: Gas–liquid chromatography of phenylthiohydantoins from selected steps of automated Edman degradation of human parathyroid hormone at successive stages of column purification. Valine and isoleucine was derived from the HPTH; the leucine and alanine represented a nonhormonal contaminant present in the P-100 preparation (A) which was eliminated by CMC chromatography (B). The identifications were carried out using a DC-560 column at 180°; isoleucine was confirmed by repeat injection onto an AN-600 column.

Figure 1) were also carried through repeat salt treatment and CCl_3COOH precipitations. This resulted in a further, though modest, enhancement of recovery (AT-2, BT-2; Table I). Overall, 24 mg of immunoassayable parathyroid hormone was recovered at the CCl_3COOH stage; hormone content of the CCl_3COOH precipitates ranged from 4 (AT-1) to 1.5% (BT-2).

The slope of the displacement curves for the various preparations at the successive steps of extraction were identical with one another (Figure 2a) and with the HPTH standard (HT-67) prepared from the earlier extracts of O'Riordan *et al.* (1971b) (Figure 2b). Nevertheless, the CCl_3COOH preparations from the various extracts were kept separate as far as was practical through the subsequent stages of purification.

Column Purification. A series of column purifications was performed using successive steps of gel filtration and ion-exchange chromatography. For simplicity the main purification procedure (CCl_3COOH preparation AT-1) is described in detail, and this is followed by consideration of the pertinent features of the purification of the CCl_3COOH preparations derived from the various reextractions.

Purification of HPTH from CCl_3COOH Preparation AT-1. Figure 3 shows the elution profile obtained from P-100 gel filtration of 273 mg of CCl_3COOH preparation AT-1. The peak of immunological activity corresponded to a K_d of 0.3, an elution position similar to that observed for bovine parathyroid hormone using the same type of column, and somewhat earlier than that for BPTH using Sephadex G-100 (Keutmann *et al.*, 1971). An aliquot of the pooled peptide from this region (tubes 113–142) was analyzed by automated Edman degradation. Two predominant amino acids were found at each cycle, in essentially equivalent yields (Figure 4A): step 1, Ser, Val; step 2, Val, Leu; step 3, Ser, His; step 4, Glu, Pro; step 5, Ile, Ala.

The pooled P-100 preparation was subjected to ion-exchange chromatography on carboxymethylcellulose; the elution profile is shown in Figure 5. All of the immunoreactive hormone eluted in a single peak at a conductivity of 7–9 mmhos, a position comparable to that observed for chromatography of BPTH in the same system. Aliquots were taken from across the peak (tubes 124, 128, and 130; Figure 5), as well as from selected tubes adjacent to the peak, for automated Edman degradation. The peptide from all tubes within the peak retained the sequence Ser-Val-Ser-Glu-Ile-, while a peptide with the sequence Val-Leu-His-Pro-Ala- was found in the eluate fractions following the peak of immunoreactive hormone. This peptide, devoid of immunoreactivity, was felt to represent a non-hormonal contaminant which was responsible for the double sequence seen in Edman degradation of the earlier P-100 pool.

The hormone fractions were pooled and lyophilized. Purity of the peptide (CMC–HPTH) in this pool was found to be 95% based on multiple steps of Edman degradation (Figure 4B). Identical profiles and results were obtained from P-100 and CMC purification of a second lot (107 mg) of CCl_3COOH preparation AT-1.

Purification of HPTH from Reextract CCl_3COOH Precipitates. A third and fourth Bio-Gel P-100 column run was carried out with the reextract CCl_3COOH precipitates, and the elution profiles were closely similar to those described for the preparation AT-1. However, the purity of the hormone in the respective pooled eluates, again assessed by automated Edman degradation, was found to be lower. In the third P-100 pool, derived principally from preparations BT-1 and

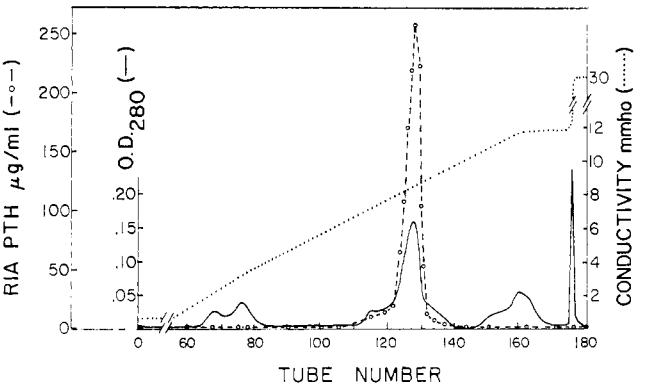

FIGURE 5: Elution profile from chromatography of a P-100 pool of HPTH (Figure 3) on a 10 × 0.9 cm column of carboxymethylcellulose using an ammonium acetate buffer gradient as described in text. Fraction size was 1.8 ml. Hormone eluted between tubes 121 and 131.

TABLE II: *In Vitro* Renal Adenylyl Cyclase Activity of Purified Parathyroid Hormone from Various Species.

Hormone	Act. (MRC Units/mg)[a]
Bovine I	3000 (2500–4000)
Porcine	1000 (850–1250)
Human	350 (275–425)

[a] Activity expressed as mean potency with 95% confidence limits.

BT-2, HPTH represented about 25% of the total peptide while in the fourth pool, comprised principally of preparation CT-1, the per cent HPTH was even lower.

CMC chromatography on a pilot scale of material from one of these reextract pools showed that, while the pattern of eluted immunoreactivity remained the same as that for extract AT-1 (Figure 5), the purity of pooled hormone was, as with the pools from gel filtration, significantly lower. In particular, the principal non-hormonal peptide (Val-Leu-His-Pro-Ala-) was found to overlap the later fractions of the immunoreactive hormone peak.

Therefore, the further purification of the reextract P-100 preparations was modified to include a step of CMC chromatography in 8 M urea. Several additional peaks of optical density were found across the profile, but the immunoreactive hormone again eluted in a single peak, at a conductivity of 4–5 mmhos. The eluate pool from this region was diluted with distilled water (to lower the ionic strength) and reapplied to CMC in order to remove the urea. When a gradient was developed using urea-free buffer, the hormone eluted in a profile again resembling that shown in Figure 5. The hormone in this product was 85–90% pure by end-group analysis. A final passage through CMC, using the same urea-free buffer system, increased the purity to a level comparable to that of the CMC–HPTH from the original AT-1 preparation.

Recovery Estimates. In the purification of the initial CCl_3COOH preparation AT-1, recovery of immunoassayable hormone was found to be similar at each step: 62% from Bio-Gel P-100 and 56% from CMC. Recoveries from P-100 gel filtration of the two reextract CCl_3COOH preparations were somewhat lower: 41 and 45%, respectively. Per cent recovery of these preparations from CMC chromatography was closely similar to the AT-1 material. It was found that use of urea–CMC chromatography was not accompanied by any significant enhancement in recovery. The total yield of highly purified CMC–HPTH, suitable for structural studies, from purification of all the CCl_3COOH preparations was 3.2 mg as calculated from the amino acid analyses.

Biological Assays. Three preparations of CMC–HPTH, quantitated by amino acid analysis, were tested in the renal-cortical adenylyl cyclase system. The assays were found to be statistically homogeneous with a mean potency of 350 MRC units/mg. The relative potencies of human, bovine, and porcine parathyroid hormone in this assay system are compared in Table II.

Amino Acid Composition. The compositional analysis of CMC–HPTH, based on duplicate hydrolysis for 24 and 72 hr, is outlined in Table III. The compositions of BPTH (Keutmann *et al.*, 1971) and PPTH (Woodhead *et al.*, 1971) are also shown for comparison. Results of additional 24-hr hydrolyses of CMC–HPTH, carried out with other preparations for calibration of peptide weight, indicated that all the CMC products (whether derived from AT-1 or from the various reextracts) had the same amino acid compositions.

Discussion

Availability of parathyroid tissue starting material has been the limiting factor in efforts to purify human parathyroid hormone. It was also evident from the earlier experience of O'Riordan *et al.* (1971b) that additional problems could also be anticipated in successfully carrying out the purification of sufficient hormone for structural analysis. In particular, it was found that (a) yields of hormone from column chromatography, particularly Sephadex gel filtration, were low and (b) purity, by radioimmunoassay, of HPTH after CCl_3COOH precipitation and gel filtration was considerably lower than that found at the corresponding stages for bovine hormone.

Accordingly, in the current work modifications were made in the later stages of the purification procedure in an effort to offset these problems. Gel filtration was carried out on polyacrylamide instead of polydextran, since in our experience recoveries from the polyacrylamide are somewhat better, perhaps owing to less adsorption of peptide to the column packing. All columns were presaturated with bovine parathyroid hormone prior to use for the human hormone.

By use of these combined maneuvers, it would appear that considerable improvement in yield, especially at the gel filtration step, was obtained, although recoveries were still lower than those found in the course of purification of larger amounts of bovine hormone (Keutmann *et al.*, 1971). Purity of the hormone after gel filtration, as assessed by end-group analysis, was also greater than the estimate of 10% based on radioimmunoassay, reported previously (O'Riordan *et al.*, 1971b).

Use of the manual Edman end-group procedure (Edman, 1960) in monitoring chemical purity of hormone at successive isolation steps proved to be of great value in our earlier work on a larger scale with BPTH (Keutmann *et al.*, 1971). In the current work, with more limited amounts of material, the automated degradation was effectively employed as a rapid, efficient means of screening aliquots across peaks as well as pooled peptide. Only 3–5 nmol of peptide was required for a degradation of several steps, permitting an accurate computation of the content of hormone *vs.* contaminating peptides. Direct chemical evidence of peptide purity was thereby obtained, eliminating the need to consume material through other types of purity assessment such as electrophoretic or chromatographic procedures. The Edman degradation also was useful in providing assurance that the use of BPTH for presaturation of columns to minimize losses was a safe procedure, since in initial pilot-scale column runs the purified HPTH fractions were found to be free from alanine, the amino-terminal residue of BPTH.

The automated Edman degradations showed that a principal contaminant, devoid of immunoassayable parathyroid hormone activity, had been eliminated by the final step of purification (Figure 4). This contaminant appeared different by amino-terminal sequence from the two contaminating peptides which were encountered in the purification of BPTH (Keutmann *et al.*, 1971). Complete purification of the bovine hormone was accomplished by use of CMC chromatography with buffers containing urea. With the principal P-100 preparations of the human hormone, CMC chromatography in the absence of urea was sufficient to yield a pure product. This may be in part a reflection of the differences in contaminating peptides. Moreover, evidence for isohormonal forms,

TABLE III: Amino Acid Composition of Human Parathyroid Hormone.

Amino Acid	Residues Found (Mol Fraction)		Residues Taken[a]			
	24 hr	72 hr	Mol/Mol of Peptide	Mol Integer	BPTH I	PPTH
Aspartic acid	0.119	0.123	9.80	10	9	8
Threonine[b]	0.017	0.017	1.40	1	0	0
Serine[b]	0.074	0.075	6.10	6	8	8
Glutamic acid	0.118	0.113	9.70	10	11	11
Proline	0.027	0.032	2.30	2	2	2
Glycine	0.056	0.057	4.65	5	4	5
Alanine	0.090	0.092	7.35	7	7	6
Valine	0.093	0.094	7.60	8	8	9
Methionine	0.022	0.015	1.80	2	2	1
Isoleucine	0.012	0.011	0.95	1	3	3
Leucine	0.119	0.116	9.80	10	8	10
Tyrosine	0.002	0.002	0.15	0	1	0
Phenylalanine	0.021	0.020	1.65	2	2	1
Lysine	0.113	0.112	9.30	9	9	9
Histidine	0.058	0.062	4.75	5	4	5
Arginine	0.059	0.059	4.80	5	5	5

[a] The limited quantities of purified peptide available precluded more detailed studies of amino acid composition based on total enzymatic hydrolysis and acid hydrolysis for 24, 48, 72, and 96 hr, to determine the extent of amidation of aspartic and glutamic acid and content of tryptophan, and to more closely examine the content of certain low-yield residues (such as threonine) found to differ in amount from integral values of moles of residue per mole of peptide. [b] Calculated after extrapolation to zero hydrolysis time.

such as those found after urea–CMC chromatography of BPTH (Keutmann *et al.*, 1971), was not found in any of the eluates of the human hormone.

Ion-exchange chromatography in the presence of urea was, however, a useful step in processing P-100 preparations of lower purity, namely, those originating from the tissue re-extractions (Figure 1). Keeping the CCl_3COOH preparations from the initial extraction and from the reextracts separate for subsequent purification is therefore clearly warranted. The final purified products from all of the respective extractions were, however, identical by radioimmunoassay and composition.

While the improved yields from the chromatographic steps were gratifying, assessment of yields of hormone from the earlier extraction stages (Table I) showed that substantial quantities of immunoassayable hormone found in the original acetone powder were not accounted for at the CCl_3COOH stage. Hence, the 394 mg of CCl_3COOH precipitate (AT-1) from the initial extraction contained 16 mg of hormone, compared with 85 mg in the acetone powder starting material. For this reason, the various reextractions of acetone powder residue and ether powder were carried out. These yielded additional amounts of hormone, but the total recovery at the CCl_3COOH stage was still only 30%.

The basis for this apparent loss remains uncertain. The greatest drop in immunoassayable hormone content appeared to occur between the acetone powder and ether powder stages. Little or no loss took place at the salt precipitation stage, but appreciable loss again occurred at the CCl_3COOH step. Relevant to this is the finding that reextraction of the original phenol residue yielded more hormone than did re-extraction of the ether powder (Table I).

Conceivably, some of the immunoreactivity in the acetone powder could represent fragments which are separated out along the way, despite the fact that the displacement curves for the hormone from the successive stages of extraction appeared to be parallel.

Prior to their independent sequence analysis of the active amino-terminal region of the molecule, Brewer *et al.* (1972) purified the human hormone starting with a similar quantity of pooled parathyroid tissue. Their procedure employed chloroform, instead of acetone, for drying and defatting of the tissue, and urea–hydrochloric acid rather than phenol for extraction (Arnaud *et al.*, 1970). Although a detailed description of their purification is as yet unavailable, their overall recovery appears to have been similar to our own.

Substitution of serine for alanine at the amino terminus may contribute to the low activity (350 MRC units/mg) of native HPTH, compared to that of the bovine hormone, when assessed by the *in vitro* renal adenylyl cyclase assay. Earlier studies had demonstrated that porcine parathyroid hormone (PPTH), which also contains an amino-terminal serine residue, likewise shows a lower activity relative to BPTH in the same assay system (Woodhead *et al.*, 1971).

The compositional data obtained in these studies (Table III) point toward significant structural features to be anticipated in analysis of the carboxyl-terminal region of the molecule. Certain similarities to porcine parathyroid hormone may be discerned, including a similar distribution of basic residues, high content of leucine, and absence of tyrosine. However, several differences from both the porcine and bovine molecules are evident as well.

Although the structure for the amino-terminal region proposed by ourselves (Niall *et al.*, 1974) differs in certain respects from that reported by Brewer *et al.* (1972), it is clear from the compositional studies that, regardless of how these discrepancies are resolved, several amino acids unique to the composition of HPTH should be found in the sequence of the carboxyl-terminal portion. For example, threonine and an aspartic acid residue remain to be located. Also, since the single

isoleucine has already been located (position 5), substitutions would be expected at the two positions occupied by residue in the carboxyl terminus of BPTH and PPTH. Presence in the carboxyl-terminal region of several sequence positions unique to HPTH could account for the low cross-reactivity observed with HPTH against a number of carboxyl-terminal directed anti-bovine antisera (Segre *et al.*, 1972).

The purification of human parathyroid hormone has not only made available peptide for structural analysis, but also has provided a standard, calibrated against multiple CMC preparations, which should be very suitable for future immunological studies. The correlations of structure, biological activity and immunoreactivity which should now be possible will represent important advances toward defining the significance of the various forms of the hormone found in the human circulation.

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Added in Proof

Subsequent evaluation of the supernatant fraction from CCl_3COOH precipitation (AT-1, Figure 1) showed that a small quantity of intact hormone (about 10% of that appearing in the precipitate) remained behind after the precipitation. This hormone was retrieved in good yield for further purification by the following procedure. The supernatant was extracted with ether to remove trichloroacetic acid. The content of acetic acid (lowered by the ether extraction) was restored to approximately 20%. The hormone was then extracted into 1-butanol, and the butanol removed by rotary evaporation.

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