Synthesis, Purification, and Chemical Characterization of the Amino-Terminal 1–34 Fragment of Bovine Parathyroid Hormone Synthesized by the Solid-Phase Procedure†

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ABSTRACT: Peptides prepared by solid-phase synthesis during a systematic study of structure–activity relations in parathyroid hormone have been subjected, after sequential purifications, to rigorous analysis of purity by a variety of analytical techniques including sequence analysis by the Edman procedure. The present paper undertakes a critical appraisal of the utility of different tests of peptide purity and the use of the procedures in guiding and monitoring optimal synthesis strategies. Sequence analysis of a peptide representing the amino-terminal 34 residues of bovine parathyroid hormone (bPTH-(1–34)) revealed the presence of at least 30% of contaminating error peptides which were undetected by other analytical procedures. The major contaminant was identified as a peptide in which glutamine at position 29 was deleted. A repeat synthesis using fluorescamine rather than ninhydrin to monitor the coupling reaction resulted in a preparation that lacked the contaminant resulting from deletion. These findings demonstrate the particular value of sequence analysis in the evaluation of purity of peptides synthesized by the solid-phase technique.

The solid-phase procedure for the synthesis of peptides, originally devised and developed by Merrifield (1962, 1963), has been successfully applied to the synthesis of a vast number of biologically interesting compounds (Meienhofer, 1973). The simplicity and convenience of the solid-phase procedure have proven useful in many laboratories in studies designed to elucidate the relationship between peptide structure and biological activity and recently have been used in large-scale semiautomated synthesis of therapeutically useful peptides (Colescott et al., 1975).

Although numerous modifications and improvements in methodology have been described in recent years, a major concern with the solid-phase procedure for peptide synthesis is the heterogeneity of the final product (Meienhofer, 1973). Heterogeneity arises because the various reaction steps in the synthesis fail to reach completion or because the peptide structure rearranges, degrades, or is modified during cleavage of the peptide from the resin and removal of the side-chain protecting groups. The successful synthesis of a peptide by the solid-phase method depends critically on the use of highly resolving purification procedures to separate the desired peptide from the by-products. Even then, a peptide which seems homogeneous after assessment by the conventional procedures of chromatography and electrophoresis may still be contaminated with peptides having relatively minor structural differences, particularly if the changes involve uncharged residues. The presence of an undetected contaminating peptide, differing in structure from the desired product in perhaps only subtle ways, may, however, significantly alter the biological effects of the preparation. This may complicate interpretation of structure–activity studies and may prove harmful in therapeutic use of the compound in medical application.

It follows that much more stringent characterization procedures are required for assessing the purity of peptides produced by the solid-phase technique.

This report describes the application of such techniques of purification and characterization, including total sequence analysis for the assessment of the purity of synthetic peptides prepared by the solid-phase method. The synthetic peptides represent the biologically active amino-terminal 1–34 fragment of bovine parathyroid hormone (bPTH). Sequence analysis has revealed a hitherto-undetected error peptide in our previously reported (Tregear et al., 1973) synthetic bPTH-(1–34) preparation (referred to as synthesis A). Identification of the contaminating peptide has enabled the appropriate modifications in the synthesis procedure to be made to avoid formation of the contaminant.

The details of synthesis techniques and results achieved to test the chemical purity and biological properties of the improved bPTH-(1–34) preparation (synthesis B) are described.

Experimental Procedure

Synthesis A: GPTH-(1–34). The biological properties of bPTH-(1–34) and an outline of the original synthesis procedure have been previously reported (Tregear et al., 1973). A major difference from the procedure used for synthesis B described below was the use of ninhydrin to monitor the completeness of the coupling reaction (Kaiser et al., 1970).

Synthesis B: bPTH-(1–34), bPTH-(2–34), and bPTH-(3–34). Details of the solid-phase synthesis protocol using the Beckman Model 990 Synthesizer and methods for purification of the solvents and reagents have been described elsewhere (Tregear et al., 1974). All reactions and washing steps were carried out in a single solvent (methylene chloride) system using 30% trifluoroacetic acid for α-amino deprotection and

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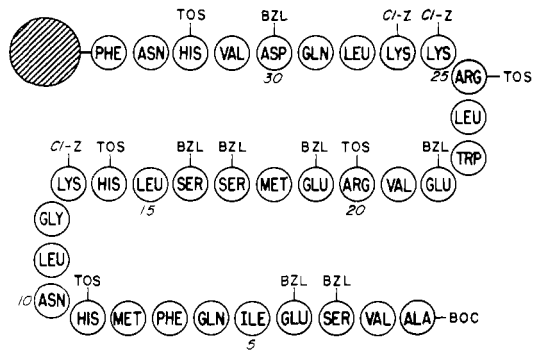

FIGURE 1: The amino acid sequence of bPTH-(1-34) showing the side-chain protecting groups used during the synthesis. The peptide chain was assembled from the phenylalanine, residue 34, attached by its carboxyl group to the solid phase.

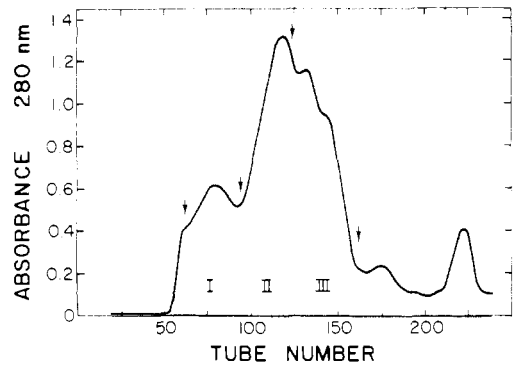

FIGURE 2: Elution pattern of crude synthetic bPTH-(1-34) after gel filtration on Bio-Gel P6 in 1 M acetic acid. Tubes 97-120 (indicated by the arrows) representing the major fraction (II) were combined and lyophilized. Bioassay using the in vitro rat adenylate cyclase assay system indicated that fraction II contained the biologically active peptide. The elution patterns of crude peptide from syntheses A and B were indistinguishable.

10% triethylamine for neutralization. After incorporation of tryptophan into the peptide sequence, 1% mercaptoethanol was added to the trifluoroacetic acid reagent and to the methylene chloride washes following the deprotection step.

The protected amino acids were purchased from Beckman Bioproducts (Palo Alto, Calif.) or Bachem (Marina Del Rey, Calif.) and, with the exception of glycine, were in the L configuration. The Boc[1] group or the Aoc group, in the case of arginine, was used for amino-terminal protection. The side-chain protecting groups used in the synthesis are shown in Figure 1.

The procedures used for esterifying the first amino acid to the resin and for extending the peptide chain have been previously described (Tregear et al., 1973, 1974). Chloromethylated polystyrene resin (Bio-Beads SX-1; Bio-Rad, Richmond, Calif.) with a capacity of 0.69 mequiv of Cl/g was used as the solid-phase and synthesis was commenced with 10 g of Boc-phenylalanine resin (2 mmol of Phe). Coupling reactions were conducted in dichloromethane with 2.5 molar ratios of Boc-amino acid and *N,N'*-dicyclohexylcarbodiimide for each amino acid incorporation, except glutamine and asparagine. These

[1] Abbreviations used are: Boc, tertiary butoxycarbonyl; Aoc, amyloxycarbonyl; TOS, toluene-*p*-sulfonyl or tosyl; BZL, benzyl; Cl-Z, chlorobenzyloxycarbonyl; TPCK, tosylamino-2-phenylethyl chloromethyl ketone; CM, carboxymethyl; bPTH, bovine parathyroid hormone.

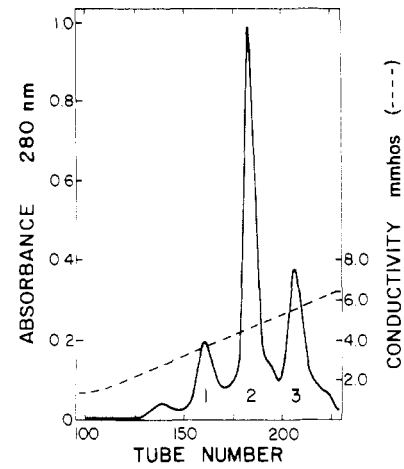

FIGURE 3: Chromatography of synthetic bPTH-(1-34) on CMC using a linear gradient of ammonium acetate in 8 M urea. Tubes 178-190 representing the major peak 2 were combined and applied to a second CMC column (see Figure 4). Peak 2 had the maximum biological activity (see Results and Discussion). Peptide from syntheses A and B gave identical elution profiles.

amino acids were introduced in dimethylformamide as the *p*-nitrophenyl "active esters" in a 5.0 molar ratio and reacted for 4 h (2×). The completeness of the coupling reaction was monitored by the fluorescamine procedure (Felix and Jimenez, 1973), and, where necessary, repeat coupling reactions were performed.

A portion (one-third) of the resin peptide was removed from the reaction vessel after completion of coupling residues number 3, 2, and 1 for the preparation of bPTH-(3-34), bPTH-(2-34), and bPTH-(1-34), respectively.

The completed peptide sequences were cleaved from the resin with simultaneous removal of the side-chain protecting groups by treatment with hydrogen fluoride for 1 h at 0 °C in the presence of anisole using the experimental procedure described by Stewart and Young (1969).

Purification. The synthetic bPTH peptides were purified by the same procedures found to be successful for the purification of the corresponding synthetic human peptides. These experimental conditions have been previously described in detail (Tregear et al., 1974) and involve gel filtration on Bio-Gel P6 (Bio-Rad, Richmond, Calif.) in 1 M acetic acid (see Figure 2) followed by ion-exchange chromatography on carboxymethylcellulose (Whatman CM-52; Reeve-Angel, Clifton, N.J.) in the presence of 8 M urea. The peptides are eluted with a linear gradient using 0.01 M ammonium acetate (pH 5.1, conductivity 2.0 mmho) and 0.1 M ammonium acetate (pH 6.2, conductivity 8.0 mmho) (see Figure 3) and then rechromatographed on CM-52 using a urea-free ammonium acetate buffer, from 1.5 to 15.0 mmho (see Figure 4). Samples were desalted by gel filtration on Bio-Gel P2 equilibrated in 0.1 M acetic acid.

Chemical Characterization. Amino acid analyses following acid hydrolysis and total enzymatic digestion (Keutmann et al., 1970) were carried out using the Beckman Model 121 automatic analyzer.

Homogeneity of the synthetic peptides was checked by thin-layer chromatography (TLC) using the following systems: R_f^a, 1-butanol-acetic acid-water-ethyl acetate, 1:1:1:1, Eastman cellulose 6064 plates; R_f^b, 1-butanol-pyridine-acetic acid-water, 15:10:3:12, cellulose 6064 plates; R_f^c, same solvent system as for b with cellulose 6065 plates; R_f^d with silica gel 6060 plates; R_f^e, ethyl acetate-pyridine-acetic acid-water,

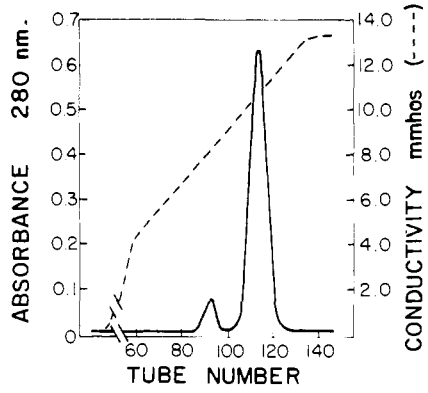

FIGURE 4: Elution of the major peak 2 from the urea-CMC profile (see Figure 3) on a second CMC column using a urea-free ammonium acetate buffer gradient. The urea in the sample passed through the column at the onset of the gradient. Tubes 105-120 were combined and desalted by gel filtration on Bio-Gel P2. As with Figures 2 and 3, the profile obtained for synthesis A peptide was identical to that for the synthesis B product and gave no hint of the presence of a significant contaminant in the final product.

5:5:1:3, silica gel 6060 plates. Thin-layer electrophoresis (TLE) was carried out at 500 V using a Camag (Switzerland) TLE apparatus. The samples were applied to cellulose plates (Eastman 6064) and the electrophoresis was run for 30 to 90 min at pH 2.0 (8% acetic acid-2% formic acid buffer) and at pH 6.5 (pyridine-acetic acid-water, 30:1:270, buffer). For both the TLC and TLE experiments, the sample load was 30 μg in 5 μL of 0.1 M acetic acid. The peptide spots were visualized by spraying the plates with Ehrlich reagent or 0.5% ninhydrin in ethanol.

The tryptophan content of the purified peptides was determined by *N*-bromosuccinimide titration according to the procedure described by Spande and Witkop (1967) and by total enzymatic hydrolysis (Table I).

Edman Sequence Analysis. Sequence analysis of the synthetic peptides (2-mg sample) was carried out in the Beckman Model 890C Automatic Sequencer using the general procedure of Edman and Begg (1967). A variation in this procedure was the use of 2-isothiocyanatonaphthalene-4,8-disulfonic acid for the initial coupling reaction (Braunitzer et al., 1971). This was carried out in a 1 M solution of dimethylallylamine in 1-propanol-water, 3:2 (v/v), pH 9. Braunitzer reagent was used for the initial coupling only, in order to enable the application of the standard protein sequencing program of the automated sequencer to the analysis of these smaller peptides. All subsequent coupling cycles were performed in the usual way using phenyl isothiocyanate, Quadrol coupling buffer, and single-step heptafluorobutyric acid cleavage. The cleaved amino acid derivatives were identified both quantitatively by gas chromatography (Pisano and Bronzert, 1969) and qualitatively by thin-layer chromatography (Edman, 1970).

With this procedure, it was possible to obtain reliable sequence information for residues 1-25. Information on the carboxyl region of the synthetic peptides was obtained by analysis of fragments derived by maleylation and tryptic digestion.

Maleylation and Tryptic Digestion. The synthetic peptide (1.6 mg) was dissolved in water (200 μL) and added to 0.2 M sodium borate buffer, pH 9.1 (1.5 mL). Maleic anhydride (5.5 mg) dissolved in dioxane (100 μL) was slowly added in 10-μL increments over a period of 15 min (Klotz, 1967). The mixture was then applied to a Bio-Gel P2 column (2 × 40 cm) previously equilibrated in 0.2 M ammonium bicarbonate buffer, pH 8.2. The peptide eluting from the column was recovered by lyophilization and redissolved in 0.2 M trimethylamine acetate buffer, pH 8.2 (500 μL). TPCK-trypsin (30 μL of a 2 mg/mL solution) was added and the mixture was incubated at 37 °C for 2 h. The reaction was stopped by lyophilization and the residue was redissolved in water and applied to a Sephadex G-15 (Pharmacia) column (1.2 × 70 cm) equilibrated in 0.01 M acetic acid. The column was monitored by UV absorption at 250 and 280 nm. The peptide fragments 1-20, 21-25, and 26-34 separated well (see Figure 5) and were recovered by lyophilization. Sequence analysis of these fragments was carried out by the manual Edman procedure using an accelerated version of the three-stage procedure (Niall and Potts, 1970).

Bioassays. Biological activity of the synthetic peptides was assessed in vitro by the activation of renal adenylate cyclase (Marcus and Aurbach, 1969) and in vivo by a method (Parsons et al., 1973) based on the increase in serum calcium in the chick after intravenous injection. A house standard of highly purified 1-84 bovine parathyroid hormone (MRC preparation 72/286) calibrated against Medical Research Council Standard A (National Institute for Medical Research, London) was used for both in vivo and in vitro assays.

TABLE I: Amino Acid Composition of Synthetic Bovine Parathyroid Hormone Peptides after Acid Hydrolysis (AH) and Total Enzymatic Digestion (EH).

		bPTH-(1-34)			
		Synthesis A		Synthesis B	
		AH[a]	EH[b]	AH	EH
Asp	1		1.00		1.10
		3.14		3.18	
Asn	2		[c]		
Ser	3	2.86		2.72	
Glu	3		2.83		2.74
		4.92		5.12	
Gln	2				
Gly	1	1.18	1.18	1.05	1.10
Ala	1	1.05	1.16	0.85	1.03
Val	3	2.88	2.30	2.82	2.65
Met	2	1.83	1.68	1.72	1.63
Ile	1	0.95	0.96	0.83	0.82
Leu	4	4.07	3.80	4.32	3.92
Phe	2	1.91	1.92	2.18	2.10
Lys	3	3.08	3.28	3.22	3.30
His	3	2.95	2.88	2.95	2.82
Arg	2	1.99	2.04	1.92	1.92
Trp	1		0.93		0.85

[a] Average of six separate hydrolyses with 5.7 N HCl at 110 °C for 24 h. [b] Average of two separate enzymatic digestions. [c] Ser, Gln, and Asn coelute in this particular analysis system.

Results

The gel filtration and ion-exchange elution patterns of the synthetic bovine 1-34 peptides from syntheses A and B (see Figures 2, 3, and 4) were similar to those obtained for the synthetic human peptide previously published (Tregear et al., 1974). The major peptide peak eluted at a conductivity of 4.4 mmho from the CM-52 column run in the presence of urea and at a conductivity of 10.2 mmho when chromatographed in the absence of urea. There was no significant difference in the elution position of the bovine 1-34 peptide from synthesis A and the 1-34, 2-34, and 3-34 peptides from synthesis B. The yield of pure peptide for both syntheses was in the range of

10–12% of the amount of crude peptide obtained or 5–6% based on the amount of phenylalanine originally esterified to the resin.

The purified bPTH-(1–34) peptides from syntheses A and B behaved identically on thin-layer chromatography. The peptides gave a single, apparently homogeneous spot with the following R_f values: R_f^a 0.66; R_f^b 0.65; R_f^c 0.54; R_f^d 0.38; R_f^e 0.24.

The syntheses A and B 1–34 peptides were also indistinguishable on thin-layer electrophoresis. The purified peptides moved as a single spot in the pH 6.5 buffer system (4.4 cm/30 min) and the pH 2.0 buffer (9.2 cm/30 min).

Both synthetic peptides gave satisfactory amino acid compositions after acid hydrolysis and total enzymatic digestion. There was no significant difference between the peptides from syntheses A and B (Table I). The yield of Glu in synthesis A (4.92), although lower than that of synthesis B (5.12), was not significantly different from theoretical calculations. The amino acid residues obtained after enzymatic digestion also established that there was no detectable contamination of the purified peptides with side-chain-protected amino acids and no evidence of deamidation of asparagine and glutamine residues (Stewart and Young, 1969).

Tryptophan titration gave a value of 1.01 mol of Trp per mol of peptide for both preparations. The UV scan of the peptides gave the characteristic tryptophan absorption pattern with a maximum at 280 mm; 1 tryptophan, mol/mol of peptide, was detected by enzymatic hydrolysis (Table I).

Automated Edman sequence analysis of the purified bPTH-(1–34) peptide from synthesis A gave reliable information through to residue 25. Qualitative identification of the cleaved residues by thin-layer chromatography confirmed that the amino acids had been assembled in the correct sequence. There was no evidence of side-chain-protected amino acids. Because the phenylthiohydantoins present at each cycle of Edman degradation can be quantitated, the proportion of deletion-containing error peptide present can be determined by the detection of a given amino acid residue at one cycle earlier than should be obtained, according to the sequence that was synthesized. Figure 6 illustrates the rationale for interpretation of preview and its cumulative nature in the analysis of failures to complete coupling during synthesis. Quantitation of the deletion at any given point in the sequence is performed in the following manner: for example, at cycle 7, the residue expected is phenylalanine, corresponding to sequence position 7. However, methionine, corresponding to sequence position 8, was also detected in significant yield. The molar ratio of methionine to phenylalanine was 0.09, indicating a 9% preview of methionine, and, hence, the presence of a 9% coupling error in the addition of phenylalanine to methionine. At cycle 11, leucine is the expected residue; however, the molar yield of glycine, corresponding to sequence position 12, to that of leucine was 0.1, i.e., a preview of 10%. Similarly, at cycle 17 the molar ratio of the phenylthiohydantoin of methionine, corresponding to sequence position 18, to that of the serine of sequence position 17 was 0.11, and at cycle 23 the yield of leucine, sequence position 24, to that of tryptophan, sequence position 23, was 0.135. Estimations of preview are best made at cycles of Edman degradation in which the phenylthiohydantoin derivatives of the residues found are stable to quantitative analysis by gas-liquid chromatography. It is not practicable, usually, to attempt estimation at each cycle. In the case of synthesis A, a cumulative preview of 13% over 25 residues corresponds to an average incompleteness of coupling of each residue to the next of 0.5%. The actual deletional error could of course be slightly

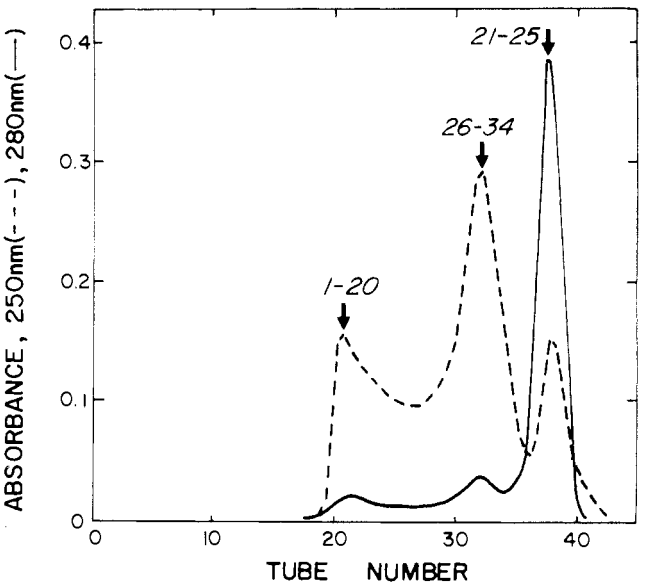

FIGURE 5: The elution profile from Sephadex G-15 of the tryptic digest of maleylated bPTH-(1–34) showing the separation of peptide fragments 1–20, 21–25, and 26–34. The column (1.2 × 70 cm) was equilibrated in 0.1 M acetic acid.

greater quantitatively at particular cycles and lower at others. Manual Edman sequence analysis of the carboxyl-terminal fragment 26–34 from synthesis A revealed a very significant preview of aspartic acid at the glutamine residue 29. Figure 7 (left) shows clearly the preview of valine obtained at residue 30. These results are consistent with the presence of a significant amount (approximately 20%) of an error peptide lacking the glutamine at position 29. When this is taken with the other less substantial deletions which were also demonstrated, it can be estimated that at least 30% of the final product consisted of error peptides.

An automated degradation of purified peptide from synthesis B gave reliable sequence information through to residue 29. As with synthesis A, the amino acids were found to be in the correct sequence and there was no evidence for the presence of side-chain protection. In marked contrast to synthesis A, the level of preview in the synthesis B peptide was very low. For example, at phenylalanine residue 7 there was only a 1.4% preview of methionine, at leucine residue 11 a 1.0% preview of glycine, at serine residue 17 a 4.0% preview of methionine, and at tryptophan residue 23 a preview of only 3.1% leucine was detected compared with 13.5% at the same position in synthesis A. Manual Edman sequence analysis of the carboxyl-terminal region of the peptide showed that at residue 30 the preview of valine was 5% (see Figure 7, right).

The figures for preview are uncorrected for "background" Pth-amino acids (see below and Discussion). Estimations of deletional errors based on preview or premature detection of given residues must be interpreted against a background of small amounts of phenylthiohydantoins of a number of residues which increases as the cycles of degradation increase. These residues reflect the problems encountered in Edman sequence analysis, namely, (1) overlap (incomplete removal of a given residue during a given cycle of degradation, followed by its release of the remaining phenylthiohydantoin residue at the next cycle of degradation), and (2) random cleavages that occur to a very small percentage due to chemical side reactions during the repetitive additions of reagents during the automated Edman procedure; since preview is cumulative, there is no difficulty in distinguishing the latter from the former.

	Peptide Sequence							Content %
Major Peptide	A - B - C - D - E - F - G - H							85
Error Peptides								
- deletion of C	A - B - D - E - F - G - H -							5
- deletion of E	A - B - C - D - F - G - H -							5
- deletion of G	A - B - C - D - E - F - H -							5
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	
Edman Yields %	A 100	B 100	C 95 D 5	D 95 E 5	E 90 F 10	F 90 G 10	G 85 H 15	

FIGURE 6: Edman degradation performed on a peptide ABCD . . . containing minor contaminants resulting from deletion errors gives rise to "preview". At the point in the sequence where deletion has occurred, the amino acid immediately following appears as "preview" in an amount corresponding to the magnitude of the deletion. As the degradation proceeds, the preview resulting from successive deletion errors is cumulative. This amplification mechanism may reveal the presence of a series of error peptides, each of which by itself may be present in only very small amounts (Niall et al., 1972).

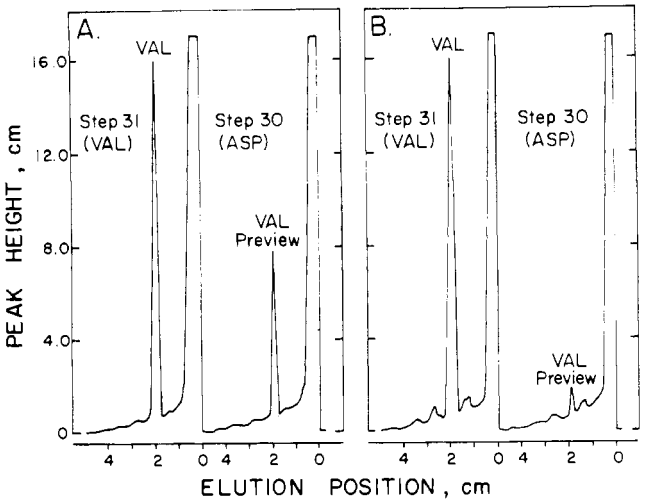

FIGURE 7: The gas-liquid chromatographic tracings of phenylthiohydantoin derivatives of residues obtained from Edman sequence analysis of synthetic bPTH-(1-34). The tracing from synthesis A (left) shows the valine at step 31 and the preview of valine at step 30. The phenylthiohydantoin derivative of aspartic acid does not elute under these particular column conditions and is analyzed separately. In the improved synthesis B (right), the preview of valine at step 30 is considerably reduced. The major early-eluting peak is diphenylthiourea, a side product of the Edman reaction. The smaller peaks seen represent trace quantities of other thiohydantoin amino acid derivatives. The chromatograms shown here were obtained with a Beckman GC-45 gas chromatograph using a 10% DC-560 column, an isothermal injection temperature of 190 °C, and a sensitivity setting of ×800.

Random cleavages plus overlap account for a yield of no more than 1 to 2% of any residue throughout the degradation.

For the peptides from both syntheses, examination of the sequence of the carboxyl-terminal fragments enabled information to be obtained through to residue 33. The presence of the carboxyl-terminal phenylalanine was confirmed by amino acid analysis.

The potency of the synthesis A, bPTH-(1-34) peptide in the in vitro rat adenylyl cyclase assay system, was 5400 (3900-8000) MRC units/mg (mean ± 95% confidence limits). In the in vivo chick hypercalcemic assay, the biological activity of the synthesis A peptide was found to be 7700 (5200 − 11 100) MRC units/mg. For synthesis B, the mean in vitro potency was 6800 (5800 − 7500) MRC units/mg and in the in vivo assay system was 4900 (3500-6900) units/mg.

The bPTH-(2-34) peptide from synthesis B was only weakly active in the in vitro rat adenylyl cyclase assay. As with the corresponding peptide from synthesis A, previously reported (Tregear et al., 1973), it gave a dose-response curve which was nonparallel to the standard. The potency estimated by comparing the activity at half-maximal response was 110 units/mg. The peptide missing the two amino-terminal residues, bPTH-(3-34), was completely devoid of biological activity in both the in vitro cyclase assay and the in vivo chick assay.

Discussion

An important fact that emerges from the experiments reported here is the obvious need for more stringent characterization procedures for evaluating the homogeneity of synthetic peptides in general, and especially those produced by the solid-phase method, for which purification is not undertaken until completion of the synthesis. Use of the conventional procedures used by many workers to date with solid-phase-synthesized peptides would have led to failure to detect the presence of at least 30% contaminating error peptides.

Total Edman sequence analysis has been found to be a very sensitive and convenient procedure for the detection of error peptides, and we suggest that analysis by this technique be an additional, necessary criterion for discussing the purity of peptides synthesized by the solid-phase technique. A preliminary report on the application of the method to the monitoring of solid-phase peptide synthesis has been presented previously (Niall et al., 1972) and the approach has also been utilized recently by Fankhauser et al., (1974).

A preparation of a synthetic bPTH-(1-34) peptide (synthesis A), apparently homogeneous as assessed by amino acid analysis, thin-layer chromatography, and electrophoresis, has been shown by total Edman sequence analysis to contain error sequences amounting to at least 30% of the total. The major contaminant was found to be an error peptide in which the glutamine at position 29 was deleted. This contaminant and the accumulation of minor amounts of other error sequences were effectively eliminated in a repeat synthesis (synthesis B). The use of fluorescamine rather than ninhydrin to monitor the completeness of the coupling reaction in synthesis B makes it easier to detect and therefore correct the deletion. The deletion of glutamine at position 29 apparently arose because the ninhydrin color test was misinterpreted. The ninhydrin color of deprotected aspartic acid at position 28 is brown rather than

blue and it was difficult to distinguish a positive test from the background color of the resin. Use of the fluorescamine test in synthesis B indicated that the coupling reaction of Glu-29 to Asp-28 was very slow and, in fact, required two repeat couplings before a negative test was obtained. The improved sensitivity of fluorescamine as a monitoring procedure in solid-phase synthesis has been previously demonstrated by Felix and Jimenez (1973) and has been convincingly confirmed in the syntheses reported here. It should be noted that several instances arose in which ninhydrin testing was positive when fluorescamine testing proved negative. However, these instances are related to the reaction of ninhydrin with dicyclohexylurea, a side product formed during the coupling reaction which occasionally is difficult to remove completely from the peptide–resin complex despite multiple washing procedures. A positive ninhydrin test under these circumstances does not signify incomplete amino acid coupling.

Sequence analysis of the bPTH-(1–34) peptide obtained from synthesis B clearly showed that the product was largely, if not totally, free from error peptides, in sharp contrast to the corresponding product from synthesis A. a precise value for total content of error peptides is difficult to estimate, since even in a degradation of a totally homogeneous peptide from natural sources, nonspecific cleavage results in the appearance of trace quantities of "background" phenylthiohydantoin-amino acid derivatives. In addition, a documented side reaction in the Edman degradation is premature cleavage, typically seen with histidine residues (Schroeder, 1967). This can lead to a preview in the degradation which would in the case of synthetic peptides be hard to distinguish from that produced by a deletion error. The only comment we can make here is that an increase in preview was not notable as the degradation reached the histidine residues at positions 9, 14, and 32 in the synthetic peptides examined. Nevertheless, the overall effect of several possible side reactions in the Edman degradation would be to increase the apparent extent of preview. Thus, the purity of the synthetic peptide may, therefore, be better even than that indicated by the face value of the preview seen. The conclusion is that it is likely that for synthesis B, deletion errors, if present at all, cumulatively represent only a few percent of the total. These figures demonstrate that the solid-phase procedure is indeed capable of producing quite pure peptides in the 30–35 amino acid range.

The yield of pure peptide was quite low—only 6% based on the amount of the first amino acid esterified to the resin. Although losses during purification account for some of the low yield, there are a number of peptides effectively separated by the purification procedure which are assumed to be peptide fragments or modifications of the desired product. The peptides represented by peaks 1 and 3 from the CM-cellulose–urea purification (see Figure 3) are biologically active albeit less than the major product, peak 2. The biological potency of peak 1 in the in vitro rat cyclase assay is 25% of peak 2, and peak 3 has 60% of the activity of peak 2. The nature of the chemical or physiochemical alterations during synthesis responsible for producing peaks 1 and 3 has not been defined. Extensive studies of the three components, peaks 1, 2 and 3, including amino acid analysis after acid and enzymatic hydrolysis, sequence analysis, thin-layer chromatographic analysis in multiple systems, and peptide mapping after tryptic and chymotryptic digestions, have been performed. The chemical clues so far uncovered are (1) the finding that the components distinguishable on CM-cellulose chromatography at pH 6.5 or electrophoresis at pH 6.5 are indistinguishable on electrophoresis at pH 2, and (2) that the UV absorbance of peak 3 at 280 nm is anomalously high. The molar extinction coefficient at 280 nm for peak 3 is 2.48 vs. 1.75 for peak 1 and 1.62 for peak 2. The former finding suggests the involvement of carboxylic acid functions or side-chain protecting groups. Experiments carried out so far have failed to confirm that peak 3 is composed entirely of glutamic acid–anisole derivatives, as would be suggested by the findings of Sano and Kawanishi (1975) and Feinberg and Merrifield (1975), although a significant portion of the peak 3 material may contain such anisole adducts. These experiments include comparison of glutamic acid content after acid hydrolysis vs. enzymatic digestion (Table I), comparison of molar extinction coefficients of peak 3 vs. peak 2, and detection of anisole adducts during Edman sequence analysis. Further studies to elucidate the nature of these by-products, such as HF cleavage in the presence of [^{14}C]anisole, are currently being carried out.

The improvement in the chemical purity of the synthetic bPTH-(1–34) peptide is not necessarily reflected in increased biological potency. The activity of bPTH-(1–34) from synthesis A, slightly lower than the corresponding product from synthesis B in the in vitro assay, may be significant; however, a higher potency in the in vivo assay was not seen with the peptide from synthesis B. The intrinsic potency of the des-Gln^{29} contaminating peptide in the in vitro and in vivo assay systems and the effect of its addition to a pure bPTH-(1–34) preparation will have to be established in a separate synthesis designed for this purpose.

The activity of 6800 units/mg obtained in the in vitro rat renal adenylyl cyclase assay for synthesis B, bPTH-(1–34), is equal to the biological potency of the full 1–84 native sequence when compared on a molar basis (Tregear et al., 1973). This finding that the synthetic 1–34 is as potent as the native molecule is consistent at least with the interesting thesis that metabolism of parathyroid hormone after release into the circulation could represent an activation process (Segre et al., 1974, Canterbury et al., 1975).

The weak nonparallel biological response of the bPTH-(2–34) and the complete lack of activity for the bPTH-(3–34) peptides obtained from the improved 1–34 synthesis (synthesis B) confirm and validate the conclusions regarding the minimum sequence-length requirement for biological activity previously reported by Tregear et al. (1973). Further studies by Goltzman et al. (1975) with the bPTH-(3–34) have shown that the peptide has, in fact, antagonist properties when tested in the rat renal adenylyl cyclase assay against the native hormone or against the synthetic 1–34 peptide. The finding that bPTH-(3–34) has inhibitory properties illustrates how the presence of contaminating peptides differing in only one or two amino acids could profoundly alter the biological activity of the peptide of interest and highlights the necessity of being aware of such contamination. Once contaminating error sequences have been identified, modifications can then be made in the synthesis procedure to prevent their formation, as removal of small amounts of error peptides from the final product by conventional purification procedures is virtually impossible.

The use of repetitive sequence analysis by the Edman procedure seems especially valuable as a probe of purity of synthetic peptides. The nature of the phenyl isothiocyanate degradation makes it particularly suitable for detection of quite small deletion errors, since these are cumulative, as illustrated in Figure 6. Since most side-chain blocking groups are stable to the conditions of Edman degradation, errors resulting from incomplete deblocking can also in principle be detected by this method. No such errors were found in the present study. Some

categories of error peptide are, of course, not revealed by the Edman technique, for example, those due to racemization, or to premature chain termination where the α-amino group becomes blocked. Our findings suggest, however, that deletion errors are probably much more common in the products of solid-phase synthesis than has been realized, and that their presence can be clearly demonstrated by Edman degradation, which should be used as a powerful addition to other analytical techniques to assess purity of synthetic peptides.

Acknowledgments

We wish to thank Dr. John Parsons of the National Institute for Medical Research, London, for data on the in vivo activity of the synthetic peptides, and Miss Joanne Murray and Mrs. Ellen Arneson for expert technical assistance. Finally, the authors would like to record that the in vitro bioassays in this study were performed by the late Mr. E. N. Callahan with the same care and dedication which he gave to all his work.

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