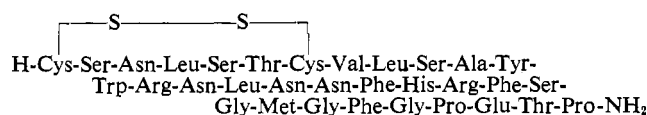


## Degradation and Structure of Porcine Calcitonin-1\*

W. F. Barg, Jr., M. E. Englert, M. C. Davies, D. F. Colucci, E. H. Snedeker, C. Dziobkowski, and P. H. Bell

**ABSTRACT:** Enzymatic and chemical methods were used to determine the sequence of the 32 amino acid residues of porcine calcitonin. The single-chain polypeptide was found to contain a disulfide bridge forming a ring of seven amino acid residues at the N-terminal end of the molecule. The C-terminal amino acid was identified as prolinamide. Enzymatic digestion and chemical degradation of isolated peptide fragments released a single glutamyl residue and four asparagyl residues accounting for all five of the dicarboxylic

acid residues in porcine calcitonin-1. On the basis of these data and from the data of peptide fragments isolated and characterized, the complete structure of porcine calcitonin-1 was derived as:



In an earlier report by Bell (1967) it was shown that acid extracts of porcine thyroid tissue contain three calcitonins. Two of these (PC-1 and PC-2)<sup>1</sup> were obtained in a high state of purity by countercurrent distribution studies and characterized (Bell *et al.*, 1970). A preliminary communication (Bell *et al.*, 1968) reported the complete structure of PC-1. Details of the structural determination of PC-1 are given in this paper.

## Materials and Methods

**Materials.** Trypsin and chymotrypsin (both three-times crystallized and salt free), leucine aminopeptidase, and carboxypeptidases A and B (all DFP treated) were obtained from Worthington Biochemicals Corp.; micrococcal aminopeptidase (10,000 units/mg) from Rohm and Haas (Darmstadt, Germany, supplied by Henley and Co., New York, New York). Ethylenimine and 2-mercaptoethanol were products of Matheson, Coleman & Bell. Other chemicals and their distributors were cyanogen bromide (Eastman Organic Chemicals), 5-dimethylaminonaphthalene-1-sulfonyl chloride (Pierce Chemical Co.), phenyl isothiocyanate (Distillation Products Industries), prolinamide (Cyclo Chemical Corp.). All other chemicals were reagent grade and were used without further purification. Polyamide sheets (15 × 15 cm) were purchased from Gallard-Schlesinger Chemical Corp.

**Starting Material.** Porcine calcitonin-1, isolated by countercurrent distribution or further purified by CM-cellulose chromatography, was used as the starting material for all enzymatic and chemical studies reported. In either case the

starting material was found to have essentially the same amino acid analysis and to contain 32 amino acid residues (Bell *et al.*, 1970).

**Amino Acid Analysis.** Samples of peptides were hydrolyzed for 16–18 hr at 110° with 6 N HCl in sealed, nitrogen-filled tubes. After hydrolysis amino acid content was determined by means of a Technicon AutoAnalyzer as described by Bell *et al.* (1970). Tryptophan was determined by the method of Spies and Chambers (1948) or was enzymatically released and then determined by the AutoAnalyzer. The cysteinyl residues were determined either by first oxidizing to cysteic acid by the method of Moore (1963) or as AE-cysteine after reduction and alkylation using the procedure of Raftery and Cole (1966).

**Digestions with Trypsin and Chymotrypsin.** The sample to be digested was first dissolved in a minimum amount of 0.001 N HCl and then brought to a concentration of about 1 mg/ml in 0.2 M NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0). The amount of enzyme used in each digestion was approximately 2% of the weight of the substrate. Digestion was carried out at 37° for 4 hr with trypsin and 16 hr with chymotrypsin. The resulting digests were acidified to pH 3.0 with 20% acetic acid and lyophilized.

**Digestions with Leucine Aminopeptidase, Carboxypeptidases A and B, or Micrococcal Aminopeptidase.** These enzymes were used primarily to release the glutamic and asparagine residues or to obtain smaller fragments from the various peptides isolated. For this purpose relatively high enzyme to substrate ratios (1:10 on a weight basis) and incubations at 37° for 16–18 hr were employed. All digestions were carried out in 0.2–0.3 M NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0). For LAP digestions the enzyme was first activated for one hr as described by Hill and Smith (1957). Under these conditions endopeptidase activity in LAP, CPA, and CPB was sufficient to split certain peptides internally. The final digestion mixtures were lyophilized, redissolved in water or weak acetic acid, and analyzed for free amino acids and peptides by paper chromatography and electrophoresis or for free amino acids by use of the Technicon AutoAnalyzer.

**Electrophoresis and Chromatography on Paper.** Electrophoresis on Whatmann No. 3MM filter paper (57 × 46 cm)

\* From the Biochemistry Research Department, Experimental Therapeutics Research Section, Lederle Laboratories, Pearl River, New York 10965. Received October 22, 1969.

<sup>1</sup> Abbreviations used: PC-1 and PC-2, porcine calcitonin-1 and -2; LAP, leucine aminopeptidase; MAP, micrococcal aminopeptidase; CPA, carboxypeptidase A; CPB, carboxypeptidase B; tryptic and chymotryptic peptides are designated as T and C, respectively. Peptides derived from CPB digestion were given the symbol B and cysteine-containing peptides alkylated with ethylenimine were given the symbol A. Other abbreviations are: AE, β-aminoethyl; DNS, 1-dimethylaminonaphthalene-5-sulfonyl; PTH, phenylthiohydantoin.

was performed at an initial temperature of 0° at three pH values; at pH 1.8 (4% formic acid), 55 V/cm, 75 min, temperature rise of about 6°; at pH 3.7 (pyridine-acetic acid-water, 1:10:289, v/v), 40 V/cm, 120 min, temperature rise about 4°; at pH 6.5 (pyridine-acetic acid-water, 5:0.2:95, v/v), 40 V/cm, 120 min, temperature rise about 4°.

The procedure for peptide mapping of tryptic and chymotryptic digests was patterned after Katz *et al.* (1959). Chromatography in the X direction, unless otherwise indicated, was in the system 1-butanol-pyridine-acetic acid-H<sub>2</sub>O (15:10:3:12, v/v). Chromatography was followed by electrophoresis at pH 3.7 in the Y direction. The relative positions of each peptide are described as per cent of the movement of the markers, leucine in chromatography and lysine in electrophoresis expressed as X, Y coordinates, respectively.

Certain peptides were also isolated from reaction mixtures and digests by chromatography on Whatmann No. 3MM paper in systems described in the text.

For visualization of amino acids and peptides after electrophoresis and/or chromatography, the sheets were dried at 60°, sprayed with pyridine, air dried, sprayed with 0.2% ninhydrin in 1-butanol saturated with 0.05 M sodium phosphate buffer (pH 7.0), and then heated at 80–85° for 15 min. If the various peptides were to be recovered from the paper after two-dimensional separation, the ninhydrin development was similar except 0.05% ninhydrin was used and the sheet was sprayed only on one side. If resolution was achieved in one dimension, the desired peptide was recovered without ninhydrin treatment by ninhydrin development of a small aliquot of the charge run as a marker. For purposes of recovery, individual peptide areas were cut out, washed with dry acetone if ninhydrin had been used, eluted with 20% acetic acid, and the individual peptides were recovered as lyophilized powders. Recovery of peptides by this procedure was about 30–40% of the initial charge. The N-terminal amino acids in the peptides isolated by the use of ninhydrin generally had low analysis values as would be expected. It is also to be noted that small amounts of amino acids, especially glycine, were eluted from the filter paper itself.

**Chromatography on Dowex 50-X2.** In order to obtain larger amounts of the various peptide fragments of PC-1, tryptic or chymotryptic digests were chromatographed on Dowex 50-X2 by the procedure of Guidotti *et al.* (1962) using pyridine-acetic acid buffers in a stepwise manner. Aliquots of collected fractions were analyzed for peptide content by a modification (Bell *et al.*, 1970) of the alkaline hydrolysis-ninhydrin methods of Hirs *et al.* (1956). Appropriate fractions from these columns were pooled and the peptides were recovered as lyophilized powders.

**Cyanogen Bromide Cleavage and Isolation of CNBr Peptides on CM-cellulose.** Cleavage at the methionine residue of calcitonin was carried out by the method of Gross and Witkop (1962). For this purpose about 5 mg of peptide was dissolved in 3.0 ml. HCl (0.1 N) and CNBr (30 mg) were added. The mixture was stirred until dissolved, allowed to stand at room temperature for 16 hr, diluted with 6 ml of water, lyophilized, redissolved in 1.0 ml of 0.062 M pyridine-acetate (pH 4.4), charged on top of a CM-cellulose column (0.6 × 73 cm), and developed with the gradient buffer system described by Bell *et al.* (1970). The C-terminal fragment, CNBr-2, was recovered in the first hold-back volume. The N-terminal

fragment was eluted behind and separated from intact calcitonin.

**Preparation and Isolation of AE-cysteine-Containing Peptides.** Cysteine-containing peptides (C1 or the peptide containing residues 1–7) partially purified by chromatography on paper or Dowex 50-X2 were treated with ethylenimine according to the procedure of Raftery and Cole (1966). The resulting AE-cysteine-containing peptide was separated from reagent reaction products and from neutral and acidic peptides by paper electrophoresis at pH 1.8.

**Amino Acid Sequence Determination of Isolated Peptides.** The phenylthiohydantoin method for the stepwise degradation of peptides (Edman, 1950) coupled to the fluorescent end-group reagent (DNS-Cl) method of Gray and Hartley (1963) and modified by Nedkov and Genov (1966) was used to determine or verify the sequences of various peptides. Thin-layer chromatography of the resulting DNS derivatives was performed on polyamide sheets as described by Woods and Wang (1967). In some cases the identity of a particular DNS-amino acid was verified by a third-dimensional technique. For this purpose the unknown was run in the conventional two-dimensional manner using the two-solvent systems of Woods and Wang (1967). After a tentative identification of the spot had been made, known DNS derivatives were spotted on the dried sheet adjacent to the unknown. Final identification was achieved by development with a solvent system capable of resolving the possibilities involved. The solvent systems found suitable for these comparisons were CHCl<sub>3</sub>-*t*-amyl alcohol-acetic acid (14:6:0.1, v/v) to resolve DNS-NH<sub>2</sub> from DNS-Ala; CHCl<sub>3</sub>-*t*-amyl alcohol-acetic acid (70:30:3, v/v) to separate DNS-Ser, DNS-Thr, DNS-Glu, and DNS-Asp; *n*-heptane-1-butanol-acetic acid (3:3:1, v/v) for separating DNS-Ser, DNS-Thr, and DNS-O-Tyr.

## Results

Peptide fragments from PC-1 used in the final structure deductions of the hormone are shown in Figure 1. During the course of peptide isolation from enzyme digests, several other peptides in low yield were identified but are not shown in Figure 1.<sup>2</sup> None of these peptides were incompatible with the deduced structure.

**Peptides Isolated from Maps.** Three major peptides were isolated from the trypsin digest maps (T1b, T2, and T3). The amino acid composition of these peptides was not sufficient to account for the entire PC-1 structure. Maps of chymotrypsin digests revealed peptides C2, C3, C4, C5, C6, C7, and C6,7. Amino acid analysis of these peptides failed to account for the same nine residues missing from the trypsin digest map. Characterization studies by Bell *et al.* (1970) had shown cysteine to be the N terminus in PC-1. The missing trypsin peptide T1a contained the two cysteine residues of PC-1 and therefore is N terminal. The absence of an Arg residue in the T1a from the trypsin digest could only be the result of a secondary nontryptic-like split in the peptide designated as T1 in Figure 1. Peptide T1a was eventually shown to be identical with C1 isolated, after alkylation with ethylenimine, as C1-A. Failure to detect the missing

<sup>2</sup> Peptide designations of this figure are not the same as used in the earlier report of Bell *et al.* (1968).

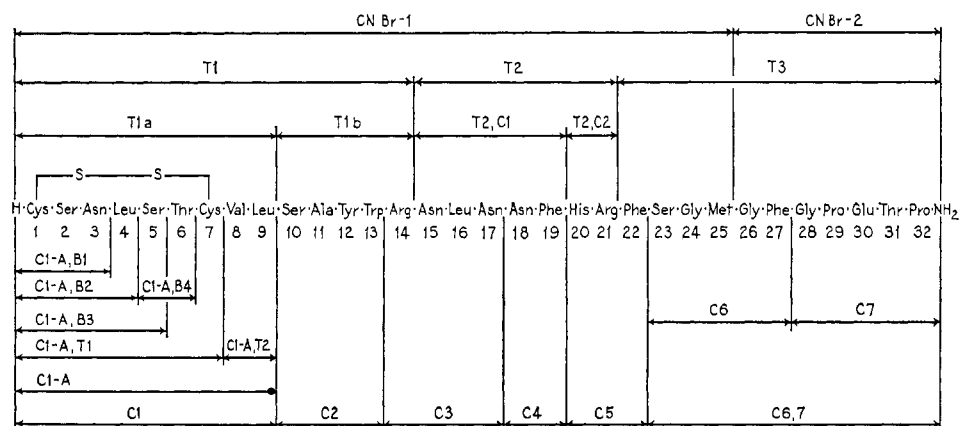


FIGURE 1: Sequences of peptides isolated from enzymatic digests and from cyanogen bromide cleavage of porcine calcitonin-1.

peptides T1a and C1 on maps by the ninhydrin method was apparently due to the low color yield from their N-terminal cysteines. Analysis of extracts of various areas of peptide maps suggested these peptides were in a region with  $X$ ,  $Y$  coordinates of 107, 36. It was deduced from the T and C peptide data that the order of trypsin peptides in PC-1 was T1a-T1b-T2-T3.

**Isolation of Peptides by Dowex 50 Chromatography.** Larger quantities of trypsin peptides were obtained by Dowex 50-X2 chromatography (Figure 2). From this procedure T1b and T2 were obtained in a state of purity suitable for final characterization. Peptide T3 formed a double peak due to partial oxidation of its methionine (see Guidotti *et al.*, 1962). Other peaks proved to be mixtures of peptides. Peptides T2 and T3 were recovered in satisfactory yields, however, T1b was obtained in only about 20% of theory. The analysis of pool 1 showed the presence of over half of the theoretical yield of T1a. The nature of this mixture was not investigated.

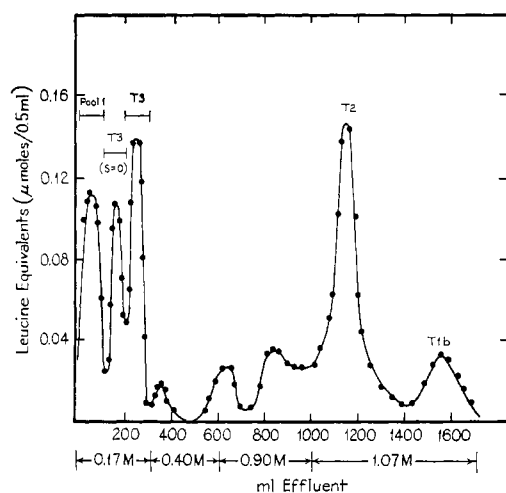


FIGURE 2: Chromatography of a trypsin digest of porcine calcitonin-1 (10 mg of peptide) on a column of Dowex 50-X2 ( $0.6 \times 65$  cm). The flow rate was 12 ml/hr (1–800 ml) and 24 ml/hr (800–1700 ml); 4-ml fractions were collected. Aliquots (0.5-ml) were hydrolyzed by alkali and analyzed by the ninhydrin method.  $S = 0$  indicates peptide T3 containing methionine sulfoxide.

**Dowex 50 chromatography of a chymotrypsin digest of PC-1 is shown in Figure 3.** Peptide C5, obtained in about 25% of theory, proved to be the only peptide obtained pure by this procedure. From the amino acid compositions of the pools, it was clear that the extent of the enzyme cleavages in the large digest used for Dowex 50 chromatography was not the same as the small digest used in the mapping procedure. Pools 1 and 2 contained little or no arginine but more than 60% of the aspartic acid, a fact inconsistent with C3 being a major product and only small amounts of C3 were found in the entire digest. Instead, the arginine of position 14 was found to be divided among at least three peptides in pools 7 and 8. Recoveries of arginine-containing peptides from Dowex 50 columns were low as has been reported by

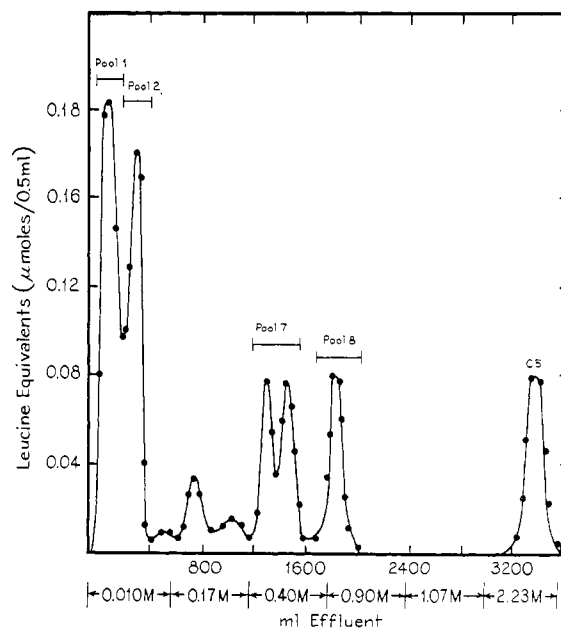


FIGURE 3: Chromatography of chymotryptic digest of porcine calcitonin-1 (28 mg) on a column of Dowex 50-X2 ( $0.6 \times 100$  cm). The flow rate was 24 ml/hr; 8-ml fractions were collected. Aliquots (0.5 ml) were hydrolyzed by alkali and analyzed by the ninhydrin method.

other investigators. Peptide C2 was eventually recovered from pool 7. All of the cysteine and valine were found to be in pools 1 and 2. Aminoethylation of pools 1 and 2 resulted in a mixture from which the AE-cysteine derivative C1-A was easily separated from the neutral peptides present.

*Trypsin-chymotrypsin digestion* was investigated to obtain further confirmation of some of the amino acid sequences deduced from the other data. PC-1 was digested by trypsin for 4 hr at 37° followed by chymotrypsin digestion for an additional 18 hr. In both cases the amount of enzyme used was 2% of the weight of the substrate. After acidification and lyophilization the double digest was resolved by peptide mapping. The data obtained for the isolated peptides (TC peptides in Peptide Summary) was found to be consistent with the structure shown in Figure 1.

*Dansyl-Edman stepwise degradation of PC-1* was carried through nine steps. The sequence obtained for the nine N-terminal amino acids by this procedure agreed with the sequence deduced from isolated peptides. The acid hydrolysis procedure used to release the DNS-amino acid from the DNS peptides led to partial oxidation of the cysteine in steps 1 and 7. Characterization of the oxidized DNS-cysteine derivative appearing on the thin-layer plates was not attempted. An equivalent fluorescent spot was obtained when bis-DNS-cysteine and DNS-oxytocin were subjected to the same conditions. The presence of a cysteinyl residue in positions 1 and 7 of PC-1 was also confirmed by dansyl-Edman studies on peptide (1-7)ox. In this case the cysteines had been first oxidized to cysteic acid and then positively identified as DNS-cysteic acid at positions 1 and 7.

*Peptide Summary.* The preparation, isolation, peptide map coordinates, amino acid analysis, dansyl-Edman degradation, and other important characterization data are given below for each peptide isolated.

#### *Proof of Sequence Peptides*

**T1b:** Ser-Ala-Tyr-Trp-Arg  
**Isolation:** Map ( $X = 92$ ,  $Y = 54$ ; yellow); Dowex 50-X2  
**Analysis:** Ser (1.0), Ala (1.0), Tyr (1.0), Arg (1.1), Trp (1.1); column preparation  
**DNS-Edman:** Ser-Ala-Tyr-

**T2:** Asn-Leu-Asn-Asn-Phe-His-Arg  
**Isolation:** Map ( $X = 54$ ,  $Y = 73$ ; blue-gray); Dowex 50-X2  
**Analysis:** Asx (3.0), Leu (1.0), Phe (1.0), His (0.9), Arg (1.1); column preparation  
**DNS-Edman:** Asx-Leu-Asx-Asx-Phe-  
**LAP:** Asp (0.3), Asn (3.0), Leu (0.9), Phe (0.8), His (1.0), Arg (1.2)

**T2, C1:** Asx-Leu-Asx-Asx-Phe  
**Preparation:** Chymotrypsin digest of T2  
**Isolation:** Paper chromatography ( $X = 92$ , purple)  
**Analysis:** Asx (2.7<sup>3</sup>), Leu (1.0), Phe (1.0), Gly (0.4)  
**DNS-Edman:** Asx-Leu-Asx-Asx-Phe (DNS-Gly from paper appeared in the first step.)

**T2, C2:** His-Arg  
**Preparation:** Chymotrypsin digestion of T2  
**Isolation:** Paper chromatography ( $X = 31$ , purple)  
**Analysis:** His (1.0), Arg (0.98)  
**DNS-Edman:** His-Arg

**T3:** Phe-Ser-Gly-Met-Gly-Phe-Gly-Pro-Glx-Thr-Pro  
**Isolation:** Map ( $X = 107$ ,  $Y = 40$ ; purple); Dowex 50-X2  
**Analysis:** Thr (1.0), Ser (1.1), Glx (1.1), Pro (1.9), Gly (3.3), Met (0.8), Phe (1.9); column preparation  
**DNS-Edman:** Phe-Ser-Gly-Met-Gly-Phe-Gly-Pro-Glx-Thr-

**C1-A:** (AE-Cys,Ser,Asx,Leu,Ser,Thr,AE-Cys,Val,-Leu)  
**Preparation:** Alkylation of pool 1 (Figure 3); paper electrophoresis at pH 1.8  
**Analysis:** Asx (1.1), Thr (1.0), Ser (2.1), Val (0.9), Leu (2.0), AE-Cys (2.0)

**C1-A, T1:** AE-Cys(Ser,Asx,Leu,Ser-Thr-AE-Cys)  
**Preparation 1:** Trypsin digest of C1-A, paper electrophoresis, pH 1.8  
**Preparation 2:** CPB digestion of PC-1, paper electrophoresis, pH 1.8, alkylation, paper electrophoresis, pH 1.8  
**Analysis:** (preparation 1) Asx (1.0), Thr (1.0), Ser (1.9), Leu (1.0), AE-Cys (1.4<sup>2</sup>)  
 (preparation 2) Asx (0.9), Thr (1.0), Ser (1.8), Leu (1.0), AE-Cys (2.1)  
**MAP:** AE-Cys (ca. 1.0)

**C1-A, T2:** Val-Leu  
**Preparation:** Trypsin digestion of C1-A  
**Isolation:** Paper electrophoresis at pH 1.8  
**Analysis:** Val (1.0), Leu (1.0)  
**DNS-Edman:** Val-Leu

**C2:** (Ser,Ala,Tyr,Trp)  
**Isolation:** Map ( $X = 109$ ,  $Y = 34$ ; gray-brown); pool 7 (Figure 3), Paper electrophoresis  
**LAP:** Ser (1.0), Ala (0.9), Tyr (1.1), Trp (1.1); paper electrophoresis preparation

**C3:** (Arg,Asx,Leu,Asx)  
**Isolation:** MAP ( $X = 48$ ,  $Y = 69$ ; purple)  
**Analysis:** Asx (2.2), Leu (1.0), Arg (0.5<sup>2</sup>)

**C4:** (Asx<sub>1-2</sub>, Phe)  
**Isolation:** Map ( $X = 61$ ,  $Y = 33$ ; purple)  
**Analysis:** Asx (1.4), Phe (1.0). The high value of 1.4 for Asx and a partial destruction by ninhydrin of the N-terminal residue could be interpreted to mean that C4 contained two rather than one Asx.

**C5:** (His,Arg,Phe)  
**Isolation:** Map ( $X = 69$ ,  $Y = 95$ ; gray-purple); Dowex 50-X2  
**Analysis:** Phe (1.0), His (0.9), Arg (1.1), from column; Phe (0.9), His (0.5<sup>2</sup>), Arg (1.1), from map

<sup>3</sup> Low value due to partial destruction of N-terminal amino acid by ninhydrin.

**C6:** (Ser,Gly,Met,Gly,Phe)  
**Isolation:** Map ( $X = 99$ ,  $Y = 32$ ; gray-brown)  
**Analysis:** Ser (0.7<sup>2</sup>), Gly (2.2), Met (0.7), Phe (1.0)

**C7:** Gly-Pro-Glx-Thr-Pro  
**Isolation:** Map ( $X = 60$ ,  $Y = 72$ ; yellow-green)  
**Analysis:** Thr (1.0), Glx (1.1), Pro (2.3), Gly (0.5<sup>2</sup>)  
**DNS-Edman:** Gly-(Pro)-Glx-Thr-

**C6,7:** (Ser,Gly,Met,Gly,Phe,Gly,Pro,Glx,Thr,Pro)  
**Isolation:** Pool 2 (Figure 3), paper chromatography ( $X = 79$ ), paper electrophoresis ( $Y = 40$ ; yellow-brown)  
**Analysis:** Thr (1.0), Ser (1.0), Glx (1.1), Pro (2.1), Gly (3.1), Met (0.8), Phe (0.9)

**CNBr-1:** (1-24 of PC-1) homoserine  
**Isolation:** CM-cellulose  
**Analysis:** Asx (4.2), Thr (1.0), Ser (3.7), Gly (1.4), Ala (1.1), Val (1.1), Cys + CysSO<sub>3</sub>H (1.7), Leu (3.1), Tyr (0.8), Phe (1.9), His (1.0), Arg (1.9), homoserine (*ca.* 0.5)

**CNBr-2:** Gly-Phe-Gly-Pro-Glu-Thr-Pro-NH<sub>2</sub>  
**Isolation:** CM-cellulose  
**Analysis:** Thr (0.9), Glx (1.1), Pro (2.0), Gly (2.1), Phe (0.9)

**DNS-Edman:** Gly-Phe-Gly-Pro-Glx-Thr-Pro-NH<sub>2</sub>; release of H-Pro-NH<sub>2</sub> after step 6 confirmed by paper chromatography and electrophoresis.

**LAP:** Glu (0.9), Pro (0.5), Gly (1.4), Phe (0.9); no Gln detected.

**C1-A, B1:** AE-Cys-Ser-Asn  
**Preparation:** CPB digestion of C1-A  
**Isolation:** Map ( $X = 11$  (5:1:4), <sup>4</sup>  $Y = 76$ ; purple)  
**Analysis:** Asx (1.2), Ser (1.0), AE-Cys (0.9)  
**DNS-Edman:** AE-Cys-Ser-Asn (free Asn chromatographically identified after second Edman)

**C1-A, B2:** (AE-Cys,Ser,Asx,Leu)  
**Preparation:** CPB digestion of C1-A  
**Isolation:** Map ( $X = 55$  (5:1:4), <sup>3</sup>  $Y = 73$ ; purple)  
**Analysis:** Asx (1.1), Ser (1.0), Leu (0.8), AE-Cys (1.1)

**C1-A, B3:** (AE-Cys,Ser,Asx,Leu,Ser)  
**Preparation:** CPB digestion of C1-A  
**Isolation:** Map ( $X = 38$  (5:1:4), <sup>3</sup>  $Y = 67$ ; purple)  
**Analysis:** Asx (1.1), Ser (1.7), Leu (1.0), AE-Cys (0.8)

**C1-A, B4:** (Ser,Thr)  
**Preparation:** CPB digest of C1-A  
**Isolation:** Map ( $X = 41$  (5:1:4), <sup>3</sup>  $Y = 42$ ; gray)  
**Analysis:** Thr (1.0), Ser (1.0)

#### Confirmatory Peptides

**TC1:** (Cys,Ser,Asx,Leu,Ser,Thr,Cys,Val,Leu)  
**Isolation:** Map ( $X = 126$ ,  $Y = 37$ ; brown)  
**Analysis:** Asx (1.1), Thr (0.9), Ser (2.0), Val (1.0), Leu (1.6), Cys (*ca.* 1.0<sup>2</sup>)

**TC2:** (Ser,Ala,Tyr)  
**Isolation:** Map ( $X = 83$ ,  $Y = 41$ ; brown)  
**Analysis:** Ser (0.9), Ala (1.0), Tyr (0.7), Trp (not determined). Synthetic Ser-Ala-Tyr,  $X = 83$  and synthetic Ser-Ala-Tyr-Trp,  $X = 100$

**TC3:** (Trp,Arg)  
**Isolation:** Map ( $X = 77$ ,  $Y = 67$ ; purple)  
**Analysis:** Arg, Trp (+)

**TC3b:** Arg  
**Isolation:** Map ( $X = 39$ ,  $Y = 90$ ; purple)  
**Analysis:** Arginine

**TC4:** (Asx,Leu,Asx,Asx,Phe)  
**Isolation:** Map ( $X = 83$ ,  $Y = 37$ ; grayish purple)  
**Analysis:** Asx (3.1), Leu (1.0), Phe (0.9)

**TC5:** (His,Arg)  
**Isolation:** Map ( $X = 31$ ,  $Y = 107$ ; bluish purple)  
**Analysis:** His (0.6<sup>2</sup>), Arg (1.0)

**TC6:** (Phe,Ser,Gly,Met)  
**Isolation:** Map ( $X = 103$ ,  $Y = 37$ ; purple)  
**Analysis:** Ser (1.0), Gly (1.1), Met (0.8), Phe (0.5<sup>2</sup>)

**TC7:** (Gly,Phe,Gly,Pro,Glx,Thr,Pro)  
**Isolation:** Map ( $X = 87$ ,  $Y = 51$ ; gray-green)  
**Analysis:** Thr (1.2), Glx (1.3), Pro (2.0), Gly (2.0), Phe (1.0)

**TC7b:** (Gly,Pro,Glx,Thr,Pro)  
**Isolation:** Map ( $X = 56$ ,  $Y = 54$ ; yellow)  
**Analysis:** Thr (1.0), Glx (1.0), Pro (1.6), Gly (0.6<sup>2</sup>)

**(1-7) ox:** CysSO<sub>3</sub>H-Ser-Asx-Leu-Ser-Thr-CysSO<sub>3</sub>H  
**Preparation:** CPB digest of PC-1, oxidized on paper sheet with performic acid.

**Isolation:** Paper electrophoresis, pH 1.8  
**Analysis:** CysSO<sub>3</sub>H (2.0), Asx (1.0), Thr (1.0), Ser (1.9), Leu (1.0)

**DNS-Edman:** Cys-SO<sub>3</sub>H-Ser-Asx-Leu-Ser-Thr-CysSO<sub>3</sub>H

#### Discussion

The peptide data summarized above are sufficient to establish an unequivocal amino sequence for PC-1. The complete structure was confirmed by at least two series of overlapping peptides. In addition to the sequence deduced from the data obtained by overlapping peptides isolated from a CPB digest and from a trypsin digest of C1-A, the sequence for the first nine N-terminal amino acids was also obtained by the dansyl-Edman procedure on intact PC-1.

Certain aspects of the studies merit comment. The presence of C-terminal prolinamide was unexpected and proved difficult to confirm. The rapid conversion of the H-Thr-Pro-NH<sub>2</sub> into its diketopiperazine in the dansyl-Edman procedure greatly reduced the yields of threonine and prolinamide. Confirmation of C-terminal prolinamide by release with carboxypeptidase A or B was not accomplished. Positive chemical identification of prolinamide at position 32 made it possible to deduce that the glutamic acid at position 30 was not in amide form since peptides C7, C6,7, and CNBr-2 were shown to be neutral in paper electrophoresis studies. The presence of proline at position 29 made it seem unlikely

<sup>4</sup> 5:1:4 refers to chromatographic system, 1-butanol-acetic acid-water.

that LAP would release glutamic acid from these peptides. Fortunately, the endopeptidase activity of the LAP preparation used made it possible to release glutamic acid in good yield from CNBr-2, thus confirming the presence of glutamic acid at position 30.

The studies of the N-terminal section of PC-1 were of particular interest. The 1-7 peptide section of PC-1 proved to be totally resistant to digestion by trypsin, chymotrypsin, LAP, MAP, CPA, and CPB so long as the disulfide bridge was intact. In fact, this cyclic peptide (residues 1-7) was prepared in good yield by a digestion of PC-1 with trypsin followed by CPB. Reduction and aminoalkylation of the cysteines at positions 1 and 7 rendered this portion of the molecule susceptible to digestion. Digestion of C1-A by CPB gave a series of peptides of value in confirming the sequence in the 1-9 position of PC-1. The presence of peptide C1-A,B4 in this digest clearly points up the presence of endopeptidase activity in the CPB used. Paper electrophoresis studies of peptides C1-A, C1-A,B1, C1-A,B2, and C1-A,B3 were only consistent with the aspartic acid in position 3 being in a nonionized form. The presence of asparagine in this position was confirmed by dansyl-Edman studies of C1-A,B1. Digestions of C1-A with MAP and CPB failed to release asparagine in any significant amounts. MAP released one AE-Cys from C1-A,T1 without any release of Ser or Asn. It would appear that the resistance to digestion by exopeptidase resides in the Ser-Asn structure. In a later study of synthetic C1 we were able to show that the papain-MAP digestion released Asn. It would appear that papain first splits the Ser-Asn bond thereby making the Asn available for the MAP digestion. It is of interest that the papain digestion was carried out on the cyclic disulfide peptide. Since this digestion was carried out in the presence of 0.02 M mercaptoethanol, it is possible that the reduced peptide was serving as the substrate.

The three aspartyl residues in positions 15, 17, and 18 of PC-1 were found to be in the amide form by analysis of a total LAP digestion of peptide T2.

A structure identical with that of porcine calcitonin-1 has been independently reported by Potts *et al.* (1968) and Neher *et al.* (1968a). Beesley *et al.* (1968) disclosed a sequence for position 8-32 in agreement with that reported for PC-1 even though these authors were unable to obtain evidence of a free N-terminal amino in their calcitonin preparations.

The minimum structural requirements for calcitonin activity cannot be deduced from present available data. Neher *et al.* (1968b) recently reported the structure of a highly active calcitonin isolated from a human medullary carcinoma. It is significant that this hormone of human origin contained a 32 amino acid structure with an N-terminal 1-7 intra-disulfide bridge and with prolinamide at the C-terminal position. Fourteen of the amino acid residues of this hormone of human origin were the same as found in porcine

calcitonin-1. The presence of 18 amino acid differences between these hormones is unexpected and merits further investigation. Neher *et al.* (1968b) also reports the isolation of a second fully active form of human calcitonin from the same tumor. Some evidence was presented to indicate that this material is an antiparallel dimer of the primary human hormone structure resulting from one to seven inter-disulfide linkages. These data along with the observations of Bell *et al.* (1970) that reduced PC-1 was active suggest that the one to seven intra-disulfide ring probably is not an absolute requirement for activity. Final confirmation of these conclusions, as well as the determination of the effects of other amino acid substitutions on biological activity, must await material made by chemical synthesis.

#### Acknowledgments

The authors wish to express appreciation to the management of our laboratories and to Dr. I. Ringler in particular for continuing support and encouragement of this study.

#### References

- Beesley, T. E., *et al.* (1968), *J. Am. Chem. Soc.* 90, 3255.
- Bell, P. H. (1967), Proceedings of Symposia on Thyrocalcitonin and the C Cells, London, England, W. Heinemann Medical Books, p 77.
- Bell, P. H., *et al.* (1968), *J. Am. Chem. Soc.* 90, 2704.
- Bell, P. H., *et al.* (1970), *Biochemistry* 9, 1665.
- Edman, P. (1950), *Acta Chem. Scand.* 4, 283.
- Gray, W. R., and Hartley, B. S. (1963), *Biochem. J.* 89, 59P.
- Gross, E., and Witkop, B. (1962), *J. Biol. Chem.* 237, 1856.
- Guidotti, G., Hill, R. J., and Konigsberg, W. (1962), *J. Biol. Chem.* 237, 2184.
- Hill, R. L., and Smith, E. L. (1957), *J. Biol. Chem.* 228, 577.
- Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem.* 219, 623.
- Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem.* 234, 2897.
- Moore, S. (1963), *J. Biol. Chem.* 238, 235.
- Nedkov, P., and Genov, N. (1966), *Biochim. Biophys. Acta* 127, 541.
- Neher, R., Riniker, B., Rittel, W., and Zuber, H. (1968b), *Helv. Chim. Acta* 51, 1900.
- Neher, R., Riniker, B., Zuber, H., and Kahnt, F. W. (1968a), *Helv. Chim. Acta* 51, 917.
- Potts, J. T., Jr., *et al.* (1968), *Proc. Natl. Acad. Sci. U. S.* 59, 1321.
- Raftery, M. A., and Cole, R. D. (1966), *J. Biol. Chem.* 241, 3457.
- Spies, J. H., and Chambers, D. C. (1948), *Anal. Chem.* 20, 30.
- Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta* 133, 369.