Isolation and Chemical Properties of Porcine Thyrocalcitonin

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SUMMARY

Thyrocalcitonin has been isolated from porcine thyroid tissue following a 40,000-fold purification. Specific biological activity is 200 MRC units per mg. The purified peptide was shown to be homogenous by multiple criteria and to consist of 32 amino acids: Arg, His, Thr, Ser, Glu, Pro, Gly, Ala, Val, Met, Asn, Leu, Phe, Tyr, Trp, half-Cys. The purified hormone is resolved by chromatography on carboxymethyl cellulose or cellulose thin layer plates into two components. The only difference between these components is that one form of the hormone contains methionine as the sulfoxide; the other component, methionine in the reduced form. Both forms have equal specific activity. The peptide contains no covalently bound carbohydrate, iodine, or other substituted amino acids. The 2 half-cystine residues are present in the hormone as an intrachain disulfide bond. Since 1 half-cystine residue is the NH$_2$-terminal amino acid, the amino-terminal portion of the molecule is in the form of a 23-membered ring. The lack of a high degree of ordered structure in the molecule is reflected in the complete solvent accessibility of the methionine, tryptophan, and tyrosine residues without prior denaturation of the polypeptide.

Thyrocalcitonin is the recently discovered hypocalcemic, hypophosphatemic peptide hormone found in the thyroid gland of man and numerous mammalian species (1–3). Recent reports have summarized the efforts of several laboratories to isolate and characterize the polypeptide, define its mode of action and physiological role, and explore its possible therapeutic implications (4–6). We have reported our earlier experience with purification of urea extracts of porcine thyroid tissue (7). These studies led to the purification of a biologically active component of high potency (200 MRC units per mg)\footnote{1 MRC unit is defined by the Division of Biological Standards of the National Institute for Medical Research, Mill Hill, London, England, with their bioassay reference standard Research Standard A (0.025 unit per mg).} with a distinctive amino acid composition; over-all purification was greater than 40,000-fold. Formidable problems were encountered in the isolation of quantities of the purified peptide sufficient for structural characterization or development of an immunological assay for the hormone. Recently, the availability of larger quantities of partially purified material prepared by acid extraction permitted the development of purification procedures suitable for yielding several hundred milligrams of pure peptide in a single fractionation. This report describes the methods ultimately developed to purify thyrocalcitonin, the evidence that the peptide product was homogeneous, and some of the chemical properties of the molecule.

MATERIALS AND METHODS

Starting Material—Partially purified thyrocalcitonin was obtained from Armour Pharmaceutical Company (Kankakee, Illinois). This material was prepared by an acid extraction method and processed by purification techniques adapted for large scale processing to yield material with an average specific activity of 15 MRC units per mg\footnote{Bioassays were performed with the method of Schlueter and Caldwell (8).}. This material is similar in potency to the urea-extracted material purified on Sephacryl G-75 (14 MRC units per mg) which was utilized in our earlier
and pharmacological studies, and is being utilized for the bio-
assay standard (Research Standard B) of the British Medical
Research Council.

**Biological Assay—**Assays were performed on 5-week-old
intact male Holtzman rats by the subcutaneous route of ad-
ministration. The assay was performed as described previously
(7) with the improved assay technique of Cooper et al. (9) and
Munson and Hirsch (10).

**Column Chromatography—**Column chromatography was per-
formed with micrograman carboxymethyl cellulose (Whatman
CM-52), and gel filtration with Sephadex G-25 (superfine) and
G-50 (fine or superfine) (Pharmacia) or Bio-Gel P-2 (Bio-Rad).

Sephadex G-25 (superfine) was suspended in water and allowed
to swell overnight. The slurry in distilled water was boiled for
2 hours and cooled, and the supernatant was decanted to remove
dissolved carbohydrate. The gel was resuspended in distilled
water to remove fine particles. The dry Bio-Gel P-2 resin
(50 to 100 mesh) was suspended in 0.1 M acetic acid and prepared
for use similarly to the Sephadex gel. The carboxymethyl
cellulose columns were developed in preliminary experiments
with a linear gradient of ammonium acetate buffer from 0.01 M
ammonium acetate (pH 5.3, conductivity 0.7 millimho) to 0.33
M ammonium acetate (pH 6.9, conductivity 17.0 millimhos).
In subsequent experiments a combined stepwise and gradient elu-
tion schedule was used, with combinations of the two ammonium
acetate buffers as described in the text. The solvent used for
developing the Sephadex columns was 0.05 M ammonium formate,
pH 3.0, and for the Bio Gel P-2, 0.1 M acetic acid. Carboxy-
methyl cellulose and Sephadex G-25 fractionations were carried
out at 4°, and Sephadex G-50 and Bio-Gel filtrations at 25°.

Thin layer chromatography was performed on precoated glass
plates (20 x 20 cm) of Avicel micro crystalline cellulose powder
obtained from Brinkmann. Samples of 0.015 to 0.1 µg peptide
were applied and the plates were developed by ascending flow
with butanol-pyridine-water-glacial acetic acid (30:20:24:6).

**Electrophoresis—**Multiphase zone electrophoresis on poly-
acrylamide gel was performed in a manner similar to that pre-
viously described (7). The gel electrophoresis in these experi-
ments were conducted with the separation gel buffer at pH 3.6
and the upper tray buffer at pH 4.4; acrylamide monomer concen-
tration was 15% (w/v). Preparative disc gel electrophoresis was
performed with a commercially available apparatus (Fraceto-
phorator) obtained from Buchler Instruments, Inc. Both preparative electrophoresis and analytic scale electrophoresis
were performed at 25° in the presence of 6 M urea. The gels were
stained with ninhydrin or Ehrlich’s solution (11).

**Amino Acid Analysis—**All analyses were made with Beckman-
Spinco automatic amino acid analyzers, model 120B, adapted
for high sensitivity (0.002 µm detection minimum) (13) and rapid
column schedule (2-hour complete analysis) (14). The results
were analyzed through the use of an in-line digital integrator
(Infotronics model CRS-12AB). Hydrolyses were performed at
24, 48, 72, and 96 hours with constant boiling 5.7 N HCl at 110°.
The samples were hydrolyzed in open tubes contained within a
clamped, sealed desiccator. The desiccator was repeatedly evac-
uated and flushed with oxygen-free nitrogen before hydroly-
sis. Selected hydrolysis samples contained mercaptoethanol,
1:2000 (v/v), with the 5.7 N HCl. This procedure has been
shown to improve recovery of methionine, tyrosine, and serine
residues during acid hydrolysis.4

Total enzymic digestion was performed with a new technique
of enzymic digestion that has been shown to provide high yields
of the constituent amino acids of a polypeptide including methio-
nine, methionine sulfide, cystine, glutamine, asparagine, and
tryptophan.4 For digestion of thyrocalcitonin, the peptide, at
a concentration of 1 to 3 mg per ml dissolved in 0.05 M am-
monium acetate, pH 5.3, containing 0.02 M mercaptoethanol,
was incubated at 37° for 2 hours with 50 µg per ml of papain.
The solution was then acidified to inactive papain, lyophilized,
and incubated for 3 hours at 37° with 1.5 mg per ml of ammino-
peptidase-M in 0.2 M trimethylamine acetate buffer, pH 8.2.
The samples were lyophilized and applied directly to the amino
acid analyzer. Asparagine, glutamine, aspartic acid, and
glutamic acid were distinguished by the use of the lithium citrate
buffer system (15).

To compare yields of amino acids in aliquots of thyrocalcitonin
hydrolyzed by the enzymic technique or by 5.7 N HCl for vary-
ing periods of time, norleucine and homogarginine were used as
internal standards. These latter amino acids have been shown
to be suitable as internal standards because they occupy unique
elution positions on the analyzer and are stable during acid or
enzymic hydrolysis.4 Thyrocalcitonin was dissolved in 0.1 M
acetic acid, internal standards were added, and aliquots for acid
or enzymic digestion were lyophilized to dryness. The samples
were redissolved in 5.7 N HCl or the ammonium acetate buffer
used for enzymic digestion. Yields of amino acids in each
aliquot after hydrolysis were then equated by comparison with
the recovery of the internal standards.

Direct tests were made for covalently bound carbohydrate,
iiodine, or other nitrogen-containing organic substances. Total
organic nitrogen of a sample of salt-free thyrocalcitonin (pre-
pared by passage over a Bio-Gel P-2 column equilibrated with
0.1 M acetic acid) was determined by the Kjeldahl method with
Nessler’s solution (16, 17). An exactly equivalent aliquot was
lyophilized, hydrolyzed, and analyzed on the amino acid ana-
lyzer to determine the micrograms of nitrogen present in the
sample as amino acid nitrogen. Another aliquot of desalted
hormone was brought to constant weight over a 5-day period by
heating at 100° in a vacuum oven. Carbohydrate analysis was
performed on this sample by the phenolsulfuric acid method (18)
and antrone reaction (19). Iodine determinations (sensitive to
0.001 µg of iodine) were made on a sample of 1 mg of peptide by
the Boston Medical Laboratory.

**Modification Reactions—**Reduction of the purified peptide was
performed at 37° for 4 hours with mercaptoethanol (Eastman)
at a final concentration of 0.1 M (molar ratio of reducing agent
to peptide, 100:1) (20). Alkylation of the reduced protein at
neutral pH was carried out for 15 min at 25° in 8 M urea (de-
ionized by passage over a column of Rexyn I-300 prior to use) and
0.2 M Tris-HCl, pH 8.5, with a 10-fold m excess of iodoacetic acid
(recrystallized from petroleum ether) over reducing agent. This
reaction was terminated by the addition of a 10-fold m excess of
mercaptoethanol over the alkylating reagent. Alkylation was
also performed on the native hormone without prior reduction;
the peptide was incubated for 15 min at 25° in 8 M urea and 0.2
M Tris-HCl, pH 8.5, with a 50-fold m excess of iodoacetic acid.

Reaction of native thyrocalcitonin with iodoacetic acid at pH

3 H. T. Keutmann and J. T. Potts, Jr., manuscript in prepara-

4 H. T. Keutmann and J. T. Potts, Jr., in preparation.
3.0 to alkylate methionine, was performed at 37° for 24 hours in either 0.1 M acetic acid alone or acetic acid made 8 M in urea; a 100-fold M excess of iodoacetic acid to peptide was used (21). After completion of the alkylation, each incubation sample was immediately applied to a Bio-Gel P-2 column equilibrated with 0.1 M acetic acid in order to separate the peptide from the reagents. The effluent from the column containing the peptide was lyophilized and subjected to enzymic or acid hydrolysis. The enzymic hydrolysis technique permitted the direct extraction of the products of cysteine alkylation with iodoacetic acid (S-carboxymethylcysteine eluted at 27 min on the rapid elution analyzer system). Completeness of alkylation of methionine at acidic pH could also be tested by the enzymic technique since the carboxymethylsulfonium salt does not revert to methionine during enzymic digestion.

Tests for free thiol groups were made by direct titration of the peptide with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (22). Titrations with 5,5'-dithiobis(2-nitrobenzoic acid) were also performed on thyrocalcitonin which had been fully reduced with mercaptoethanol and on another aliquot treated with a chelating agent (2 mg of thyrocalcitonin dissolved in 1.0 ml of 0.1 M EDTA and incubated at 25° for 30 min). Following treatment with reducing agent or EDTA, an aliquot of the peptide was passed over Bio-Gel P-2 in 0.1 M acetic acid and immediately reacted with 5,5'-dithiobis(2-nitrobenzoic acid).

Thyrocalcitonin was treated with 2-hydroxy-5-nitrobenzyl bromide (Sigma) to modify tryptophan (23). The peptide (3.5 mg per ml in 0.1 M acetic acid adjusted to pH 3.0) was incubated for 15 min at 25° with a 50:1 M excess of 2-hydroxy-5-nitrobenzyl bromide. A similar incubation with 2-hydroxy-5-nitrobenzyl bromide was performed in 0.1 M acetic acid made 8 M in urea. The precipitate formed by reaction of excess reagent with solvent was removed by centrifugation at the end of the incubation. The reagents were removed from the peptide by gel filtration on Bio-Gel P-2, and the extent of the reaction was estimated by amino acid analysis after total enzymic digestion. In addition, the absorbance change at 410 mn was measured following the addition of 2.0 M NaOH to increase the pH of the solutions containing the thyrocalcitonin derivative to 12.5.

**Performic Acid Oxidation**—The performic acid reagent was prepared by the addition of 2 parts 30% hydrogen peroxide (Merek) to 18 parts 98% formic acid (Baker Analyzed Reagent) (24). This solution was kept at 25° for 1 hour. The dry protein sample (2 to 3 mg) was oxidized for 2 hours at 0° by the addition of 0.5 ml of the performic acid reagent. Following oxidation, the sample was diluted by the addition of 10 volumes of cold-distilled water, and lyophilized twice before hydrolysis. Care was taken to avoid melting of the sample during lyophilization.

**End Group Analysis**—Phenylisothiocyanate degradation was performed by the three-stage procedure of Edman (25, 26) on both reduced and alkylated and performic acid-oxidized thyrocalcitonin. The phenylthiohydantoin derivative from alkylated thyrocalcitonin was identified by gas-liquid chromatography (27) and thin layer analysis (26); the derivative from performic acid-oxidized hormone was identified by mass spectrometry.

**Ultraviolet Spectrum, Extinction Coefficient, and Difference Spectroscopy**—Measurements of optical density of solutions of thyrocalcitonin were made in a Cary model 15 recording spectrophotometer with matched sets of quartz cuvettes for test and reference solutions. For difference spectroscopy, 0.75 to 1.5 mg of protein were added to 3 ml of 0.01 M trimethylamine acetate buffer adjusted to pH 4.8 with glacial acetic acid. The

![Graph](https://example.com/graph.png)

**Fig. 1.** Gel filtration of 300 mg of porcine thyrocalcitonin (15 MRC units per mg) on Sephadex G-50 (fine). Column size, 2.5 × 96 cm; flow rate, 100 ml per hour; aliquot, 10 ml; buffer, 0.05 M ammonium formate, pH 3.0. Biological activity (MRC units per mg) of successive pools (A to G) of the column effluent is indicated by A---A.

The pH of the solution was adjusted by serial additions of 1 to 2 μl of 1.0 M NaOH. Similar additions of distilled water were added to an identical solution of protein which served as the blank. Measurements of pH after each addition of alkali were made directly in the cuvette with the small KCl-AgCl electrode used with the Beckman pH meter (Zeromatic II). Absorbance change at 245 and 295 mn was measured.

**RESULTS AND DISCUSSION**

**Purification**—The acid-extracted starting material was initially purified by filtration on Sephadex G-50 (fine). One major peak eluted from the column at a Kg of approximately 0.7 (Fig. 1). Bioassay of successive pools of the column effluent showed the biological activity to be coincident with the major peak. Repetitive fractionation experiments used in preparing thyrocalcitonin for further purification resulted in patterns of elution closely similar to that shown in Fig. 1. The region containing biological activity was invariably the dominant component. The active product in a series of these fractionations had an average potency of 50 MRC units per mg (range, 40 to 90 MRC units per mg). Examination of this material by thin layer chromatography and analytical disc gel electrophoresis revealed numerous peptides. When further purification of this material was undertaken by chromatography on carboxymethyl cellulose with the linear gradient of ammonium acetate, two discrete peaks containing peptide eluted from the column. The biological activity was confined to the second discrete peak; however, this fraction was not homogeneous when analyzed by disc gel electrophoresis and thin layer chromatograms. Numerous basic peptides co-eluted with the thyrocalcitonin in the second peak of protein.

In order to obtain homogeneous material two further procedures were necessary. First, the material purified on Sephadex G-50 was further purified on Sephadex G-25 to eliminate a large number of the minor contaminating peptides prior to CM-cellulose chromatography. Sephadex G-25 provided the greatest theoretical resolution of thyrocalcitonin and contaminating peptides. The abbreviation used is: CM-cellulose, carboxymethyl cellulose.
Fig. 2. Gel filtration of 75 mg of Sephadex G-50 purified thyrocalcitonin on Sephadex G-25 (superfine). Column size, 2 × 130 cm; flow rate, 50 ml per hour; aliquot, 5 ml; buffer, 0.05 M ammonium formate, pH 3.0. Bioassay, thin layer chromatography, and disc gel electrophoresis (Fig. 3) indicated that thyrocalcitonin was confined to the middle peak. Arrows refer to the excluded and included volumes of the column, respectively.

Fig. 3. Acrylamide disc gel electrophoresis at pH 4.4 (15% acrylamide concentration) of successive aliquots of the thyrocalcitonin preparation fractionated on Sephadex G-25 (Fig. 2). Gels contain (left to right): 1, starting material; 2 and 3, aliquots of the first peak; 4 to 7, aliquots of the biologically active middle peak; 8 and 9, aliquots of the third peak. The major band in Gels 4 to 7 is thyrocalcitonin. Bands not detected in the starting material are readily detected in Gels 2, 3, 8, and 9 through concentration during gel filtration.

Most importantly, the first and third peaks contained essentially all of the more basic peptide contaminants that had co-eluted with thyrocalcitonin in the initial CM-cellulose purification.

A subsequent single preparative column (5 × 150 cm) of 260 mg of Sephadex G-50 purified material yielded 175 mg of thyrocalcitonin in the middle peak. This fraction was lyophilized and bioassayed; specific activity was 130 MRC units per mg. The 175 mg of thyrocalcitonin purified on Sephadex G-25 were then applied to a column of carboxymethyl cellulose, 2 × 40 cm. The column was developed with both continuous and gradient}

Fig. 4. Fractionation on carboxymethyl cellulose of 175 mg of Sephadex G-25 purified thyrocalcitonin with continuous followed by gradient elution. Column size, 2 × 40 cm; flow rate 25 ml per hour; aliquot size, 4 ml; column initially equilibrated with 0.01 M ammonium acetate, pH 5.3, conductivity 0.7 millimho. The column was eluted with an ammonium acetate buffer of pH 6.5, conductivity 7.2 millimhos until tube 120. Elutions were then begun with a linear gradient (pH 6.56, conductivity 11.8 millimhos to pH 7.0, conductivity 15.5 millimhos). Biological activity was detected between tubes 120 to 330.

Fig. 5. Acrylamide disc gel electrophoresis at pH 4.4 (15% acrylamide concentration) of successive aliquots from the CM-cellulose column (Fig. 4). Left to right, Gel 1 (tube 130, Fig. 4) contains an aliquot from the leading edge of the biologically active peak; Gels 2 (tube 150, Fig. 4, and Table I, Channel 4, Fig. 6), 3, 4 (tube 220, Fig. 4, Table I, Channel 6, Fig. 6), 5 to 6, and 7 (tube 288, Fig. 4, Table I, Channel 9, Fig. 6) represent aliquots of the biologically active peak, pure thyrocalcitonin.
elution. The elution was initially performed with an ammonium acetate buffer of pH 6.5, conductivity 7.2 millimhos, which was chosen since it represented the position in the gradient which had eluted the first biologically inactive peak in the preliminary carboxymethyl cellulose experiment. The column was developed with this buffer of intermediate ionic strength until elution of protein had ceased. Elution was then begun with a narrow linear gradient of higher ionic strength (pH 6.56, conductivity 11.8 millimhos to pH 7.0, conductivity 15.5 millimhos). The elution profile of the final CM-cellulose column is shown in Fig. 4.

Disc gel analysis of Fractions 150 through 288 showed only a single band (Fig. 5), indicating that the hormone had been recovered in homogeneous form. Analysis indicated that Fractions 120 through 150 (Fig. 1, Fig. 5) contained contaminants; these had been eluted just ahead of the major portion of the thyrocalcitonin peptide by the use of the shallow gradient.

Amino acid analysis by acid hydrolysis of successive aliquots from the biologically active second peak revealed an essentially constant amino acid composition (Table I). The agreement found in the relative content of each amino acid in successive fractions was ±6% for most residues.

Specific biological activity of successive fractions was also constant. Four separate bioassays of the contents of tubes 150, 190, 220, and 261 (the protein concentration of these tubes was determined by amino acid analysis, Table I) indicated that there was no statistically significant difference in the specific biological activity of these fractions; specific activity was 213 ± 17 MRC units per tube. The percentage of the total biological activity from the biologically active second peak revealed an essentially constant amino acid composition (Table I). The agreement found in the relative content of each amino acid in successive fractions was ±6% for most residues.

Thyrocalcitonin, therefore, had been monitored to a stage of constant specific biological activity and amino acid composition, and had been shown to be homogeneous by disc gel electrophoresis.

Table I

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<th>Amino acid</th>
<th>Thyrocalcitonin (mole fraction)</th>
<th>Agreement</th>
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<th>Tube 220</th>
<th>Tube 261</th>
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Despite the evidence of homogeneity, the column effluent containing the hormone peak contained two discrete components (Fig. 4). Thin layer analysis of successive fractions of the hormone peak also showed two components (Fig. 6). The leading edge of the CM-cellulose peak contained the slower component on thin layer chromatography (designated B1), and the trailing edge of the peak, the faster component (designated B2). A combination of both B1 and B2 was present in the center of the peak. Total enzymic digestion of a fraction of pure B1 (0.04 μmole of peptide) revealed that in B1 the methionine was present as methionine sulfoxide (0.04 μmole of methionine sulfoxide, 0.00 μmole of methionine); digestion and analysis of pure B2 indicated that methionine was present as methionine (0.04 μmole of methionine, 0.00 μmole of methionine sulfoxide). In addition, pure B1 (0.04 μmole of peptide) was alkylated at acidic pH, performed acid-oxidized, hydrolyzed, and analyzed (28). Methionine sulfone was found (0.038 μmole). After similar treatment of B2 no methionine sulfone was found. This latter procedure confirmed the findings by enzyme digestion that in B1 methionine was present as the sulfoxide, whereas in B2 the methionine was present as methionine (29). The equivalent biological activity of B1 and B2 in thyrocalcitonin is unusual; hormones such as corticosterin or parathyroid hormone lose biological activity after oxidation of methionine residues in the peptide (30-32).

An unexplained feature of the disc gel electrophoresis patterns obtained with the purified thyrocalcitonin, in view of the high resolving properties of the method, was that only one electrophoretic band was detected with analysis of B1, B2, or a mixture

![Fig. 6. Thin layer chromatograms of successive fractions of the effluent from carboxymethyl cellulose (Fig. 4). Channels 1 to 2 contain aliquots of the starting material; Channel 3 an aliquot of the first, biologically inactive peak; Channels 4 to 9 contain the purified thyrocalcitonin peptide. Channels 4 to 9 correspond to disc Gels 2 to 7 of Fig. 5. The plate is stained with Ehrlich's solution. Purified thyrocalcitonin stains poorly with ninhydrin, and other staining procedures must be used in order to visualize the peptide adequately.](http://www.jbc.org/issue/1968/36/Issue)
logical activity was confined to the area occupied by the peptide. Thin layer chromatography fractions revealed that all of the bio-
activity had been recovered in the protein peak. Therefore, despite fractionation in organic solvents or electrophoresis in the presence of 6 M urea, all biological activity remained coincident with the purified peptide identified as thyrocalcitonin. The studies provide strong evidence that there is no noncovalently bound cofactor in thyrocalcitonin which is important for biological activity.

**Characterization of 2 Half-cystine Residues**—Amino acid analysis of thyrocalcitonin preparations has consistently shown cysteine. In order to clarify the exact content of sulfur-containing amino acids in the purified peptide, a sample was subjected to performic acid oxidation and amino acid analysis. Two moles of cysteic acid and 1 mole of methionine sulfone were found per mole of thyrocalcitonin. In order to eliminate the possibility that only 1 half-cystine (cysteine) was present in peptide linkage through disulfide linkage, an additional sample of peptide was oxidized with performic acid. The oxidized peptide was then passed over a Bio-Gel P-2 column in order to separate thyrocalcitonin from any noncovalently bound cysteic acid. The effluent containing the peptide was lyophilized, hydrolyzed, and analyzed. Two moles of cysteic acid per mole of thyrocalcitonin were found, thus indicating that the 2 moles of half-cystine had both been present in peptide linkage.

In order to characterize further the state of the 2 half-cystine residues in the native molecule, an aliquot of the purified peptide was subjected to total enzymatic digestion. Amino acid analysis of the digest revealed 2 moles of half cystine as cysteine, i.e. 1 mole of cystine per mole of peptide (Table III). In addition, reduction and alkylation of 0.040 µM of peptide consistently showed 2 moles (0.078 µM) of S-carboxymethylcysteine in both B₁ and B₂. Direct alkylation (without prior reduction of thyrocalcitonin), followed by hydrolysis and analysis, indicated that no S-carboxymethylcysteine residues had been found in either B₁ or B₂. No absorbance change in either B₁ or B₂ at 410 µM was detected on titration of unreduced polypeptide with Ellman's reagent (even after prior treatment with 0.1 M EDTA to remove any metals). After reduction with mercaptoethanol absorbance changes at 410 µM were found equivalent to reaction of 1.8 moles of free thiol per mole of peptide.

To test the possibility that the disulfide represented an interchain bridge, performic acid-oxidized thyrocalcitonin was subjected to gel filtration on Sephadex G-25. A Kₐ of 0.4 was found for the oxidized product as compared to a Kₐ of 0.5 found for the native molecule. This change, if significant, indicated only a slight increase rather than a decrease in apparent molecular volume. These studies, therefore, indicated that the single disulfide of the molecule is in the form of an intrachain bridge.

**Amino Acid Composition**—In order to obtain a more definitive amino acid composition of the hormone and to eliminate the
Table III

**Amino acid composition of thyrocalcitonin**

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Mole fraction after hydrolysis for</th>
<th>Average or extrapolated value</th>
<th>Observed residues per mole</th>
<th>Enzymatic digestion (A)</th>
<th>Mole fraction</th>
<th>Observed residues per mole</th>
<th>24-hr acid hydrolysis ( ^{b} ) (B)</th>
<th>Yield, ( ^{b} ) enzymatic digestion (A/B \times 100)</th>
<th>24-hr acid hydrolysis in mercaptoethanol</th>
<th>Residues per mole combined results ( ^{c} ) results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>48 hrs</td>
<td>72 hrs</td>
<td>96 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.003</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>His</td>
<td>0.067</td>
<td>0.065</td>
<td>0.060</td>
<td>0.050</td>
<td>0.008</td>
<td>0.002</td>
<td>1.03</td>
<td>0.035</td>
<td>1.05</td>
<td>91.1</td>
</tr>
<tr>
<td>Arg</td>
<td>0.070</td>
<td>0.070</td>
<td>0.082</td>
<td>0.078</td>
<td>0.078</td>
<td>0.070</td>
<td>2.22</td>
<td>0.075</td>
<td>2.25</td>
<td>92.8</td>
</tr>
<tr>
<td>Trp</td>
<td>0.089</td>
<td>0.089</td>
<td>0.090</td>
<td>0.089</td>
<td>0.090</td>
<td>0.091</td>
<td>2.22</td>
<td>0.075</td>
<td>2.25</td>
<td>92.8</td>
</tr>
<tr>
<td>Asp</td>
<td>0.141</td>
<td>0.140</td>
<td>0.140</td>
<td>0.140</td>
<td>0.144</td>
<td>0.138</td>
<td>4.11</td>
<td>0.135</td>
<td>3.53</td>
<td>93.5</td>
</tr>
<tr>
<td>Asn (^{a})</td>
<td>0.066</td>
<td>0.063</td>
<td>0.064</td>
<td>0.060</td>
<td>0.068</td>
<td>0.064</td>
<td>2.06</td>
<td>0.061</td>
<td>1.93</td>
<td>90.8</td>
</tr>
<tr>
<td>Thr (^{a})</td>
<td>0.129</td>
<td>0.116</td>
<td>0.109</td>
<td>0.100</td>
<td>0.137</td>
<td>0.112</td>
<td>3.60</td>
<td>0.120</td>
<td>3.60</td>
<td>93.1</td>
</tr>
<tr>
<td>Ser (^{a})</td>
<td>0.083</td>
<td>0.081</td>
<td>0.083</td>
<td>0.086</td>
<td>0.083</td>
<td>0.082</td>
<td>1.03</td>
<td>0.083</td>
<td>0.90</td>
<td>90.6</td>
</tr>
<tr>
<td>Gla</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.00</td>
<td>0.000</td>
<td>0.00</td>
<td>0.000</td>
</tr>
<tr>
<td>Gln</td>
<td>0.066</td>
<td>0.069</td>
<td>0.067</td>
<td>0.069</td>
<td>0.067</td>
<td>0.050</td>
<td>1.61</td>
<td>0.062</td>
<td>1.87</td>
<td>80.4</td>
</tr>
<tr>
<td>Pro (^{a})</td>
<td>0.104</td>
<td>0.102</td>
<td>0.103</td>
<td>0.100</td>
<td>0.103</td>
<td>0.085</td>
<td>2.73</td>
<td>0.101</td>
<td>3.08</td>
<td>84.0</td>
</tr>
<tr>
<td>Gly</td>
<td>0.033</td>
<td>0.031</td>
<td>0.033</td>
<td>0.036</td>
<td>0.033</td>
<td>0.032</td>
<td>1.03</td>
<td>0.033</td>
<td>0.99</td>
<td>96.6</td>
</tr>
<tr>
<td>Ala</td>
<td>0.061</td>
<td>1.97</td>
<td>1.97</td>
<td>1.97</td>
<td>1.97</td>
<td>0.057</td>
<td>1.67</td>
<td>117.2</td>
<td>0.056</td>
<td>1.93</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.034</td>
<td>0.034</td>
<td>0.034</td>
<td>0.034</td>
<td>0.034</td>
<td>0.034</td>
<td>0.97</td>
<td>0.034</td>
<td>0.97</td>
<td>88.9</td>
</tr>
<tr>
<td>Cysteic acid (^{a}) (^{b})</td>
<td>0.011</td>
<td>0.116</td>
<td>0.115</td>
<td>0.123</td>
<td>0.116</td>
<td>0.108</td>
<td>3.46</td>
<td>0.110</td>
<td>3.29</td>
<td>99.1</td>
</tr>
</tbody>
</table>

---

\( ^{a} \) Cysteic acid value was obtained after acid hydrolysis of performic acid oxidized thyrocalcitonin in a separate experiment.

\( ^{b} \) Yield calculated from asparagine/aspartic acid and cystine/cysteic acid; all values related to internal standards.

\( ^{c} \) Mole fraction extrapolated to zero hydrolysis time.

\( ^{d} \) Following acid hydrolysis of native thyrocalcitonin in mercaptoethanol, cystine is converted to cysteine; the reduced product co-elutes with proline, falsely elevating the true proline value.

Possibility of residues detectable only after extensive hydrolysis, the polypeptide was subjected to hydrolysis in 5.7N HCl at 110° for 24, 48, 72, and 96 hours, hydrolysis in acid with added 1:2000 (v/v) mercaptoethanol, and total enzymic digestion. The combined results from each method of hydrolysis are shown in Table III. These results indicated that thyrocalcitonin consists of 32 amino acids. Isoleucine and lysine were absent despite hydrolysis for as long as 96 hours; extrapolation of the values found for serine and threonine to zero time permitted correction for slight destruction of these amino acids.

Acid hydrolysis in mercaptoethanol provided integral values for tyrosine and methionine, correcting for the slightly submolar recovery of these amino acid residues achieved on analysis after acid hydrolysis without the presence of the reducing agent. The technique of total enzymic hydrolysis showed that the 4 aspartic acid residues found after acid hydrolysis were present as asparagine and that the single glutamic acid residue was present as the dicarboxylic amino acid rather than as glutamine. No trace of aspartic acid or glutamine was found. The enzymic technique also indicated the presence of 1 mole of tryptophan per mole of hormone.

Several tests were performed to detect the presence of any covalently bound organic or inorganic substituent in thyrocalcitonin. Direct analysis revealed that less than 0.3% of the weight of thyrocalcitonin was carbohydrate (as mannose); therefore, the carbohydrate found was less than that necessary to account for 6% substitution of one amino acid in the molecule. Iodine analysis revealed that less than 0.02% of the weight of the hormone was iodine—this indicated that less than 0.67% of tyrosine, histidine, or other residue could contain iodine. Analysis in duplicate of an aliquot of thyrocalcitonin, for total nitrogen, revealed 45 μg of Kjeldahl nitrogen; amino acid analysis of the exactly equivalent aliquot accounted for 43 μg of nitrogen as amino acid nitrogen (nitrogen content of thyrocalcitonin is 18%).

The good agreement achieved between enzymic and acid hydrolysis and the absence of unusual amino acids on the analyzer chromatograms after enzymic digestion provided further assurance that thyrocalcitonin consists of only unsubstituted amino acids. The amino acid composition of purified thyrocalcitonin isolated by these techniques is identical with the composition reported by Putter (6), Bell (5), MacIntyre (5), Kahnt (30), and Franz (33).

**End Group Analysis**—Although it has been previously reported that the amino terminal amino acid of thyrocalcitonin has a dantoin derivative of cysteic acid had been formed after reaction with thiocyanate ion, and that the single amino terminal amino acid of thyrocalcitonin was examined by the Edman procedure, a high yield of the phenylthiobenzoyl derivative of S-carboxymethylcysteine was found. Analysis by mass spectrometry revealed that the phenylthiobenzoyl derivative of cysteic acid had been formed after reaction
of oxidized thyrocalcitonin with phenylisothiocyanate. Thus, the intrachain disulfide bridge is amino-terminal. In addition, the finding of only a single amino-terminal amino acid provided further proof of the purity of the isolated peptide.

**Chemical Properties**—An estimate was made of the molecular weight of thyrocalcitonin, based on the Sephadex G-50 elution characteristics of the hormone compared with peptides of known molecular weight (Fig. 8). Thyrocalcitonin eluted shortly after adrenocorticotropic hormone, mol wt 4500, and much earlier than characteristics of the hormone compared with peptides of known molecular weight (Table III), 3604 containing 1 mole of tyrosine and 1 mole of tryptophan per mole of peptide.

The physical state of the thyrocalcitonin molecule in solution was examined by studying the solvent accessibility of tyrosine, methionine, and tryptophan.

The solvent accessibility of methionine and tryptophan in thyrocalcitonin was evaluated by reacting the molecule with iodoacetic acid or 2-hydroxy-5-nitrobenzyl bromide respectively in the absence as well as the presence of 8 M urea. Total enzymic digestion and analysis of 0.04 μM of each product formed in the absence of urea revealed less than 5% residual methionine or tryptophan. In addition, an aliquot of the tryptophan-modified derivative formed without urea was treated with 2 N NaOH to increase the pH of the solution to 12.5. The absorbance increase at 410 μM was equivalent to 0.9 mole of tryptophan adduct per mole of thyrocalcitonin. Therefore, the tryptophan and methionine residues of the molecule were completely modified with specific reagents at pH 3 without, as is often necessary with highly folded proteins (23), exposure of the molecule to strongly denaturing solvents such as urea. These preliminary findings suggest that a high degree of ordered structure does not exist in the region of the molecule adjacent to these residues. On the other hand, thyrocalcitonin undoubtedly does not exist in solution as a completely unfolded, random coil, devoid of any specific conformation since some degree of ordered structure must be imposed on the molecule by the presence of the intrachain disulfide bridge forming a 23-membered ring at the amino terminus of the molecule. Further detailed studies will be necessary to evaluate the conformation of the thyrocalcitonin molecule in aqueous solution.

**CONCLUSION**

Recently, the complete amino acid sequence of thyrocalcitonin was reported by this laboratory utilizing material purified by the methods outlined above (37). An identical amino acid sequence has also been independently reported by Bell et al. (38). Now that the amino acid sequence of thyrocalcitonin has been determined, the synthesis of fully active hormone and structural analogues of the peptide should shortly follow. In fact, considerable progress has been made in synthesis efforts but details of this work are not reported (38).

However, recent studies have emphasized that highly purified thyrocalcitonin may lose biological activity rapidly with simple incubation in aqueous solvents particularly at alkaline pH; furthermore, differences have been noted in the stability of certain preparations of the hormone initially of equivalent potency (39, 40). The information reviewed above, that thyrocalcitonin consists only of 32 unsubstituted amino acids in normal peptide linkage, devoid of a highly
ordered three-dimensional structure or any important cofactor, covalently or noncovalently bound, suggests that synthetic
material should have biological and chemical properties exactly
identical with the native molecule. However, particularly in
view of these recent reports concerning the stability of the biolo-
gical activity of thyrocalcitonin (39, 40) full appreciation of the

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REFERENCES
2. HIRSCH, P. F., GAUTHIER, G. F. AND MUNSON, P. L., Endo-
   crinology, 73, 244 (1963).
6. PUTTER, L., KACZKA, E. A., HARMAN, R. E., RICKES, E. L.,
   KREMY, A. J., CHAETZ, L., ROTHERICH, J. W., WASE, A. W.,
7. POSTS, J. T., JR., REIFIELD, R. A., HIRSCH, P. F., WASTED,
8. SCHLUETER, J. R., AND A. L. CATIONW, Jr., Endocrinology,
   81, 854 (1967).
9. COOPER, C. W., HIRSCH, P. F., TOVEYD, S. U., AND MUNSON,
   P. L., Endocrinology, 81, 610 (1967).
    (1967).
12. CHRAMBACH, A., REIFIELD, R. A., WYCOFF, M., AND ZACCARI,
13. HUBBARD, R. W., AND KRAMER, A. M., Anal. Biochem., 12,
    293 (1965).
    (1965).
15. BENSON, J. Y., JR., GORDON, M. J., AND PATTERSON, J. A.,
    Anal. Biochem., 18, 228 (1967).
17. VANNIER, W. E., AND CAMPBELL, D. H., J. Allergy, 32, 36
    (1961).
18. DUBOIS, M., GILLIS, K. A., HAMILTON, J. K., RECKERS, P.
19. KEBAT, E. A., AND MAYER, M. M., Experimental immuno-
    chemistry, Ed. 2, Charles C Thomas, Publisher, Springfield,
    (1961).
21. NEUMANN, N. P., MOORE, S., AND STEIN, W. II., Biochemistry,
    1, 68 (1962).
    242, 5771 (1967).
26. EDMAN, P., AND BENDZ, G., Experientia, J. Biochem., 1, 80
    (1907).
27. PISANO, J. J., VANDENHEEVE, W. J. C., AND HORNING, E. C.,
28. NEUMANN, N. P., MOORE, S., AND STEIN, W. II., Biochemistry,
    1, 68 (1962).
29. BREWER, H. B., JR., KEUTMANN, H. T., REIFIELD, R., MUN-
    SON, P., SCHLUETER, J. R., AND POSTS, J. T., Jr., Fed. Proc.,
    27, 690 (1968).
30. KAHNT, K. W., RINKER, B., MACINTYRE, I., AND NEHER, R.,
31. DEDMAN, M. L., FARMER, T. H., AND MORRIS, C. J. O. R.,
33. BELL, J. F., BARE, W. F., JR., COLUCCI, D. F., DAVIES, M. C.,
35. KAHNT, K. W., RINKER, B., MACINTYRE, I., AND NEHER, R.,
36. DEDMAN, M. L., FARMER, T. H., AND MORRIS, C. J. O. R.,
37. MACINTYRE, I., FOSTER, G. V., AND KUMAR, M. A., in P. J.
38. MACINTYRE, I., FOSTER, G. V., AND KUMAR, M. A., in P. J.
39. MACINTYRE, I., FOSTER, G. V., AND KUMAR, M. A., in P. J.
Isolation and Chemical Properties of Porcine Thyrocalcitonin
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