*THE ISOLATION AND CHARACTERIZATION OF THYROCALCITONIN**

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Much recent evidence[1-6] indicates that the thyroid gland contains a hypocalcemic principle, thyrocalcitonin, which may be released from the gland under appropriate circumstances and play an important role in calcium homeostasis.

The extraction and partial purification of this principle has been reported.[4, 5] These reports imply, but do not establish, that thyrocalcitonin is a polypeptide or small protein, the biological activity of which is readily lost.

The purpose of the present communication is to describe the isolation and characterization of a thyrocalcitonin from porcine thyroid glands. Our evidence indicates that thyrocalcitonin is a single polypeptide chain with a molecular weight of approximately 8700.

Experimental.—Freshly collected, porcine thyroid glands were frozen with solid CO_2, stored up to 3 weeks at −15°C, and ground through a meat grinder into cold acetone containing sodium EDTA 30 mg/l. The ground material was extracted 10 times with cold acetone, 6 times with cold chloroform, and again 10 times with cold acetone. This dehydrated and defatted powder was carefully dried at room temperature and then stored at 4°C. It was the starting material for subsequent isolation work. Extraction was carried out using a method developed in this laboratory for the extraction of polypeptides from bovine parathyroid glands.[7] Dried, defatted thyroid powder was treated initially with a solution of urea-HCl-cysteine, followed by solvent and salt fractionation, precipitation of the active material with trichloroacetic acid (TCA), and recovery of this TCA precipitate as a lyophilized powder (TCA powder).

This TCA powder was further purified by gel filtration on Sephadex. Initially, columns of Sephadex G-100 were employed, but better resolution was obtained with Sephadex G-75. Five hundred mg were dissolved in 5 ml of buffer and applied to a 2.5 × 130-cm column of G-75 Sephadex (bead form, Pharmacia). The developing buffer (0.2 *M* ammonium acetate, pH 4.6) was allowed to flow by gravity through the column at a flow rate of 48 ml/hr. Fractions were collected at 8-min intervals. Column and gel preparation, fraction analysis, and starch gel electrophoresis were performed as previously described.[7] Amino acid analysis was done with a Technicon amino acid analyzer (Technicon Inc., Chauncey, N. Y.). Performic acid oxidation was carried out as described by Moore.[8] The N-terminal amino acid was determined by the methods of Sanger,[9] and by the Konigsberg and Hill modification of the Edman procedure.[10] Tryptophan was measured by the method of Goodwin and Morton.[11] Sulfhydryl titration was done by a modification of the method of Benesch, Lardy, and Benesch.[12] Molecular weight was determined by equilibrium centrifugation according to a modification of the method of Yphantis,[13] assuming a partial specific volume of 0.7.

Thyrocalcitonin activity was assayed by one of two methods: either the procedure of Hirsch, Voelkel, and Munson,[4] or by a method developed in this laboratory.[14] In the latter technique, the preparation to be tested was infused at a constant rate into the jugular vein of a conscious rat. One and one-half hr after the start of the infusion a sample of blood was obtained by heart puncture and was analyzed for its content of calcium and phosphate as previously described.[15] The decrease in the plasma calcium concentration from control levels was found to be a precise and direct index of hormonal activity, and a satisfactory dose response relationship was obtained.

Results.—The extraction of 200 gm of the dried acetone-chloroform-treated porcine thyroid tissue yielded 1.2–1.4 gm of TCA product. This material possessed the hypocalcemic activity as illustrated in Figure 1. Gel filtration of 500 mg of the TCA product on Sephadex G-75 led to the elution pattern illustrated in Figure 2.

The highest specific biologic activity was found in the fraction marked by the tubes 92 through 102. The biological activity of this material was 5–15 times greater than that of the TCA powder (Fig. 1). The reason for the nonparallelism of the log dose plots of the crude versus the purified material is not clear at the present time.

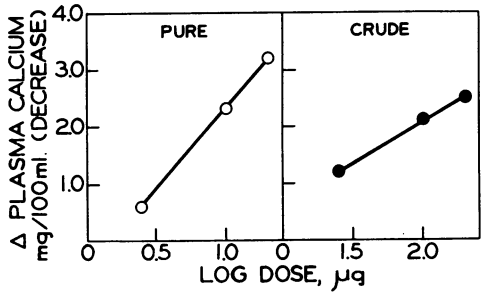

FIG. 1.—A plot of log dose of thyrocalcitonin versus response in 100-gm rats. On the right is shown the curve obtained with a crude TCA powder, and on the left that obtained with the purified hormone. Ten μg (log 1.0) of the purified material (*left*), gave a response similar to 100 μg (log 2.0) of crude material (*right*).

The Sephadex product appeared to be homogeneous when subjected to starch gel electrophoresis (Fig. 3) and had a single N-terminal threonine, as determined by the method of Sanger. Edman degradation, followed by amino acid analysis of the residual peptide, showed loss of a residue of threonine as well as a lysine; however, no di-DNP lysine was found by the Sanger procedure. The average values of 6 amino acid analyses (duplicate studies of the pure products of each of 3-column runs) of 22-hr hydrolyzates and the average values of two analyses of a 72-hr hydrolyzate are recorded in Table 1. From these data, a tentative empirical formula was calculated: Lys_8, His, Arg_4, Thr_1, Ser_4, Glu_{10}, Pro_4, Gly_5, Ala_8, Val_4, Meth, $^1/_2$ Cys, $Ileu_3$, Leu_9, Tyr_2, Phe_2, Trypt, and $(-CONH_2)_7$. The molecular weight estimated from the amino acid composition was 8,666, whereas that obtained by sedimentation analysis in the presence of mercaptoethanol was 8,700. A simultaneous determination in the absence of mercaptoethanol gave a value of 9,700. The presence of a free sulfhydryl group could not be detected by amperometric titration at pH 7.0 in the presence or absence of 7 *M* urea. However, the amino acid analyses of both the native and performic acid-oxidized peptide (Table 1) indicated the presence of a single cysteine residue.

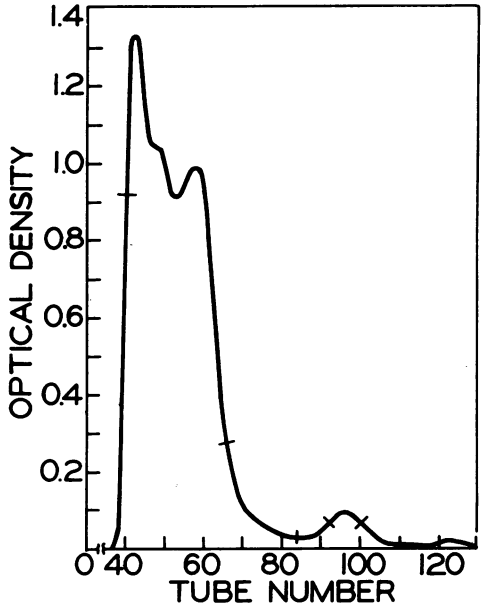

FIG. 2.—Gel filtration of crude thyroid extract on a Sephadex G-75 column 2.5 × 130 cm. The eluant was 0.2 *M* ammonium acetate, pH 4.6. The peak observed in tubes 92–102 contained the major portion of the biological activity.

Discussion.—On the basis of present evidence, the polypeptide isolated appears relatively homogeneous. The molecular weight estimates from equilibrium centrifugation and amino acid analysis are in good agreement. The material appears homogeneous upon starch gel, gives a single N-terminal amino acid by the Sanger method, and gives amino acid analyses which are reproducible from one preparation

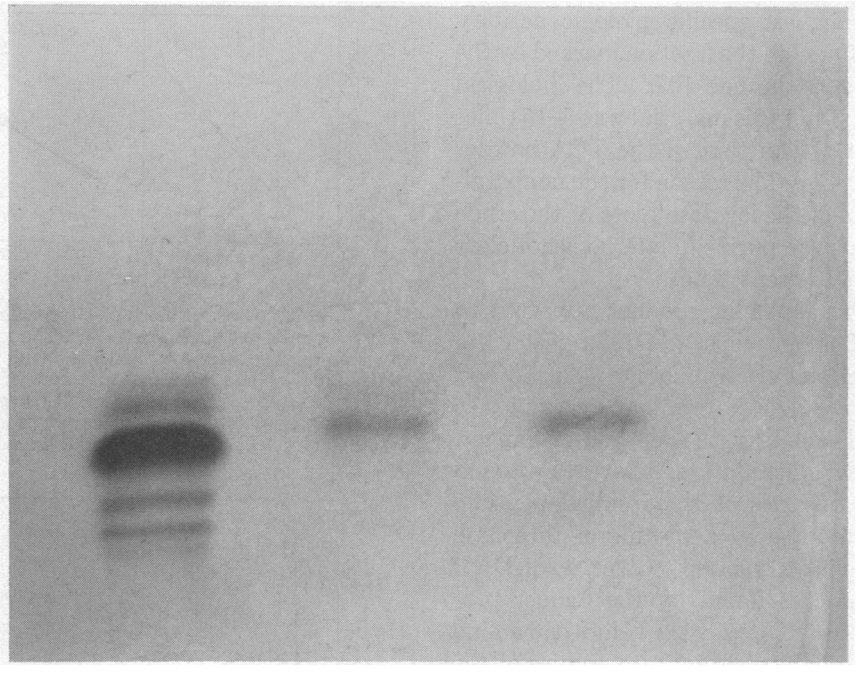

FIG. 3.—The patterns obtained on starch gel electrophoresis when crude and purified preparations of thyrocalcitonin were analyzed. *Left*, crude TCA powder; *middle*, active fraction from gel filtration on G-100; *right*, active fraction from gel filtration on Sephadex G-75. The material on the right is the substance which was further characterized.

to the next. There remains the fact that, by the method of Konigsberg and Hill,[10] a lysine as well as a threonine residue was lost during cyclization and hydrolysis. However, in view of the fact that the epsilon-amino group of lysine reacts with the Edman reagent and that complete regeneration of free lysine is not always obtained,[10] the presence of a single N-terminal threonine is highly probable.

Of particular interest is the finding of a single half-cystine residue or, after performic acid oxidation, a single cysteic acid residue. This led naturally to the attempt to demonstrate a free sulfhydryl group. However, it was not possible to do so even if the peptide was dissolved in 7 *M* urea. Three alternatives appear possible: either (1) the SH group is present but becomes oxidized rapidly; (2) the sulfur atom of the cysteine residue is in a thioester linkage or thiazoline ring structure; or (3) the cysteine is linked to a mercaptan in an S-S linkage similar to that recently found in the protease from streptococci.[16] The first possibility is made unlikely by the fact that incubation of the peptide with N-ethylmaleimide at pH 6.0 under nitrogen did not lead to any change in biological activity. The other two possibilities are being investigated.

Thyrocalcitonin is highly active biologically in that 50 mμg/min infused into a 100-gm rat led to a significant (1 mg/100 ml) decrease in the concentration of the plasma calcium within 60 min. In addition to lowering the concentration of the plasma calcium, infusion of the purified material also led to a fall in the concentration of the plasma phosphate. A similar fall in plasma phosphate has been observed after the administration of crude extracts of porcine thyroid tissue.[4]

TABLE 1

AMINO ACID COMPOSITION OF THYROCALCITONIN

	Residues per Molecule				
Amino acid	22 Hr	72 Hr	22 Hr oxidized	Calculated	Peptide residue after Edman degradation
Cysteic acid	—	—	1.07	1	—
Aspartic acid	7.06	6.86	—	7	6.83
Threonine	3.69	3.26	3.95	4	3.26
Serine	3.76	3.00	3.48	4	4.06
Glutamic acid	9.72	9.25	—	10	9.51
Proline	4.36	4.86	—	4	4.89
Glycine	5.22	5.06	5.42	5	5.60
Alanine	7.82	7.83	7.95	8	7.69
Valine	4.24	4.20	4.25	4	4.12
Cystine	0.50	—	—	0.50	—
Methionine	0.90	0.67	—	1	1.08
Isoleucine	2.79	2.76	2.84	3	2.71
Leucine	9.15	8.96	9.27	9	8.98
Tyrosine	2.22	2.00	—	2	2.03
Phenylalanine	2.13	2.20	1.99	2	2.28
NH_3	8.03	10.16	—	7	—
Lysine	8.38	8.29	8.08	8	7.08
Histidine	1.22	1.27	1.02	1	1.32
Arginine	3.60	3.48	3.62	4	3.66
Tryptophane	1.00	—	—	1	—

The terms in the column headings are the intervals of hydrolysis. The column headed "22 Hr oxidized" is the analysis of the performic acid-oxidized sample. The residues recorded in the column headed "Calculated" represent values corrected by extrapolation to 0 time. The final column represents the amino acid analysis of the peptide carried through one Edman degradation cycle.

The availability of a homogeneous preparation of thyrocalcitonin now makes possible a systematic investigation of its mode of action and an immunochemical approach to its measurement in biologic fluids. Hopefully, such studies will lead to a better understanding of the role of this agent in calcium homeostasis.

Summary.—The isolation and characterization of thyrocalcitonin from porcine thyroid tissue is described. The hormone is a polypeptide with a molecular weight of 8,700, a single N-terminal threonine, and contains a single cysteine residue in an unusual but as yet unidentified form.

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*CRYSTALLINE BACTERIAL LUCIFERASE FROM PHOTOBACTERIUM FISCHERI**

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Bacterial luminescence has been shown to involve $FMNH_2$, a straight-chain fatty aldehyde, O_2, and bacterial luciferase.[1-3] Progress has also been made recently on the nature of some of the intermediates involved in the bioluminescent oxidation of $FMNH_2$ by bacterial luciferase.[4]

We wish to report the crystallization of bacterial luciferase by methods which result in high yields of the enzyme from starting material that can be stored for years without loss of activity. Evidence for the existence of active subunits and some properties of the enzyme are also reported.

Methods.—Photobacterium fischeri, American Type Culture Collection no. 7744, was routinely cultured at 25°C in a medium containing the following amounts of components per liter: 30 gm NaCl, 10 gm $Na_2HPO_4 \cdot 7H_2O$, 12 gm KH_2PO_4, 8 gm nutrient broth, and 3 ml glycerol. The resulting medium was brought to pH 7.2 by the addition of NaOH. Cells were grown in a 40-liter Biogen culture apparatus, and acetone powders were prepared from the cells as previously described.[5] The acetone powder may be stored for years at −20°C without loss of activity.

Preparation of crude extract and purification of luciferase: The following is a brief outline of the purification procedure, the details of which will be published elsewhere. Routinely, 200 gm of acetone powder were extracted with 3500 ml of 0.05 *M* Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, which contained 0.001 *M* mercaptoethanol (phosphate-MSH). The extraction was carried out by grinding with sand in a mortar at 2–4°C. The extract was centrifuged at 14,000 × *g* for 30 min and the supernatant referred to as "crude extract." Purification of crude extract involved addition of streptomycin sulfate to a final concentration of 1.75% (w/v) followed by centrifugation at 14,000 × *g* to remove nucleic acids. The supernatant was incubated for 30 min at 25°C in the presence of 20 mg each of DNAase and RNAase. The protein was precipitated with ammonium sulfate (0–80% saturation)[6] and dissolved in phosphate-MSH. When this enzyme solution was then fractionated with saturated ammonium sulfate, pH 6.6, the bulk of luciferase activity appeared in the 45–65% saturation fraction. The stock solution of saturated ammonium sulfate was prepared at 2–4°C. The 0–45 and 65–80 fractions were recombined and refractionated, and the 45–65 fraction thus obtained was combined with the original 45–65 fraction. The combined 45–65 fractions were dissolved in, and dialyzed overnight against 0.01 *M* Tris-HCl buffer, pH 7.3, which contained 0.005 *M* magnesium acetate, 0.001 *M* EDTA (ethylene diamine tetraacetic acid), and 0.001 *M* mercaptoethanol (Tris-MSH). The total volume was 300 ml. This dialyzed enzyme was chromatographed on a 3.5 × 50-cm DEAE-cellulose column. Elution of luciferase from the column was achieved by use of a linear gradient consisting of Tris-MSH in the mixing chamber and an equal volume of the same buffer containing 1 *M* NaCl in the reservoir. Ten-ml fractions