Porcine Calcitonin. Simple Procedure for Isolation in High Yield*

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ABSTRACT: A procedure for the isolation of calcitonin from porcine thyroid glands which offers the advantages of simplicity, economy, and high yield (40–60%) is presented. It involves three major steps: (1) extraction of calcitonin from dried defatted glands with 1-butanol–acetic acid–water (12:3:5), followed by batchwise adsorption of contaminants with silicic acid and then trichloroacetic acid precipitation of biologic activity, (2) gel filtration of the trichloroacetic acid precipitate on Bio-Gel P-6, and (3) gel filtration on Bio-Gel P-2. The final product has a biopotency and an amino acid composition essentially identical with those previously reported for porcine calcitonin (Beesley, T. E., Harman, R. E., Jacob, T. A., Homnick, C. F., Bitali, R. A., Veber, D. F., Wolf, F. J., Hirschman, R., and Denkewalter, R. G. (1968), *J. Am. Chem. Soc. 90*, 12; Bell, P. H. Barg, W. F., Jr., Colucci, D. F., Davies, M. C., Dziobkowski, C., Englert, M. E., Heyder, E., Paul, R., and Snedeker, E. H. (1968), *J. Am. Chem. Soc. 90*, 2704; Franz, J., Rosenthaler, J., Zehnder, K., Doepfner, W., Huguenin, R., and Guttmann, S. (1968), *Helv. Chim. Acta 51*, 218; Kahnt, F. W., Riniker, B., MacIntyre, I., and Neher, R. (1968), *Helv. Chim. Acta 51*, 214; Potts, J. T., Jr., Niall, H. D., Keutmann, H. T., Brewer, H. B., Jr., and Deftos, L. J. (1968), *Proc Natl. Acad. Sci. U. S. 59*, 1321). This material migrates as two components on disc gel electrophoresis but preliminary studies indicate that these components represent oxidoreductive changes, not involving the methionine residue, of a single component. The ease and economy with which procine calcitonin can be isolated by the procedure described make feasible the production of gram quantities of the hormone under industrial conditions, which would satisfy present research and potential future therapeutic needs.

Calcitonin is a hypocalcemic, hypophosphatemic principle which has been extracted from thyroid glands of all mammalian species studied (Tenenhouse *et al.*, 1968); recently it also has been extracted from the ultimobranchial glands of birds (Tauber, 1967) and amphibians and teleost fish (Parkes and Copp, 1968). Its role as a hormone in the regulation of calcium and phosphate metabolism has been recently reviewed (Tenenhouse *et al.*, 1968). The major biologic action of calcitonin is probably the inhibition of bone resorption, and serious consideration has been given to the hormone as a therapeutic agent for the treatment of osteopenic bone disease.

The covalent structure and complete amino acid sequence of porcine calcitonin have been recently elucidated (Beesley *et al.*, 1968; Bell *et al.*, 1968; Neher *et al.*, 1968; Potts *et al.*, 1968; Franz *et al.*, 1968). As extracted from porcine thyroid glands, it is a single-chain polypeptide with 32 amino acids and a calculated molecular weight of 3700. There is a 1–7 intrachain disulfide bridge and the carboxy-terminal amino acid is prolinamide. Synthesis of the entire molecule has been accomplished (Guttmann *et al.*, 1968; Rittel *et al.*, 1968). However, it is not anticipated that sufficient quantities of the synthetic polypeptide will be available for general experimental and therapeutic use in the near future. Poor yields and prolonged and complex isolation techniques have characterized all of the published procedures for the preparation of highly purified calcitonin from porcine thyroid glands (Tenenhouse *et al.*, 1968).

The present report gives a detailed description of a new procedure for the isolation of calcitonin from porcine thyroid glands. The method is relatively simple and inexpensive to perform, is capable of being scaled up for larger quantities, and has high yields. The final product has an amino acid composition which is the same as that previously reported for porcine calcitonin but it migrates as two components on disc gel electrophoresis. Preliminary work indicates that these two components represent oxidoreductive changes of a single component.

Experimental Procedure

Preparation of Dried Defatted Tissue. Fresh porcine thyroid glands were quickly frozen in solid carbon dioxide as soon as they were excised and were stored for up to 1 year at −15°. They were finely ground in the frozen state, lyophilized, extracted ten times with five volumes of cold chloroform and once with cold acetone, and then dried at room temperature overnight. The dried defatted powders were stored at −15° and were used as needed for all subsequent extraction procedures.

Extraction Methods. No modification other than the drying and defatting of thyroid glands was made in the 8 M urea–0.2 M HCl–trichloroacetic acid procedure described by Tenenhouse and associates (1965) for the extraction of calcitonin from porcine thyroid tissue. This material, "crude urea–trichloroacetic acid ex-

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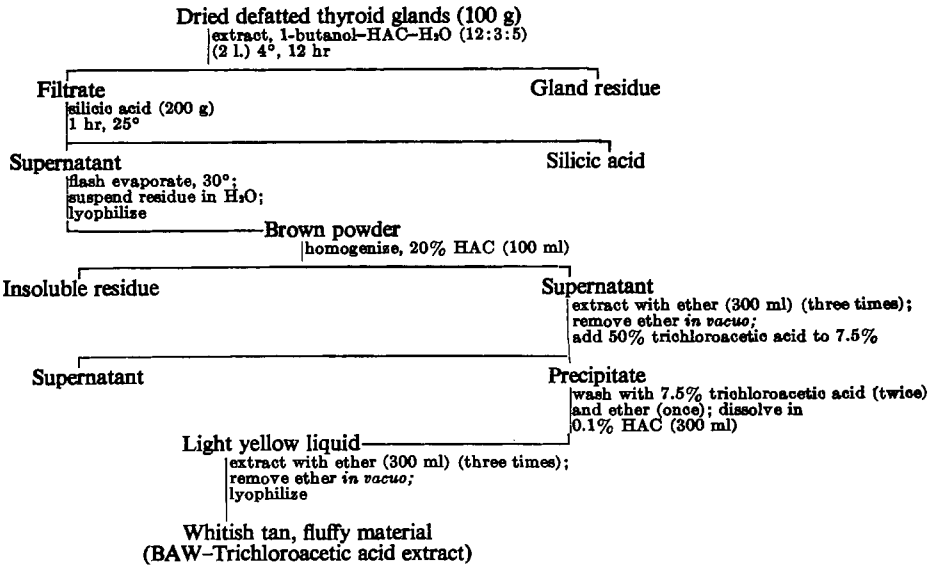
SCHEME I: 1-Butanol–Acetic Acid–Water Extraction of Calcitonin from Porcine Thyroid Glands.



tract," was used in comparison studies with the extract prepared by the newly developed procedure.

The observation that thin-layer chromatography of partially purified (urea–trichloroacetic acid extracted) calcitonin preparations on silica gel in a 1-butanol–acetic acid–water solvent system resulted in rapid mobility of biologic activity whereas the majority of contaminating proteins remained at the site of application suggested that this solvent system might be a useful extractant of calcitonin biologic activity from thyroid tissue and that silicic acid used in a batchwise fashion might serve to remove contaminants when the hormone was in an acidic alcohol environment. Accordingly, an extraction scheme (Scheme I) was devised based on these principles with the addition of a trichloroacetic acid precipitation step. The following description of the procedure indicates quantities of reagents used for the extraction of 100-g aliquots of dried defatted glands, but as much as 10.0 kg of starting material, has been successfully processed for us by the Upjohn Co. All reagents are reagent grade; 1-butanol is redistilled; ether is fresh and peroxide free; and water is glass distilled.

Dried defatted gland powder is homogenized in a Waring blender for 0.5–1 min with 2 l. of butanol–glacial acetic acid–water (12:3:5). The mixture is mechanically stirred for 12–24 hr at 4° and then filtered under strong manual pressure through three layers of cheesecloth. The gland residue is discarded. The remainder of the procedure except for column chromatography is carried out at room temperature. Silicic acid (200 g, Bio-Sil, 100–200 mesh) is then added to the dark brown filtrate and stirred mechanically for 1 hr. The silicic acid, which becomes tannish brown, is then removed by centrifugation. The 1-butanol is removed by flash evaporation at 30° with frequent addition of water. The result of this procedure is a mass of dark brown material which is suspended in water, shell frozen, and lyophilized.

The lyophilized brown powder is suspended in 100 m of 20% acetic acid and the mixture is homogenized in a Virtis "45" homogenizer for 1 min at "medium" setting. The mixture is centrifuged at 5000*g* for 15 min and the supernatant is removed by careful aspiration and saved (20% acetic acid extract 1). The dark brown residue is reextracted with 300 ml of 20% acetic acid in the same way (20% acetic acid extract 2). These supernatants are translucent, deep yellow, and, depending upon the care with which centrifugation and aspiration are carried out, more or less free of fine particulate brown material. They are processed in the same way thereafter, but separately (see Results for rationale).

First they are extracted three times with two volumes of ether and the residual ether is removed by rotary vacuum evaporation. Trichloroacetic acid, 50%, is then added slowly with mechanical stirring to a final concentration of 7.5%. A fine whitish gray precipitate forms and is collected by centrifugation at 5000*g* for 15 min. The translucent, yellow supernate is discarded. The precipitate is washed twice with 7.5% trichloroacetic acid and once with a small amount of ether and is then dissolved in 300 ml of 0.1 N acetic acid. The resulting translucent, light yellow solution is extracted three times with an equal volume of ether. The ether is removed by rotary vacuum evaporation and the solution is shell forzen and lyophilized. The final dry material, termed "BAW[1]–trichloroacetic acid extract," is whitish tan and fluffy.

Fractionation on Porous Polyacrylamide Gels. Pyrex glass columns (2 × 200 cm) were used after they were coated twice with dimethyldichlorosilane (1% in benzene) as suggested by the manufacturer of Bio-Gel materials. Dry Bio-Gel P-6 (100–200 mesh) was washed with 0.2 M ammonium acetate buffer at pH 4.6 (eluting

[1] "BAW" stands for butanol–acetic acid–water.

buffer) three to five times and then equilibrated with it at 4° for 24 hr. Columns were poured as a slurry and washed with eluting buffer for 24 hr before use. Flow was maintained by hydrostatic column of about 70 cm.

Both the urea–trichloroacetic acid and the BAW–trichloroacetic acid extracts were homogenized in 10–15 ml of 0.1 N acetic acid with a Virtis homogenizer because they did not dissolve well in eluting buffer (urea–trichloroacetic acid extract, usually 50 mg/ml, and BAW–trichloroacetic acid extract, 10–20 mg/ml). These mixtures were centrifuged at 5000*g* for 15 min and the residue was reextracted with 5 ml of 0.1 N acetic acid. The supernatants were pooled, applied to the column, and washed in three separate portions of equal volume of buffer. Column eluates were read at 277 mμ in a Zeiss PMQ II spectrophotometer. All column preparation and chromatography was done at 4°. Preparation of columns of Bio-Gel P-2 was done in the same way except that column dimensions were 2 × 100 cm and the eluent was 0.1 N acetic acid. Samples were dissolved in 5 ml of 0.1 N acetic acid and applied in a concentration of 10 mg of protein/ml.

Carboxymethylcellulose Chromatography. After removal of fines, carboxymethylcellulose (Whatman microgranular CM 32) was prepared as suggested by the manufacturer. It was equilibrated by washing with 0.1 M ammonium acetate. Columns (0.9 × 100 cm) were poured as a slurry. Samples which had been desalted on columns of Bio-Gel P-2 were lyophilized, dissolved in 1–2 ml of 0.1 M ammonium acetate, and applied to these columns. Linear gradients were developed by using two vessels, one containing 150 ml of 0.1 M ammonium acetate and one containing 150 ml of 1 M ammonium acetate.

Biologic Assay for Calcitonin. Hypocalcemic activity of calcitonin was determined in 60–80-g male Sprague–Dawley rats bred and raised at this institution and given a commercial low-calcium diet (General Biochemicals) for 1.5–3 days before use. Test materials and standard preparations were injected into the external jugular vein in 0.5 ml of a 1% bovine serum albumin–20 mM NaCl solution while the animals were lightly anesthetized with ether. Blood was obtained by cardiac puncture 70 min later. The calcium concentration in the plasma was measured by atomic absorption spectrophotometry.

Urea–trichloroacetic acid extract (0.5 MRC unit/mg) served as a standard and was calibrated in four separate four-point assays against MRC research standard A.[2] At least three doses (2.5, 5, and 10 munits) of this calibrated standard were included in all assays except those in which column fractions were being scanned for biologic activity. Assay results were highly reproducible and an index of precision, λ, of 0.11 was obtained in a series of 40 consecutive experiments. Specific biologic activity is expressed in terms of MRC units per milligram weight of protein. Protein was determined by the method of Lowry and associates (1951).

TABLE I: Comparison of Yields and Specific Biologic Activities of Crude Porcine Calcitonin Extracts: Urea–Trichloroacetic Acid *vs.* BAW–Trichloroacetic Acid Procedures.

Extraction Method[a]	Yield (mg)	Sp Act. (MRC units/mg)	Yield (MRC units)
Urea–trichloro-acetic acid[a]	900	0.4	360
	1020	0.3	306
	920	0.4	368
Mean	947	0.37	345
BAW–trichloro-acetic acid[b]	110	7.4	814
	105	8.0	840
	115	7.1	817
Mean	110	7.5	824
Difference factor[c]		20.3	2.4

[a] Both methods were applied to three 200-g aliquots of the same batch of dried defatted porcine thyroid powder. [b] From 20% acetic acid extract 1. [c] Ratio BAW–trichloroacetic acid product/urea–trichloroacetic acid product.

Analytical Disc Gel Electrophoresis. Of the several procedures and buffer systems studied, the system described by Reisfeld and coworkers (1962) produced the most consistent and reproducible results with the highest resolution. It was routinely used without modification except for the introduction of 6 M urea into all of the gel solutions. Fifteen per cent running gels (pH 4.3) were used. Samples were usually dissolved in the upper gel solution before application. At 6–7 mA/tube, running times ranged from 1 to 1.2 hr. Gels were always run in duplicate with between 100 and 400 μg of sample per tube. Staining was done with Amido Black for 1 hr, and destaining was accomplished by electrophoretic means.

Amino Acid Analysis. Amino acid analyses were done using a Beckman–Spinco automatic amino acid analyzer, Model 120B. Hydrolysis of samples was performed either with 6 N HCl at 110° for 22 hr after performic acid oxidation according to the method of Hirs (1967) or by the alkaline method of Neumann (1967) for the analysis of methionine sulfoxides. Tryptophan was determined by the method of Goodwin and Morton (1946).

Results

Extraction Procedure. The new method described herein results in a crude product (BAW–trichloroacetic acid extract) which has specific biologic activity 15–30 times greater and yields of approximately 2–3 times greater than provided by the urea–trichloroacetic acid procedure (Table I). This comparison is based on the

[2] Specific biologic activity of calcitonin is given in terms of an established standard preparation "Research Standard A" (Division of Biological Standards, National Institute for Medical Research, Mill Hill, London). According to convention, units of activity are designated MRC-A units.

TABLE II: Recovery of Protein and Biologic Activity at Major Stages of BAW–Trichloroacetic Acid Extraction.

Stage	Total Protein (mg)	Sp Act. (MRC units/mg of protein)	Total Biol Act. (MRC units)	Biol Act. Lost (%) per Step	Purifcn Factor, Over-all
Filtrate of saline extraction	22,000	0.007	154		
Filtrate of BAW extraction	857	0.4	343		57
Supernatant after silicic acid treatment	90	3.0	270	21	430
BAW–trichloroacetic acid extract	27	9.4	254	6	1340

BAW–trichloroacetic acid product derived from the first extraction with 20% acetic acid (20% acetic acid extract 1). Additional activity can be obtained from 20% acetic acid extract 2. The specific biologic activity of this latter material is less but still is 5–10 times that of the urea–trichloroacetic acid product and represents an activity yield at least equal to that obtained in a single extraction of thyroid powder by the urea–trichloroacetic acid method. If the total yield of the BAW–trichloroacetic acid procedure is calculated on the basis of the products of both acetic acid extracts, it approximates four times that of the urea–trichloroacetic acid procedure. Most of our purification work has been done with the BAW–trichloroacetic acid product of high specific activity, but preliminary studies suggest that the material with lower specific activity is a mixture of the same protein components and behaves the same as the high specific activity product when subjected to gel filtration. The disc electrophoretic pattern of the product from 20% acetic acid extract 1 is shown in Figure 1A.

The biologic activity in the product of each major step of the BAW–trichloroacetic acid extraction procedure was assayed to determine the efficiency of the method (Table II). In these experiments, three 50-g aliquots of dried defatted thyroid powder were carried separately but simultaneously through to one of three stages: filtrate after extraction of glands with 1-butanol–acetic acid–water, supernatant after removal of contaminants with silicic acid, and BAW–trichloroacetic acid extract. For assay, the intermediate products were flash evaporated, lyophilized, homogenized with 20% acetic acid, extracted extensively with ether, and then relyophilized. In the BAW–trichloroacetic acid procedure, it is striking

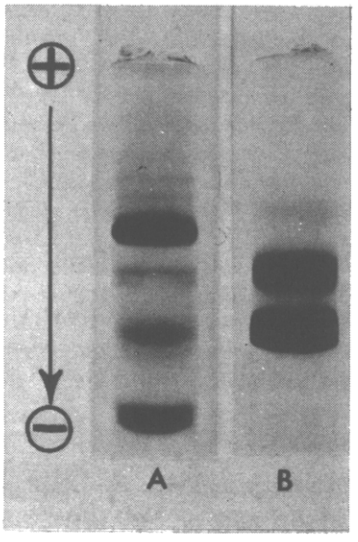


FIGURE 1: Analytical polyacrylamide disc gel electrophoresis of (A) 100 μg (protein) of BAW–trichloroacetic acid extract and (B) 200 μg (protein) obtained from any of the 27-ml pools of fraction III of the elution pattern shown in Figure 2.

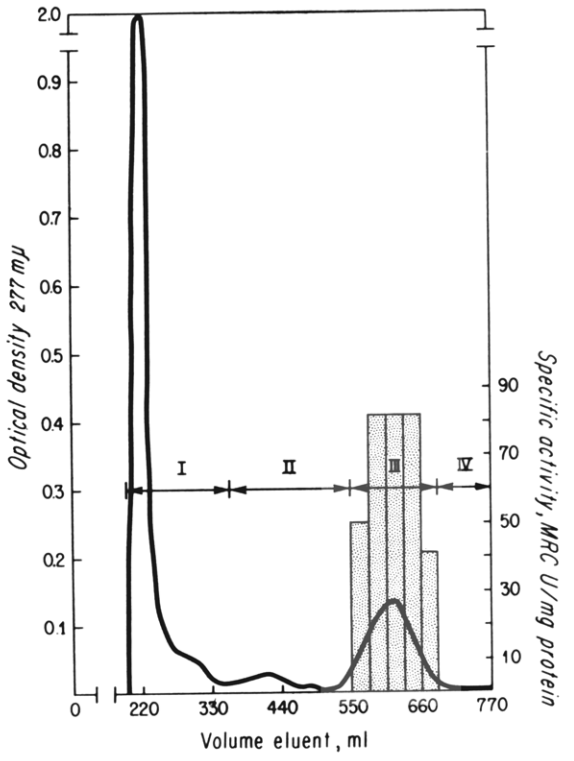


FIGURE 2: Elution pattern obtained when BAW–trichloroacetic acid extract (72 mg) was subjected to gel filtration on 2 × 200 cm column of Bio-Gel P-6. Conditions: eluent, 0.2 M ammonium acetate (pH 4.6); flow rate, 0.38 ml/min; fraction size, 5.5 ml; hydrostatic pressure head, 70 cm; temperature, 4°. Solid line represents absorbance at 277 mμ; numerals I–IV are lyophilized fractions; histogram shows specific activity values of sequential 27-ml pools.

TABLE III: Protein and Biologic Activity after Gel Filtration of BAW–Trichloroacetic Acid Extract on Bio-Gel P-6.

Fraction (Figure 2)	Total Protein (mg)	Sp Act. (MRC units/mg of protein)	Total Act. (MRC units)	% Act. Recovd in Fraction	Purifcn Factor
BAW–trichloroacetic acid extract	72	9.0	648		
I	57.0	0.2	11	2	−45
II	4.9	12.0	59	10	+1.3
III	8.3	60.0	498	86	+6.6[a]
IV	1.4	7.0	10	2	−0.78
Total	71.6		578		
Recovery (%)	99.5		89		

[a] Expected purification on basis of protein = 8.7; actual on basis of specific activity increase = 6.6.

that simple extraction of the glands with acidic alcohol produces a product which has a specific biologic activity comparable with the final product of the urea–trichloroacetic acid procedure.

Although the batchwise silicic acid adsorption of contaminants leads to the greatest loss of total biologic activity (21%), a 7.5-fold increase in specific biologic activity is achieved; without this step, the final product has a greatly decreased specific biologic activity. Practically all of the biologic activity lost at this step can be recovered by eluting the silicic acid with water. The material recovered has a specific biologic activity of 0.004 MRC units/mg of protein. We have not yet characterized this material further but have used it as a source of biologic activity in a number of physiologic screening experiments.

There is a negligible loss of total biologic activity between the silicic acid adsorption step and the final product, but a further increase in specific biologic activity of 3.1-fold is achieved. Total recovery of biologic activity has ranged from 50 to 80%.

Fractionation on Porous Polyacrylamide Gels. A typical elution pattern is shown in Figure 2. Superimposed is a histogram showing the specific biologic activities for five-tube pools (P-6 product). Data concerning recoveries, specific activities, and purification factors for each lyophilized fraction from this column are shown in Table III. The specific biologic activities of the three five-tube pools between elution volumes 577 and 660 ml were constant at 82 MRC units/mg of protein, and the disc gel electrophoretic patterns were identical (Figure 1B). Two major components are clearly visible: a darkly staining band with an R_F value (calculated from the mobility of methyl green) of 0.54 and a more lightly staining band with an R_F value of 0.4. Two barely visible components are also present, with R_F values in the range of 0.2–0.3. Recovery of virtually all protein and 89% of biologic activity and an actual purification factor of 6.6, compared with a theoretical factor of 8.7 on the basis of protein, was achieved with this procedure (Table III). Comparable results have been obtained in many similar experiments.

A comparison between the results obtained when 500 mg (250 MRC units) of urea–trichloroacetic acid extract and 72 mg (648 MRC units) of BAW–trichloroacetic acid extract were subjected to gel filtration on the same column of Bio-Gel P-6 indicated that (1) the total percentage recovery of biologic activity was greater with BAW–trichloroacetic acid extract (89 *vs.* 55%) and (2) a far greater proportion of biologic activity was re-

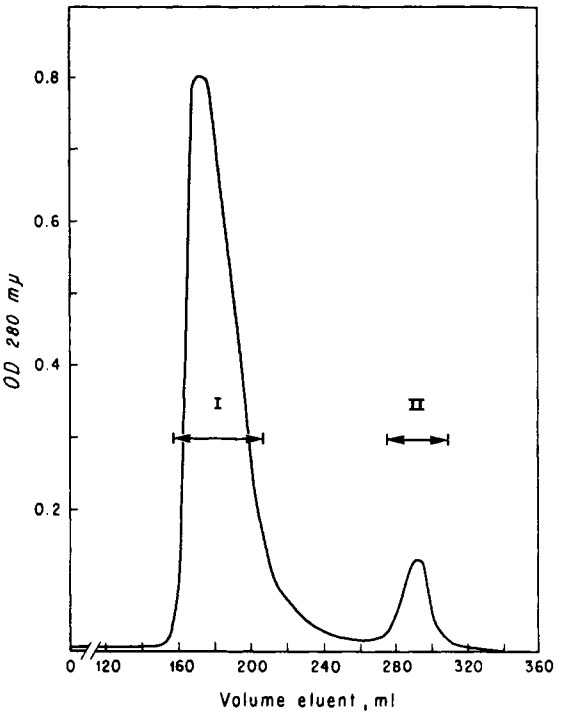


FIGURE 3: Elution pattern obtained when 60 mg of fraction III of Bio-Gel P-6 fractionation (Figure 2) was subjected to gel filtration on Bio-Gel P-2. Most of the biologic activity was recovered in fraction I.

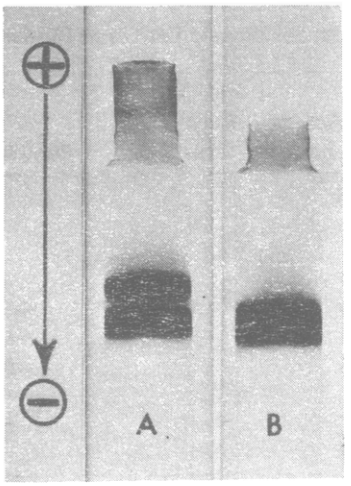


FIGURE 4: Analytical polyacrylamide disc gel electrophoresis of (A) fraction I from Bio-Gel P-2 fractionation (Figure 3) and (B) material obtained after cysteine reduction of fraction I.

covered in the low specific activity, nonretained fraction with the urea–trichloroacetic acid extract (67 *vs.* 2%). This is of considerable importance because, in our experience, it is only the retained fraction which can be further purified.

The elution pattern which was obtained when the P-6 product was run on columns of Bio-Gel P-2 is shown in Figure 3. Essentially all of the biologic activity applied to the column was recovered in fraction I (P-2 product). Polyacrylamide disc gel electrophoresis of this material (Figures 4A and 6A) showed that the two minor components of slow mobility present in the P-6 product (Figure 1B) had been removed. Fraction II from this column contained these two components. The P-2 product had a specific biologic activity of 120–150 MRC units/mg weight of protein.

Carboxymethylcellulose Fractionation of P-2 Product. The elution pattern which was obtained when the P-2 product was subjected to carboxymethylcellulose chromatography using a linear ionic gradient from 0.1 to 0.55 M ammonium acetate is shown in Figure 5. Two distinct protein peaks were obtained but separation was not complete. The results of polyacrylamide disc electrophoresis of the materials contained in fractions in the regions of 172 and 192 ml, respectively, are shown in Figure 6. Partial separation of the two components observed on the gel electrophoresis of the P-2 product (Figure 6) was achieved. The specific biologic activities of these two fractions were identical.

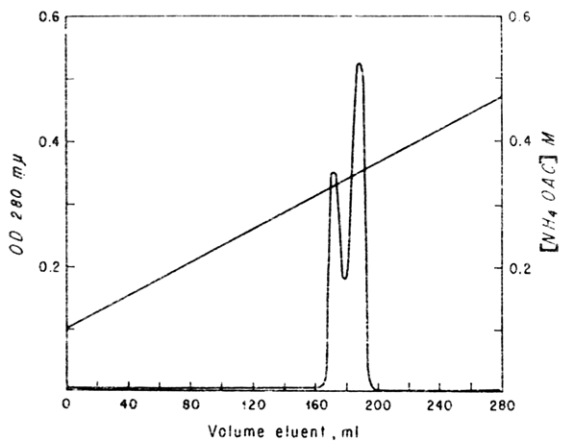


FIGURE 5: Elution pattern obtained when 20 mg of protein of fraction I of Bio-Gel P-2 fractionation (Figure 3) was subjected to carboxymethylcellulose chromatography. Solid diagonal line denotes linear gradient of ammonium acetate eluent.

TABLE IV: Amino Acid Composition of Oxidized Porcine Calcitonin.

Amino Acid	Residues		
	Present Procedure		Lit. Values[a]
His	1.0	1	1
Arg	2.0	2	2
Asp	4.1	4	4
Thr[b]	1.5	1–2	2
Ser[b]	2.2	2	4
Glu	1.1	1	1
Pro	2.1	2	2
Gly	2.9	3	3
Ala	1.0	1	1
Cys (O_3H)	1.6	2	0
Half-$(Cys)_2$	0.0	0	2
Val	1.0	1	1
Lys	0.0	0	0
Met (O_2)[c]	0.9	1	0
Met	0.0	0	1
Ile	0.004	0	0
Leu	3.0	3	3
Tyr	0.3	1	1
Phe	3.1	3	3
Trp	1.0	1	1

[a] Taken from Beesley *et al.* (1968), Bell *et al.* (1968), Franz *et al.* (1968), Kahnt *et al.* (1968), and Potts *et al.* (1968). [b] Not corrected for destruction. [c] Analysis of an alkaline hydrolysate of the P-2 product showed no methionine sulfoxides.

Amino Acid Analysis. The finding that the two electrophoretic components of the P-2 product possessed the same specific biologic activities suggested that they were chemically similar. Accordingly, amino acid analyses were done on the P-2 product after performic acid oxidation. The results of these analyses and, for comparison, the amino acid composition of porcine calcitonin previously reported by five separate groups are shown in Table IV. The amino acid composition of the P-2 product is essentially identical with that previously reported for porcine calcitonin except (1) the values for serine and threonine which have not been corrected for destructive losses and (2) the value for tyrosine, some of which was probably destroyed by the performic acid

TABLE V: Summary of Purification of Calcitonin Preparations.

Step	Sp Biol Act. (MRC A units/ mg of protein)	Potency Increase	Yield (%) per Step
Dried defatted glands	0.007[a]		
BAW–trichloroacetic acid extract[b]	10.00	1428	50–80
Bio-Gel P-6	80.00	8	80–90
Bio-Gel P-2	140.00	1.75	90–100
Over-all purification = 20,000			

[a] MRC A units/mg weight. This value is 1.6 times higher than that previously reported (Potts *et al.*, 1967) and is calculated on the basis of butanol–acetic acid–water extraction of dried glands (Table II). This accounts in part for the lower over-all purification value for the present procedure as compared with previously reported isolation techniques (Potts *et al.*, 1967). [b] These data are for material prepared in our laboratory. The yield is slightly lower under industrial conditions.

oxidation. Alkaline hydrolysis of the P-2 product in Teflon vessels and subsequent amino acid analysis showed no methionine in either the sulfoxide or sulfone form.

Preliminary Study of Cysteine Reduction of P-2 Product. Reduction of the P-2 product was carried out by incubating 2 mg of this material in 0.1 M cysteine hydrochloride (pH 1.6) at 37° for 12 hr. The disc gel electrophoresis patterns of this material and of unreduced P-2 product are compared in Figure 4. Treatment with cysteine converted the material which migrated as two components into material which migrated as a single component. The R_F of the single component was the same as that of the more mobile of the two components, suggesting that the less mobile component is an oxidation product of the more mobile component.

Discussion

The procedure which we have described for the isolation of calcitonin from porcine thyroid glands offers the advantages of simplicity, economy, and high yield (Table V). Three stages are involved. In the first, a rapid, easily performed method for the extraction of calcitonin from porcine thyroid glands yields a product with a specific biologic activity 15–30 times that of the acid–urea extraction procedure of Tenenhouse and associates (1965) and 6–10 times that of the recently reported butanol–acetic acid–water method of MacIntyre (1968). The second is a gel filtration step using Bio-Gel P-6 which carries the crude extract to approximately two-thirds purity. The third, another gel filtration step using Bio-Gel P-2 yields a product which has an amino acid composition (Table IV) essentially identical with that previously reported for porcine calcitonin. This similarity and the fact that this empiric formula has been confirmed by the determination of the amino acid sequence (Bell *et al.*, 1968; Neher *et al.*, 1968; Potts *et al.*, 1968) and by the complete synthesis (Guttmann *et al.*, 1968; Rittel *et al.*, 1968) of porcine calcitonin support the premise that the P-2 product is relatively homogeneous.

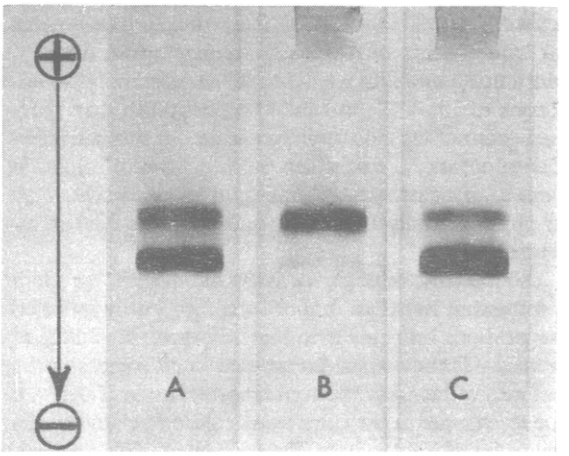


FIGURE 6: Disc gel electrophoresis of (A) fraction I from Bio-Gel P-2 fractionation (Figure 3) and material obtained from the regions of 172 (B) and 192 ml (C) of carboxymethylcellulose fractionation of fraction I (Figure 5).

Little precise information is available concerning the yields of previously described procedures for the isolation of porcine calcitonin. However, it is clear from our experience and that of others (Hawker *et al.*, 1967; Tenenhouse *et al.*, 1968) that they are poor at best (1–10%). The procedure which we have developed has an over-all yield of about 40–60%. The reasons for these dramatic differences in yield are not entirely clear; however, the method of initial extraction of the glands is probably at least partly responsible.

The question of why the P-2 product migrates as two components on disc gel electrophoresis is intriguing. The observation that treatment with cysteine results in the conversion of the two-component material to one-component material strongly suggests that the P-2 product represents an oxidoreductive alteration of a single component. Oxidoreductive changes in the methionine residue of porcine calcitonin have been reported to result in derivatives which have different mobilities in liquid–liquid partition systems, but these derivatives migrated as a single component on disc gel electrophoresis and had the same biologic activity (Kahnt *et al.*, 1968). However, the alteration in the P-2 product is probably not in the methionine residue because no methionine sulfoxide or sulfone was detected after alkaline hydrolysis of the peptide. Although alternative explanations can be offered, it seems likely that intermolecular disulfide interchange is responsible for the production of these two components. This notion is supported by preliminary studies in which oxidation of the P-2 prod-

uct with peroxide resulted in the production of at least five components on disc gel electrophoresis, none of which had a mobility greater than the most mobile component of the P-2 product. The possibility that polymerization of the calcitonin molecule *via* disulfide interchange occurs *in vivo*, either within its cell of origin, in plasma, or at its site of binding in target tissues, may be physiologically important and warrants further investigation.

The specific biologic activity of the P-2 product is somewhat less than that of some previously reported preparations but similar to that of others. It is difficult to know if these variations are due to differences in the methods of bioassay between laboratories or if they are due to changes in the calcitonin molecule related to the means by which the hormone is isolated. This problem can be solved only when the various preparations are subjected to comparative bioassay and immunoassay (Arnaud *et al.*, 1968; Deftos *et al.*, 1968) in a number of different laboratories.

Starting with dried defatted thyroid glands, we have been able to produce, in our laboratory, milligram quantities of the P-2 product with a few days' work. Scale-up studies in collaboration with the Upjohn Co. have shown that as much as 10 kg of dried defatted thyroid glands can be brought successfully to the "brown powder" stage (Scheme I) with minor modification of the original extraction procedure. Further purification of this material in our laboratory has resulted in the production of a P-2 product identical in all respects with that previously obtained and it appears entirely feasible to produce gram quantities of calcitonin.

The need for a standardized, relatively homogeneous preparation of calcitonin which is available in quantities adequate for use in physiologic, biochemical, and clinical investigation is pressing and immediate. A commercial source of a synthetic calcitonin would be the ideal solution to this problem but this is not likely to become a reality until calcitonin is demonstrated to be therapeutically effective in common metabolic bone disorders. The ease and economy with which porcine calcitonin can be isolated by the procedure we have described could make possible, within a short time, the production of a standard preparation of peptide which would satisfy the research needs of all interested investigators. It should also be possible, with further scale-up refinements in technique, to produce quantities of calcitonin by the present procedure sufficient for potential future therapeutic requirements.

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