Characterization of the Immunochemical Forms of Calcitonin Released by a Medullary Thyroid Carcinoma in Tissue Culture

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ABSTRACT Immunoreactive calcitonin released by a medullary thyroid carcinoma in tissue culture has been found to exhibit heterogeneity when analyzed by gel chromatography and radioimmunoassay, in a pattern analogous to that seen in the circulation of the patient from whom the neoplasm was removed. To examine the cause of the heterogeneity, the immunoreactive material released by the tumor into tissue culture medium was further analyzed by gel electrophoresis in the presence of the protein denaturant 8 M urea, by gel chromatography after reduction and alkylation, by affinity chromatography on concanavalin A-agarose, and by bioassay in a renal adenylyl cyclase system of enhanced sensitivity. The results suggest that the larger immunochemical forms of calcitonin described in the circulation of patients with medullary thyroid carcinoma may be released directly from the neoplasm and need not derive from peripheral metabolism of the monomer. It could be demonstrated that a major proportion of the immunochemical enlargement is dependent upon intermolecular disulfide bridge formation whereas aggregation or non-convalent protein binding account for a smaller component of the heterogeneity. In view of the absence of binding of the immunoreactive material to the lectin agarose, carbohydrate side chains, at least of the α-D glucosyl variety, do not seem to contribute significantly to calcitonin enlargement. Additionally, the studies indicate that, at least by in vitro assay, the larger immunochemical forms of calcitonin, representing the majority of the immunoreactivity released by a medullary thyroid carcinoma, are biologically inactive.

INTRODUCTION

Calcitonin in several species is known to be a 32-amino acid polypeptide hormone (1–6) containing an intrachain disulfide bridge between sequence positions 1 and 7 at the NH_2-terminal end of the molecule and an amide group on the COOH-terminal proline. The human hormone (hCT)[1] is secreted by normal (7–12) and neoplastic C cells (7–15), as well as by non-C cell neoplasms (16–18). It has been established that immunochemical heterogeneity of calcitonin exists (19–21) in body fluids of patients with medullary thyroid carcinoma and non-C cell neoplasms. This heterogeneity, defined by gel chromatography and radioimmunoassay techniques, consists mainly of larger forms of the conventional calcitonin monomer. There is little information, however, as to the basis of this heterogeneity; that is, it is unknown whether the "larger" immunochemical forms represent aggregates of the monomer, non-covalent binding of the monomer to another protein, high molecular weight complexes of calcitonin formed by disulfide bridge formation, or covalent extensions of the monomer. Furthermore, there is no information as

This work was presented in part at the Annual Meeting of the Canadian Society for Clinical Investigation, Toronto, Canada, 26 January 1977 and has appeared in abstract form in 1976. *Clin. Res.* **24**: 658A.

Dr. Goltzman is a recipient of a scholarship from the Medical Research Council of Canada. Dr. Tischler was supported by Training Grant GM 00568 in Experimental Pathology from the National Institutes of Health.

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Received for publication 4 May 1977 and in revised form 13 October 1977.

[1] *Abbreviations used in this paper:* hCT, human calcitonin; K_d, distribution coefficient of a solute within a gel, estimated as the difference between the elution volume of the solute and the void volume, divided by the difference between the salt volume and the void volume.

to the biological activity of these other molecular species. The present studies were undertaken to characterize the forms of immunoreactive calcitonin released into culture medium by a medullary thyroid carcinoma maintained in tissue culture. Both physicochemical and biological properties of the immunoreactive material were investigated.

METHODS

Source of samples. Plasma samples and medullary thyroid carcinoma tissue were obtained at the time of surgery from a 55-year-old male with familial medullary thryoid carcinoma as a part of the multiple endocrine neoplasia syndrome (22). Tumor tissue was minced in calcium- and magnesium-free Earle's balanced salt solution, and the cells were dissociated mechanically or with 0.25% trypsin diluted in the same solution. Aliquots of 10^4–10^6 viable cells were plated in 60-mm polystyrene tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in McCoy's 5A medium (modified) with 20% (vol/vol) fetal calf serum. Cultures were incubated at 37°C in a mixture of 5% CO_2 and 95% air. Tissue culture medium for each dish of cultured cells was changed every 72 h and saved after each change. In preliminary studies of medullary thyroid carcinoma culture, it was determined that immunoreactive calcitonin declined as a function of time in tissue culture; however, gel filtration profiles of the immunoreactive material were essentially unchanged. Consequently, medium from the first 10 days culture was pooled for subsequent use. In control experiments, synthetic calcitonin monomer (kindly supplied by Ciba-Geigy Ltd., Basel, Switzerland) was incubated with outdated blood bank plasma (known to contain no detectable immunoreactive calcitonin) at 37°C for 15 min, or with 10^4–10^6 viable fibroblast cells (known to release no immunoreactive calcitonin) in McCoy's 5A medium (modified) with 20% (vol/vol) fetal calf serum at 37°C at 72 h. Experimental and control plasma and tissue culture medium were frozen in aliquots and stored at −70°C before analysis.

Radioimmunoassay. The radioimmunoassay for calcitonin employed in these studies is similar to that previously described (23, 24). Antiserum was raised in rabbits to synthetic human calcitonin monomer. The same synthetic peptide was also used for radioimmunoassay standard and for radioiodination. Radioimmunoassay buffer consisted of a solution of 0.03 M sodium phosphate buffer at pH 7.0, 0.001 M disodium EDTA, 0.005% (wt/vol) merthiolate, and 10% (vol/vol) athyreotic plasma or outdated blood bank plasma (previously tested for absence of immunoreactive calcitonin and extent of damage to tracer). Unknown samples were assayed in duplicate or triplicate and often in multiple dilutions. Each 0.5-ml incubation volume contained no more than 20% (vol/vol) plasma. Synthetic peptide fragments employed for characterization of the antiserum were kindly supplied by Drs. W. Rittel and R. Maier, Ciba-Geigy Ltd., and Dr. F. R. Singer, University of Southern California.

Gel filtration. Gel filtration studies were performed at 4°C (for bioassay studies) or 22°C (for studies employing guanidine-HCl) with 1.5 × 70-cm columns of Bio-Gel P-100 (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) eluted with 0.1 M ammonium acetate buffer, pH 5.0. ^{131}I-labeled calcitonin monomer and ^{131}I-Na were added to each 1-ml sample of plasma or 4-ml sample of culture medium immediately before gel filtration to serve as internal markers of the hormone monomer and salt peak respectively. ^{131}I-counts were determined for each eluted 0.9-ml fraction, in a gamma well spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Each fraction was then lyophylized, reconstituted in 0.2 ml radioimmunoassay buffer, and assayed in duplicate. For studies analyzing reduced and alkylated hormone, column calibration was performed with the tritiated *S*-carboxymethylcysteine derivative of calcitonin monomer (*vida infra*) and no ^{131}I-labeled internal standards were added. ^{3}H radioactivity in 0.1-ml aliquots of each eluted fraction was determined in a liquid scintillation spectrometer (Packard Instrument Co., Inc.), whereas the remainder of the aliquots was lyophilized and then assayed for immunoreactivity. For assessment of apparent molecular weight, ovalbumin, chymotrypsinogen A, ribonuclease A (all from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Uppsala, Sweden), bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), human growth hormone (courtesy of the National Pituitary Agency), and bovine parathyroid hormone (courtesy of Dr. J. T. Potts, Jr., Massachusetts General Hospital) were employed for column calibration.

Gel electrophoresis. 0.2-ml aliquots of fractions obtained by gel filtration of medullary carcinoma tissue culture medium were combined into four pools (the pool sizes having been determined by radioimmunoassay of the remainder of each fraction). The pools were then lyophilized, and each pool was reconstituted in 0.2 ml of a solution of 8 M urea-0.1 M acetic acid, and incubated with intermittent stirring for 30 min at 22°C before electrophoresis at pH 4.4 on 8% urea-polyacrylamide gels at 4°C. ^{131}I-labeled calcitonin monomer was employed as a marker. Gels were cut into 1-mm slices, and each slice was shaken for 24 h at 4°C in 0.2 ml of radioimmunoassay buffer. Each 0.2-ml sample was then assayed for ^{131}I radioactivity and analyzed by radioimmunoassay.

Reduction and alkylation. Lyophilized pools of immunoreactive calcitonin obtained by gel filtration of medullary carcinoma tissue culture medium or lyophilized synthetic human calcitonin monomer were each dissolved in 0.2 ml of a solution of 6 M guanidine-HCl in 0.05 M Tris-HCl, pH 8.5, gassed with N_2, sealed, and then incubated with shaking at 37°C for 1 h to denature the protein. Dithiothreitol (Sigma Chemical Co.) was then added to the mixture to a final concentration of 0.0015 mM, and the mixture was gassed with N_2 and shaken for 4 more h at 37°C. A solution of 0.2 ml of 0.0075 mM iodoacetic acid (Sigma Chemical Co.) in 2 M Tris-HCl, pH 8.5, was then added to the calcitonin-guanidine–dithiothreitol mixture, and the combined mixtures were incubated in the dark at 22°C for 30 min. Then the reaction was stopped by the addition of 0.1 ml β-mercaptoethanol. The entire mixture was chromatographed on Bio-Gel P-100, and each fraction was radioimmunoassayed as described previously.

In initial experiments involving synthetic human calcitonin monomer, 10–20 × 10^6 cpm of [2-^{3}H]iodoacetic acid (specific activity 64 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was added to the alkylating solution. The *S*-carboxymethylcysteine derivative of the calcitonin monomer with incorporated ^{3}H, was desalted on a column of Bio-Gel P-2 (100–200 mesh, Bio-Rad Laboratories) and was employed as a column marker for the alkylated monomer in subsequent reduction and alkylation studies with immunoreactive pools. In preliminary experiments, it was determined that synthetic human calcitonin monomer, after reduction and alkylation by the procedure just described, reacted with the antiserum used in these studies in parallel with the synthetic human calcitonin monomer standard.

Affinity chromatography. Lyophilized pools of immunoreactive calcitonin obtained by gel filtration of medullary carcinoma tissue culture medium or lyophilized synthetic

human calcitonin monomer were each dissolved in 0.5 ml of 1.0 M ammonium acetate buffer, pH 6.0, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Each 0.5-ml sample was applied separately at 22°C to an 0.3-ml column of concanavalin A-agarose (Sigma Chemical Co.), prepared in a Pasteur pipette. The column was then eluted with an 0.5-ml vol of buffer followed by nine 1-ml aliquots of buffer. Four 1-ml vol of buffer containing 0.2 M α-methyl D-mannopyranoside (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were then added to elute specifically-bound material. The column was then washed with 4 more 1-ml aliquots of buffer without glycoside. ^{131}I-labeled calcitonin monomer, or ^{131}I-labeled human thyroid-stimulating hormone (National Pituitary Agency) was added to each sample chromatographed to serve as an internal control. ^{131}I radioactivity was determined in each eluted fraction which was then lyophilized, reconstituted in radioimmunoassay buffer, and radioimmunoassayed for calcitonin.

Adenylyl cyclase assay. Adenylyl cyclase activity was determined in purified renal membranes prepared from the kidneys of male albino (New Zealand) rabbits, 15–17 kg, or of male Sprague-Dawley rats, 150–200 g, according to a method previously described (25, 26). In preliminary experiments, the adenylyl cyclase assay when performed in membranes prepared from rabbit kidneys, was found to be 13-fold more sensitive to synthetic hCT monomer than was the assay performed in conventional rat renal membranes; consequently, rabbit membranes were employed for subsequent studies. Adenylyl cyclase activity was determined (27) after a 10-min incubation at 37°C. The incubation mixture contained, in a 0.1-ml vol, 50 mM Tris-HCl, pH 7.4; 0.84 mM ATP; 0.8–2.0 × 10^6 cpm of [α-^{32}P]ATP (ICN Pharmaceuticals, Inc., Irvine, Calif.); 9 mM theophylline; 4.2 mM $MgCl_2$; 26 mM KCl; 0.118% (wt/vol) albumin; 5 mM creatine phosphate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); 0.1 mg/ml creatine phosphokinase, and 30–80 μg of membrane protein determined by the method of Lowry (28). [α-^{32}P] cyclic AMP was isolated by the method of Salomon (29). Synthetic human calcitonin monomer, Lot K692 -2, (115 MRC U/mg; MRC, Medical Research Council, Great Britain) used as a standard in the adenylyl cyclase assay, was kindly supplied by Dr. J. L. Hughes, Armour Pharmaceutical Co., Kankakee, Ill.

RESULTS

Antiserum specificity. The synthetic hCT fragment representing sequence positions 17–28 produced minimal inhibition of binding of ^{125}I-labeled hCT (1–32), the intact monomer, to the antiserum (Fig. 1). The hCT fragment extended towards the NH_2-terminus by six residues and, in representing sequence positions 11–28, produced more marked, but still incomplete, inhibition of hCT monomer binding to the antiserum (Fig. 1). However, inclusion of the four COOH-terminal residues, in the fragment representing sequence positions 17–32, resulted in marked inhibition of binding of the intact labeled monomer to the antiserum (Fig. 1). Nevertheless, twice the concentration of this latter fragment as of the intact monomer was required to produce 50% inhibition, and the displacement was nonparallel. Inclusion of both the six more NH_2-terminal residues, as well as the four residues at the COOH-terminus, in the fragment representing sequence positions 11–32, achieved inhibition of binding of labeled intact monomer to the antiserum which was both parallel and equimolar with the unlabeled intact monomer standard (Fig. 1).

Consequently, the antiserum appeared to require primarily a major antigenic site within sequence positions 29–32 at the COOH-terminus of hCT monomer, and a minor antigenic site in the middle of the molecule within sequence positions 11–16. Whether the residues within these sites interact directly with antibody sequences or produce a molecular conformation appropriate for antibody recognition could not be distinguished by these studies.

Gel filtration of plasma and tissue culture medium calcitonin. Patient plasma and medullary carcinoma tissue culture medium contained 21 and 48 ng/ml of immunoreactive calcitonin, respectively, and recoveries of total immunoreactivity after gel filtration were 63 and 71%, respectively. Both elution profiles demonstrated multiple "peaks and shoulders" of immunoreactivity (Fig. 2, panels a and b) but appeared qualitatively similar in the overall pattern of distribution of eluted immunoreactivity. In each case, only one peak of immunoreactivity co-eluted with the ^{131}I-labeled hCT monomer; the remaining immunoreactivity eluted before the monomer. However, quantitative differences in the major eluted regions of immunoreactivity were apparent with, for example, a greater proportion of the total immunoreactivity of plasma than of tissue culture medium co-eluting with ^{131}I-labeled hCT monomer (28% vs. 24%, respectively). In contrast, in control experiments incubating synthetic human calcitonin monomer with either calcitonin-free plasma or with non-calcitonin-producing cells (fibroblast cells) in tissue culture, virtually all immunoreactivity co-eluted with the hCT monomer marker (Fig. 2, panels c and d).

For further analysis of the calcitonin immunoreactivity released into the medullary carcinoma tissue culture medium, the immunoreactive material obtained by gel chromatography was combined into four major pools, I–IV (Fig. 2b): pool I included material having apparent molecular weights > 60,000 (K_d 0–0.11); pool II included material having apparent molecular weights in the 13,000–40,000 range (K_d 0.21–0.48); pool III included material having apparent molecular weights in the 4,000–13,000 range (K_d 0.48–0.72); and pool IV included material coeluting with calcitonin monomer (K_d 0.72–0.83). Although any of these four major pools in themselves may have represented heterogeneous entities, the absence of biochemical information regarding their precise identity seemed to minimize any advantage which might have been gained by further subfractionating them. Consequently, these pools were used as the starting

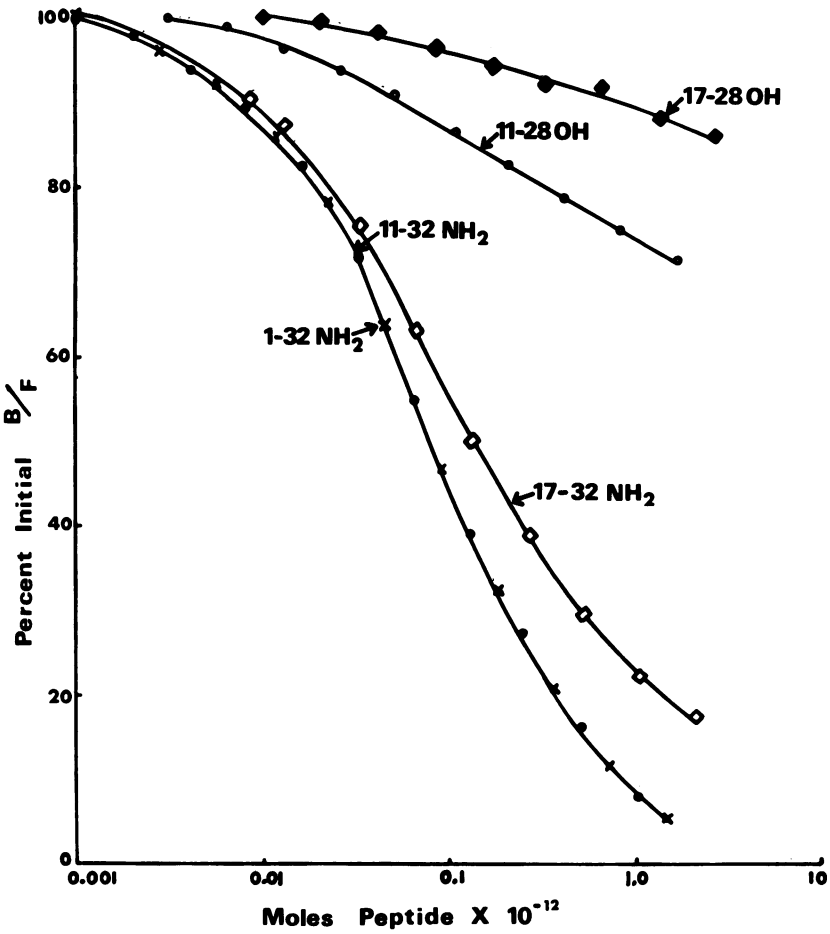

FIGURE 1 Comparison of the inhibition of binding of ^{125}-I-labeled hCT monomer to anti-calcitonin antiserum by increasing concentrations of intact hCT monomer, 1-32 (X), and by the synthetic hCT fragments represented by sequence positions 17–28 (◆), 11–28 (○), 17–32 (◇) and 11–32 (●). OH represents the free carboxyl group at the COOH-terminus, and NH_2 an amide group on the COOH-terminal residue. B and F refer to quantities of antibody-bound and free ^{125}I-labeled hCT, respectively. Initial B/F was 0.74.

point for the characterization of the calcitonin immunoreactivity released by the tumor.

Serial dilutions of aliquots of medullary carcinoma culture medium before gel filtration and of aliquots of each eluted pool obtained by gel filtration inhibited ^{125}I-labeled HCT monomer binding to the antiserum in parallel with the hCT monomer standard (Fig. 3). Consequently, quantitation of each pool was possible in terms of nanograms of hCT monomer. Each of pools I–IV represented 13, 15, 38, and 24%, respectively, of recovered immunoreactivity. The binding inhibition in parallel with hCT standard displayed by each eluted pool of immunoreactivity, indicated that each pool contained sufficient antigenic determinants to satisy at least the spatially discrete sites required for the interaction of the antiserum with hCT monomer.

Electrophoretic analysis. When each pool of calcitonin immunoreactivity obtained by gel filtration of medullary carcinoma medium was analyzed by polyacrylamide gel electrophoresis and radioimmunoassay, each pool was found to be heterogenous; that is, the immunoreactivity did not resolve into a single peak comigrating with the ^{125}I-labeled calcitonin monomer despite the presence of the protein denaturant 8 M urea (Fig. 4). Nevertheless, in pools I–III, a significant proportion of the immunoreactivity co-migrated with the hCT monomer marker (23, 26, and 16% of recovered immunoreactivity, respectively); only in pool IV, however, did the bulk of the immunoreactivity (79%) co-migrate with the monomer marker. Consequently, despite analysis in the presence of the protein denaturant, immunochemical heterogeneity persisted (Fig. 4).

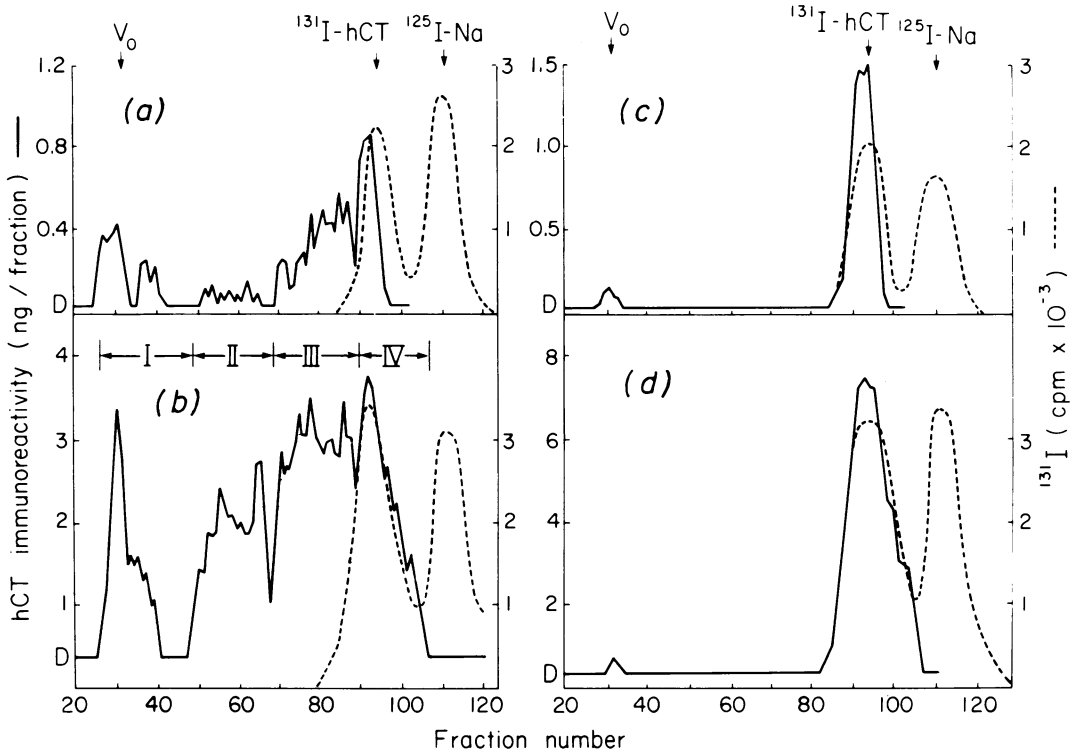

FIGURE 2 Gel filtration profiles of calcitonin immunoreactivity obtained by chromatography on Bio-Gel P-100 of patient plasma (a), of culture medium from the patient's medullary thyroid carcinoma maintained in tissue culture (b), of calcitonin-free plasma incubated with synthetic hCT monomer (c), and of culture medium from fibroblasts incubated with synthetic hCT monomer (d). Eluting buffer was 0.1 M ammonium acetate, pH 5.0. Vertical arrows represent, from left to right, the void volume (Vo), the elution positions of the labeled monomer ^{131}I-hCT, and the salt peak, ^{131}I-Na. D represents the detection limit of the radioimmunoassay. After chromatography of the tissue culture medium, the column effluent was divided into four pools, represented by regions I–IV, for subsequent analysis.

Reduction and alkylation studies. After reduction with dithiothreitol in the presence of the denaturant 6 M guanidine, and alkylation with iodoacetic acid, each pool of calcitonin immunoreactivity was re-chromatographed on the same column, and the eluted fractions were re-immunoassayed. With this procedure, pool IV co-chromatographed with the *S*-carboxymethylcysteine derivative of hCT, but only a proportion of pools II and III (21 and 18% of recovered immunoreactivity) now eluted in this position (Fig. 5). Pool I, which had previously eluted close to or in the void volume (Fig. 2), now eluted entirely in the position of the alkylated derivative of the hCT monomer.

Affinity chromatography of immunoreactive calcitonin. Pools II and III, the majority of which had failed to resolve into a single molecular species identifiable as hCT monomer or its alkylated derivative, despite subjection to protein denaturants and reducing agents, were then analyzed by affinity chromatography on concanavalin A-agarose to detect possible glycoprotein content. Recoveries of immunoreactivity in pools II and III and for synthetic hCT were 83, 84, and 74%, respectively. Neither one of the pools nor synthetic hCT monomer assessed by radioimmunoassay or labeled with ^{131}I, bound detectably to the concanavalin A-agarose. Despite this, 86% of ^{131}I-labeled thyroid-stimulating hormone did bind by the procedure employed.

Bioassay of immunoreactive calcitonin. Each pool of immunoreactive calcitonin obtained by gel filtration of medullary carcinoma culture medium was bioassayed in an in vitro rabbit renal adenylyl cyclase assay. The assay incubations, performed in duplicate contained either 5 or 2.5 ng of pool I, 9 or 4.5 ng of pool II, 12 or 6 ng of pool III, or 8 or 4 ng of pool IV. Of the four pools bioassayed, only pool IV significantly stimulated adenylyl cyclase activity above basal, in each of the two concentrations employed (Fig. 6).

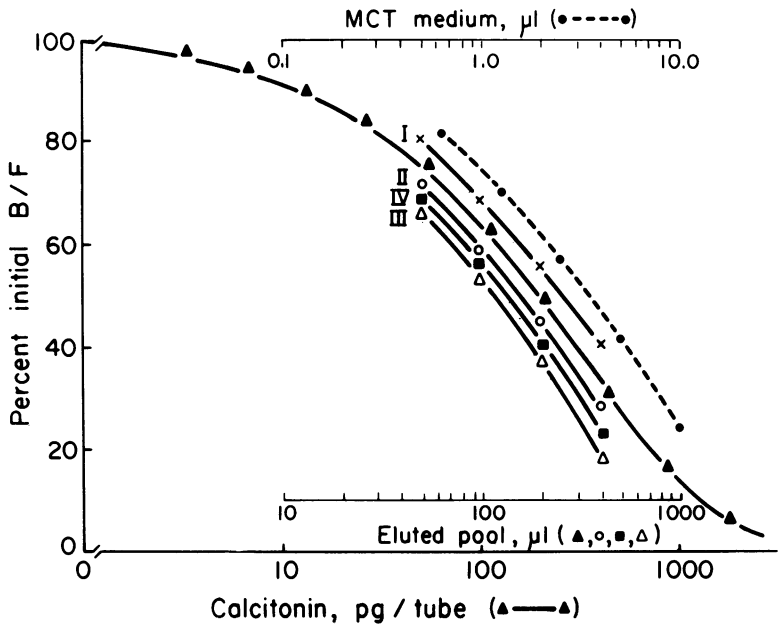

FIGURE 3 Immunoreactive calcitonin in serial dilutions of medullary thyroid carcinoma (MCT) tissue culture medium (●) and in serial dilutions of pools I (X), II (○), III (△), and IV (■) obtained by fractionating the immunoreactive material in the effluent after gel filtration of the culture medium. B and F refer to antibody-bound and free ^{125}I-labeled calcitonin tracer. Initial B/F was 0.81. Concentration is expressed in picograms of human calcitonin monomer (▲).

DISCUSSION

There has been considerable interest in the immunochemical heterogeneity of calcitonin in view of the possibility of deriving further insight into peptide hormone biogenesis and action, and because of the potential for improved detection of neoplastic states for which the hormone is a marker (17–23). Yet, there is little information regarding the characterization of this heterogeneity. The origin of the heterogeneity has not been clarified, although it has been suggested that synthetic human calcitonin monomer continues to circulate as the monomer after administration to humans and does not contribute to calcitonin heterogeneity by peripheral conversion (19). The present studies, by demonstrating analagous patterns of immunochemical heterogeneity in the circulation of a patient with medullary thryoid carcinoma and in the culture medium of the same neoplasm maintained in tissue culture, support the thesis that the heterogenous forms of calcitonin are released directly by the neopolasm and need not be derived from peripheral metabolism of secreted monomer (30).

The protein denaturant 8 M urea failed to convert the bulk of the larger immunochemical forms of calcitonin obtained by gel filtration into species identifiable (by polyacrylamide gel electrophoresis) with the monomer. Thus, aggregation of the monomer or noncovalent binding of the monomer to a high molecular weight protein appears not to be responsible for the majority of the heterogeneity observed.

However, after reduction and alkylation, the largest molecular weight pool of calcitonin immunoreactivity did resolve into a single peak co-eluting with the monomer on gel filtration. Consequently, this material seemed to be either a high molecular weight polymer of calcitonin resulting from intermolecular disulfide bridge formation, or a high molecular weight complex formed by disulfide bridge linkage of the monomer to an unrelated protein. For these studies the use of an antiserum with demonstrable absence of antigenic binding sites within the NH_2-terminal ring of the monomer, permitted continued detection of calcitonin immunoreactivity despite considerable interaction involving this region of the molecule.

To date only small quantities of the antiparallel dimer of calcitonin have been isolated from medullary thyroid carcinoma tissue (31), and intermolecular disulfide bridge formation has not been previously implicated in circulating heterogeneity. However, in previous analyses, the conditions employed for reduction of heterogeneous circulating calcitonin forms did not include a protein denaturant to facilitate exposure of the disulfide bonds, were not performed under nitrogen, and did not include irreversible alteration of the exposed thiol groups to prevent recombination during subsequent analysis (19–21).

That only small quantities of pools II and III co-

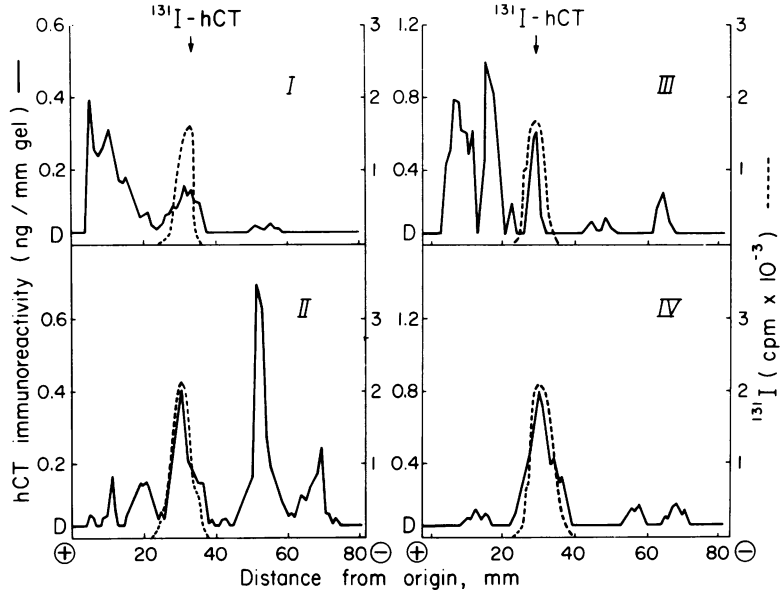

FIGURE 4 Immunoreactive profiles of pools I to IV, after electrophoresis on polyacrylamide gels at pH 4.4 in 8M urea. The vertical arrow represents the migration position of ^{131}I-labeled hCT used as a marker for the hormone monomer. D represents the detection limit of the radioimmunoassay. Vertical scales to the immediate left of the panels refer to immunoreactivity and vertical scales to the immediate right refer to ^{131}I radioactivity.

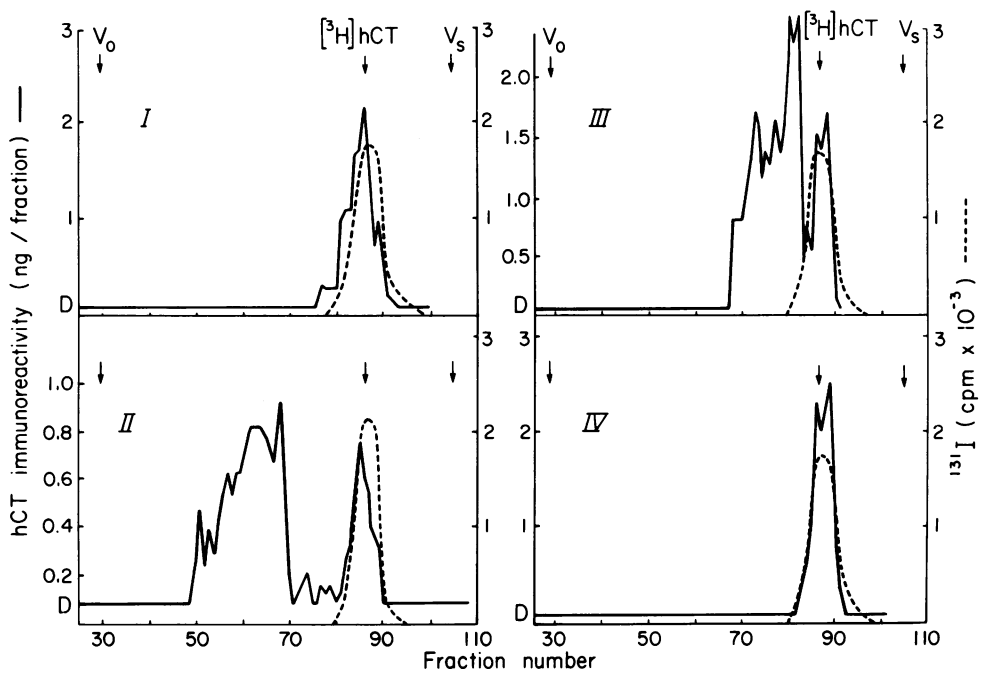

FIGURE 5 Gel filtration profiles of calcitonin immunoreactivity after chromatography on Bio-Gel P-100 of reduced and alkylated pools I to IV. Each pool initially obtained by gel filtration of medullary carcinoma tissue culture medium, was reduced with dithiothreitol in 6 M guanidine-HCl under N_2 at 37°C and then alkylated with iodoacetic acid before rechromatography. Vertical arrows, from left to right, represent the void volume (Vo), the elution position of the reduced and alkylated hCT monomer labeled with [2-^{3}H]iodoacetic acid ([^{3}H]hCT), and the salt peak (V_s). D represents the detection limit of the radioimmunoassay. Vertical scales to the immediate left of the panels refer to immunoreactivity and vertical scales to the immediate right refer to ^{3}H radioactivity.

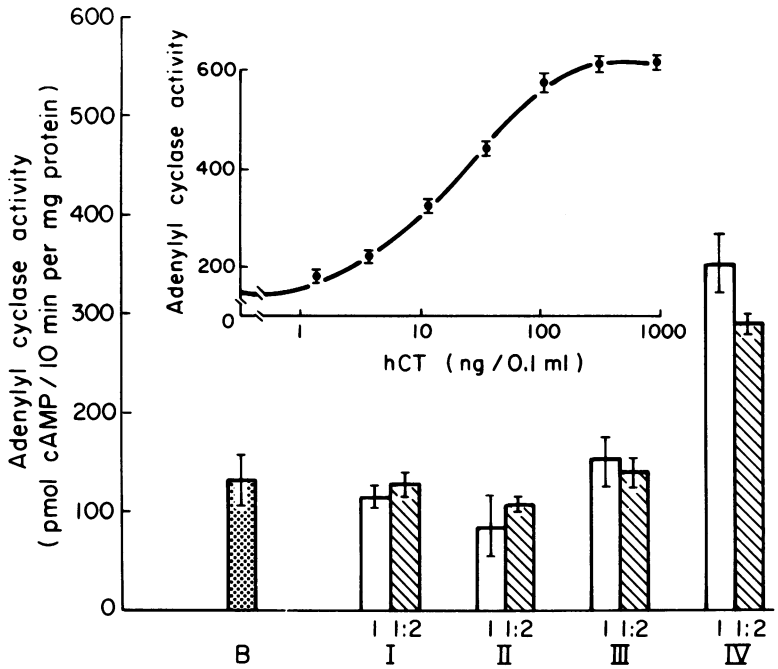

FIGURE 6 Assay of adenylyl cyclase activity in rabbit renal membranes and of pools I–IV obtained by gel filtration of medullary carcinoma culture medium. Each pool was assayed in two concentrations, and each bar represents the mean of duplicate determinations ± SE. B represents the basal (unstimulated) activity of the membranes. The adenylyl cyclase activity stimulated by the hCT monomer used as the assay standard is depicted in the inset.

eluted with the monomer after reduction and alkylation in the presence of 6 M guanidine, was commensurate with that proportion of these pools comigrating with the monomer in the presence of 8 M urea. Consequently, the fraction of these pools identifiable with the monomer may have existed as an aggregate or non-covalently bound to another protein before disruption with the denaturant. Disulfide bridge disruption need not be invoked here. As a result, the nature of the bulk of pools II and III remained unresolved by that technique.

In view of recent reports (32, 33) identifying larger molecular weight forms of other peptide hormones as glycoproteins, the capacity of pools II and III to bind to concanavalin A was assessed. No binding of pools II and III to the lectin was observed, however, indicating that as a minimum they contained no carbohydrate side chains with α-D-mannosyl or sterically related residues. However, these pools did react in a parallel fashion with the antiserum employed, which is known to require at least two spatially separate sites for interaction with the monomer; consequently, these molecular species contain a significant proportion of the calcitonin sequence, most likely covalently extended.

Calcitonin is known to enhance urinary excretion of electrolytes and cyclic AMP in vivo (34) and has been demonstrated to stimulate adenylyl cyclase in membranes prepared from rat kidneys in vitro (35, 36). Consequently, this defined property of calcitonin of stimulating in vitro adenylyl cyclase was used as a bioassay in the present studies. Rabbit renal membranes were employed rather than rat membranes in view of their enhanced sensitivity to human calcitonin. Only pool IV, corresponding to the human calcitonin monomer, could be demonstrated to stimulate adenylyl cyclase significantly. Consequently, insofar as the assay reflects in vivo activity, the majority of the immunoreactivity released by a medullary thyroid carcinoma, at least in vitro, may be biologically inactive or of markedly reduced activity relative to the monomer.

Studies correlating calcitonin structure with function have demonstrated that an intact disulfide bridge between sequence positions 1 and 7 of the monomer is essential for biological activity (37), and, indeed, the dimer previously extracted from tumor tissue was demonstrated to have little activity until converted to monomer (38). This, therefore, may explain the absence of detectable activity in pool I in which the intramolecular disulfide bridge would no longer have remained intact.

The precise nature of the majority of pools II and III remain undefined, although immunoreactivity

within these pools would be the most likely to represent covalent extensions of calcitonin monomer possibly functioning as biosynthetic precursors. Biosynthetic precursors of peptide hormones are frequently reported to have reduced biological activity relative to the hormonal products (39, 40). Of the two studies which have reported the existence of precursors of calcitonin, one did not assess the biological activity of the precursor (molecular weight 7,000) derived from trout ultimobranchial glands (41), but the other study did report reduced activity (relative to monomer) of a precursor (molecular weight about 13,000) derived from chicken ultimobronchial glands (42). Consequently, the discordance between immunologic and biologic activity seen in our studies may be related to the observations regarding bio-activity reported for peptide hormone precursors including calcitonin precursors.

Further studies will be required to determine more precise chemical and biological properties of the larger forms, and development of specific antisera to them may eventually lead to more sensitive indices of the activity of neoplastic and perhaps of non-neoplastic C cells.

ACKNOWLEDGMENTS

This work was supported by grants MA-5775 and MA-5860 from the Medical Research Council of Canada, and by grant 760012 from the Quebec Medical Research Council.

REFERENCES

1. Neher, R., B. Riniker, H. Zuber, W. Rittel, and F. Kahnt. 1968. Thyrocalcitonin. II. Struktur von α-Thyrocalcitonin. *Helv. Chim. Acta.* **51:** 917–924.
2. Potts, J. T. Jr., H. D. Niall, H. T. Keutmann, H. B. Brewer, and L. J. Deftos. 1968. The amino acid sequence of porcine thyrocalcitonin. *Proc. Natl. Acad. Sci. U. S. A.* **59:** 1321–1328.
3. Bell, P. H., W. R. Barg, Jr., D. F. Colucci, M. C. Davies, C. Dziobkowski, M. E. Englert, E. Heyder, R. Paul, and E. H. Snedeker. 1968. Purification and structure of porcine calcitonin. *J. Am. Chem. Soc.* **90:** 2704–2706.
4. Neher, R., B. Riniker, W. Rittel, and H. Zuber. 1968. Menschliches Calcitonin. III. Structur von Calcitonin M und D. *Helv. Chim. Acta* **51:** 1900–1905.
5. Niall, H. D., H. T. Keutmann, D. H. Copp, and J. T. Potts, Jr. 1969. Amino acid sequence of salmon ultimobranchial calcitonin. *Proc. Natl. Acad. Sci. U. S. A.* **64:** 771–778.
6. Brewer, H. B. Jr., and R. Ronan. 1969. Amino acid sequence of bovine thyrocalcitonin. *Proc. Natl. Acad. Sci. U. S. A.* **63:** 940–947.
7. Samaan, N. A., C. S. Hill, Jr., J. R. Beceiro, and P. N. Schultz. 1973. Immunoreactive calcitonin in medullary carcinoma of the thyroid and in maternal and cord serum. *J. Lab. Clin. Med.* **81:** 671–681.
8. Silva, O. L., R. H. Snider, and K. L. Becker. 1974. Radioimmunoassay of calcitonin in human plasma. *Clin. Chem.* **20:** 337–339.
9. Tashjian, A. H., Jr., H. J. Wolfe, and E. F. Voelkel. 1974. Human calcitonin: immunologic assay, cytologic localization, and studies on medullary thyroid carcinoma. *Am. J. Med.* **56:** 840–849.
10. Heynen, G., and P. Franchimont. 1974. Human calcitonin radioimmunoassay in normal and pathological conditions *Eur. J. Clin. Invest.* **4:** 213–222.
11. Parthermore, J. G., and L. J. Deftos. 1975. The regulation of calcitonin in normal human plasma as assessed by immunoprecipitation and immunoextraction *J. Clin. Invest.* **56:** 835–841.
12. Wolfe, H. J., E. F. Voelkel, and A. H. Tashjian, Jr. 1974. Distribution of calcitonin containing cells in the normal adult human thyroid gland: a correlation of morphology with peptide content. *J. Clin. Endocrinol. Metab.* **38:** 688–694.
13. Melvin, K. E. W., and A. H. Tashjian, Jr. 1968. The syndrome of excessive calcitonin produced by medullary carcinoma of the thyroid. *Proc. Natl. Acad. Sci. U. S. A.* **59:** 1216–1222.
14. Milhaud, G., M. Tubiana, C. Parmentier, and G. Coutris. 1968. Epithelioma de la thyroide sécrétant de la thyrocalcitonine. *C. R. Hébd. Séances Acad. Sci. Ser. D. Sci. Nat.* **266:** 608–610.
15. Clark, M. B., G. W. Boyd, P. G. H. Byfield, and G. V. Foster. 1969. A radioimmunoassay for human calcitonin M. *Lancet.* **II:** 74–77.
16. Milhaud G., C. Calmatte, J. P. Raymond, J. Bignon and M. S. Moukhtar. 1970. Carcinoide sécrétant de la thyrocalcitonine, *C. R. Hébd. Séances Acad. Sci. Ser. D* **270:** 2195–2198.
17. Coombes, R. C., C. J. Hillyard, P. B. Greenberg, and I. MacIntyre. 1974. Plasma immunoreactive calcitonin in patients with non-thyroid tumors. *Lancet.* **I:** 1080–1083.
18. Silva, O. L., K. L. Becker, A. Primack, J. Doppman, and R. H. Snider. 1974. Ectopic secretion of calcitonin by oat-cell carcinoma. *N. Engl. J. Med.* **290:** 1122–1124.
19. Singer, F. R., and J. F. Habener. 1974. Multiple immunoreactive forms of calcitonin in human plasma. *Biochem. Biophys. Res. Commun.* **61:** 710–716.
20. Deftos, L. J., B. A. Roos, D. Bronzert, and J. G. Parthemore, 1975. Immunochemical heterogeneity of calcitonin in plasma. *J. Clin. Endocrinol. Metab.* **40:** 409–412.
21. Sizemore, G. W., and H. Heath III. 1975. Immunochemical heterogeneity of calcitonin in plasma of patients with medullary thyroid carcinoma. *J. Clin. Invest.* **55:** 1111–1118.
22. Melvin, K. E. W., A. H. Tashjian, Jr., and H. H. Miller. 1972. Studies in familial (medullary) thyroid carcinoma. *Recent Prog. Horm. Res.* **28:** 399–470.
23. Goltzman, D., J. T. Potts, Jr., E. C. Ridgway, and F. Maloof. 1974. Calcitonin as a tumor marker: use of the radioimmunoassay for calcitonin in the postoperative evaluation of patients with medullary thyroid carcinoma. *N. Engl. J. Med.* **290:** 1035–1039.
24. Deftos, L. J. 1971. Immunoassay for human calcitonin. I. Method. *Metab. Clin. Exp.* **20:** 1122–1128.
25. Goltzman, D., A. Peytremann, E. N. Callahan, G. V. Segre, and J. T. Potts, Jr. 1976. Metabolism and biological activity of parathyroid hormone in renal cortical membranes. *J. Clin. Invest.* **57:** 8–19.
26. Marx, S. J., S. A. Fedak, and G. D. Aurbach. 1972. Preparation and characterization of a hormone responsive renal plasma membrane fraction. *J. Biol. Chem.* **247:** 6913–6918.
27. Goltzman, D., A. Peytremann, E. Callahan, G. W. Tregear, and J. T. Potts, Jr. 1975. Analysis of the requirements

for parathyroid hormone action in renal membranes with the use of inhibiting analogues. *J. Biol. Chem.* **250:** 3199–3203.
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
29. Salomon, Y., C. Londos, and M. Robdell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58:** 541–548.
30. Bailey, R., P. Scarpace, D. Stein, A. Frelinger, and B. A. Roos. 1977. Rapid in vivo catabolism of calcitonin monomer to very large covalent immunoreactive calcitonin moieties. Program, 59th Annual Meeting, The Endocrine Society. J. B. Lippincott Co., Philadelphia. Abstract no. 259, 186.
31. Riniker, B., R. Neher, R. Maier, F. W. Kahnt, P. G. H. Byfield, T. V. Gudmundsson, L. Galante, and I. MacIntyre. 1968. Menschliches Calcitonin. I. Isolierung und Characterisierung. *Helv. Chim. Acta.* **51:** 1738–1742.
32. Eipper, V. A., R. A. Mains, and D. Guenzi. 1976. High molecular weight forms of adrenocorticotrohic hormone are glycoproteins. *J. Biol. Chem.* **251:** 4121–4126.
33. Orth, D. N., and W. E. Nicholson. 1977. High molecular weight forms of human ACTH are glycoproteins. *J. Clin. Endocrinol. Metab.* **44:** 214–217.
34. Ardaillou, R., R. Isaac, M. P. Nivez, J. M. Kuhn, J. L. Cazor, and J. P. Fillastre. 1976. Effect of salmon calcitonin on renal excretion of adenosine 3′5′ monophosphate in man. *Horm. Metab. Res.* **8:** 136–140.
35. Heersche, J. N. M., R. Marcus, and G. D. Aurbach. 1974. Calcitonin and the formation of 3′5′—AMP in bone and kidney *Endocrinology.* **94:** 241–247.
36. Marx, S. J., C. J. Woodard, and G. D. Aurbach, 1972. Calcitonin receptors of kidney and bone. *Science (Wash. D. C.).* **178:** 999–1001.
37. Rittel, W., R. Maier, M. Brugger, B. Kamber, B. Riniker, and P. Sieber. 1976. Struktur-Wirkungzbeziehungen beim menschlichen Calcitonin III. Die biologische Aktivität verkurzter oder an den kettenden modifizierter synthetischer Analoger. *Experientia (Basel).* **15:** 246–248.
38. MacIntyre, I. 1970. Human calcitonin: practical and theoretical consequences *In* Calcitonin 1969: Proceedings of the Second International Symposium. S. Taylor, and G. V. Foster, editors. William Heinemann Medical Books Ltd. London. 1–13.
39. Kitabchi, A. E. 1970. The biological and immunological properties of pork and beef insulin, proinsulin, and connecting peptide. *J. Clin. Invest.* **49:** 979–987.
40. Peytremann, A. D. Goltzman, E. N. Callahan, G. W. Tregear, and J. T. Potts, Jr. 1975. Metabolism and biological activity of proparathyroid hormone and synthetic analogues in purified kidney membranes. *Endocrinology.* **97:** 1270–1280.
41. Roos, B. A., K. Okano, and L. J. Deftos. 1974. Evidence for a pro-calcitonin. *Biochem. Biophys. Res. Commun.* **60:** 1134–1140.
42. Moya, F., A. Nieto, and J. L. R-Candela. 1975. Calcitonin biosynthesis: evidence for a precursor. *Eur. J. Biochem.* **55:** 407–413.