

Immunochemical Heterogeneity of Calcitonin in Plasma of Patients with Medullary Thyroid Carcinoma

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ABSTRACT Marked discrepancies (values up to four times higher in one assay than in the other) were observed when the plasma concentration of immunoreactive human calcitonin (iCT) was measured by two radioimmunoassays in 18 patients with medullary thyroid carcinoma. The two antisera used had different binding affinities for the NH₂- and COOH-terminal regions of synthetic calcitonin monomer (CT 1-32). Except for this difference, the assays were identical and reacted equally with CT 1-32. Plasma samples from patients with medullary thyroid carcinoma were gel filtered on columns of Bio-Gel P-150, and the immunoreactivity in column effluent fractions was measured with both assays. The one utilizing the antiserum with prominent NH₂-terminal binding affinity (and giving higher iCT values) recognized at least five molecular species that eluted with or before CT 1-32. The other assay, utilizing the antiserum with a COOH-terminal binding affinity, recognized two of these molecular species—one eluting with CT 1-32 and the other in a position consistent with a dimer. A mixture of athyretic plasma and added CT 1-32 contained a single immunologic species that was recognized equally by both antisera. No forms smaller than CT 1-32 were detected in any study. The results suggest that iCT circulating in the plasma of patients with medullary thyroid carcinoma is heterogeneous. The absolute iCT concentration measured by radioimmunoassays depends on recognition of these distinct molecular species as well as on the specific binding affinities of the antiserum used to detect them. These observations may partially explain the variations among iCT values reported by different laboratories.

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INTRODUCTION

Active debate continues concerning the immunoreactive serum or plasma calcitonin (iCT)¹ concentration in fasting, healthy persons. An appreciable number have been reported to have undetectable iCT concentrations, and the measurable iCT concentrations in the unextracted serum or plasma of normal humans have ranged from 0.010 ng/ml to as high as 1.000 ng/ml in reports from several laboratories (1-7). There also is some controversy about the control of human calcitonin (hCT) secretion in vivo. For example, we have reported (8) that calcitonin secretion may increase more after oral calcium ingestion than after i.v. calcium infusion; Heynen and Franchimont (7) observed increases in iCT levels in two of five subjects receiving calcium gluconate orally; and Silva, Snider, and Becker (9) reported no increase in iCT levels. Agreement has been better in studies of iCT concentrations in patients with medullary thyroid carcinoma (MTC), a tumor that contains and secretes large quantities of calcitonin.

Several problems common to the radioimmunologic measurement of peptide hormones may be partially responsible for this variability. These include methodologic differences, selection of an appropriate acalcitoninemic serum or plasma to be used as a control for the "nonspecific" interference with binding of radioiodinated calcitonin tracer to the antiserum, and the absolute sensitivity of the different antisera used by each laboratory. There also is the possibility that immunochemical heterogeneity of circulating hCT may be responsible for some of the variation in reported iCT values. There is

¹Abbreviations used in this paper: bPTH, bovine parathyroid hormone; CT 1-32, synthetic human calcitonin; hCT, human calcitonin; iCT, immunoreactive human calcitonin; MTC, medullary thyroid carcinoma.

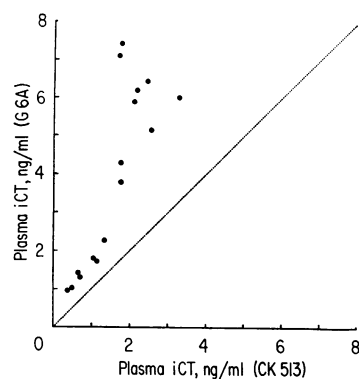


FIGURE 1 Comparison of plasma iCT concentrations, in patients with MTC, measured with two antisera. iCT concentrations are consistently higher when measured with G6A antiserum.

ample precedent for such a postulate in the experience with other peptide hormones, particularly parathyroid hormone (10–13). Furthermore, different antisera against hCT are known to have different immunochemical specificities (7, 14, 15). If multiple species of calcitonin circulate in human plasma and if the various antisera being used to measure iCT preferentially recognize different circulating species, a major discordance in iCT values might be anticipated.

This study demonstrates immunochemical heterogeneity of the calcitonin in the plasma of patients who have MTC and shows that radioimmunoassays with antisera having different sequence-specific calcitonin recognition sites give different values for the absolute iCT concentration in the plasma samples of these patients.

METHODS

Plasma samples. Heparinized plasma samples were obtained from 18 patients (ages 17–62 yr) with either sporadic or familial (16, 17) forms of MTC; the plasma was frozen within 1 h after collection and was stored at -20°C . All patients had histologically proved MTC; eight patients were studied before primary thyroidectomy, and the remainder were studied after one or more operations to remove the tumor. The plasma samples used in gel filtration studies were obtained from a 62-yr-old man (patient A) and a 59-yr-old woman (patient B); both patients had generalized metastasis involving the liver and lumbar vertebrae. An additional sample (plasma C) consisted of plasma from two totally thyroidectomized patients to which synthetic human calcitonin (CT 1–32) had been added.

Gel filtration. The method was similar to that of Benson, Riggs, Pickard, and Arnaud (18). The same 2.5 by 80-cm column of Bio-Gel P-150 (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif.) at 4°C was used for all studies, with an eluting buffer of 0.2 M ammonium acetate, pH 4.6. Fraction size was 1.1–3.2 ml. The plasma samples (7.6–9.5 ml) were centrifuged at 4°C for 20 min at 28,000 *g* before they were added to the column. Each sample was added to the column within 30 min after reaching room

temperature. To each column we also added the following molecular markers: highly purified ^{125}I -labeled parathyroid hormone extracted from bovine parathyroid glands (bPTH [1–84]), 20,000 cpm; ^{125}I -labeled CT 1–32, 20,000 cpm; and ^{125}I -labeled iodide, 15,000 cpm. A portion (0.5 ml) of each sample that was applied to the column was immediately refrozen for subsequent immunoassay. Aliquots of peak four (see below) were rechromatographed in a similar manner on 1.0 by 85-cm columns of Bio-Gel (Bio-Rad Laboratories) P-10 with eluting buffers of either 0.2 M ammonium acetate or 0.2 M ammonium acetate and 10 mM β -mercaptoethanol; the following molecular markers were used: ^{125}I -labeled CT 1–32 (20,000 cpm) and ^{125}I -labeled CT 1–32 dimer (20,000 cpm).

Radioactivity was measured in a gamma-ray scintillation counter (Nuclear-Chicago, Chicago, Ill.) equipped with a pulse-height analyzer. Optical density was determined at 277 nm in a Carl Zeiss PMQ II spectrophotometer. The void volume was marked by the elution position of the largest plasma proteins. [^{125}I]Iodide was used to determine the elution position of the salt volume. Recoveries of ^{125}I -labeled CT 1–32 and iCT added to each column were estimated by summation of the counts in the ^{125}I -labeled CT 1–32 peak and by summation of the total immunoreactivity in all column eluates. The elution position of peaks is identified as *K_d* which is defined as: (elution volume of iCT peak or labeled marker peak minus void volume)/(elution volume of salt peak minus void volume).

Radioimmunoassay. iCT was measured in plasma samples and in column effluents by the previously described radioimmunoassay (4). For purposes of this report, some features of the assay procedure require further elaboration. Radioiodination of hCT was accomplished by Tashjian's (19) modifications of the Hunter and Greenwood procedure (20). The ^{125}I -labeled CT 1–32 produced in this manner had specific activities of 96–216 $\mu\text{Ci}/\mu\text{g}$. It was further purified by chromatography on 1 by 30-cm columns of Bio-Gel P-10 (Bio-Rad Laboratories) with 0.1 M Tris, pH 7.8.

Two different antisera were used; otherwise the immunoassay systems were identical. Antiserum G6A, produced in a goat by repeated intradermal injections of CT 1–32 (Ciba-Geigy Corporation, Basel, Switzerland), was donated by Dr. Jan Fischer (Zurich, Switzerland). Antiserum CK-513 was produced in a chicken by repeated intradermal injections of a conjugate of the same calcitonin and human serum albumin by the method of Goodfriend, Levine, and Fasman (21) with the following modifications: human serum albumin instead of bovine, 10 mg of CT 1–32, and reagents dissolved in 1.0 ml of water. This antiserum was obtained from a different bleeding than that used for a previous study (4). The absolute sensitivities of the immunoassays were 5 pg and 10–15 pg with G6A and CK513 antisera, respectively, depending on the individual preparation of ^{125}I -labeled CT 1–32 used.

The amount of plasma sample added to each assay tube varied from 5 to 50 μl (total incubation volume, 0.5 ml). The addition of comparable amounts of athyreotic plasma did not produce nonspecific effects on the immune reactions.

Values were calculated as previously described (22). Interassay variation for duplicate analyses has been less than 22% in the G6A immunoassay system and less than 20% in the CK513 immunoassay system.

The iCT concentration in the column effluents was measured by adding 100- μl aliquots of dilutions (1:10–1:1,000) of each original fraction (dilutions made with assay diluent) to both immunoassays. The immunoreactivity was measured

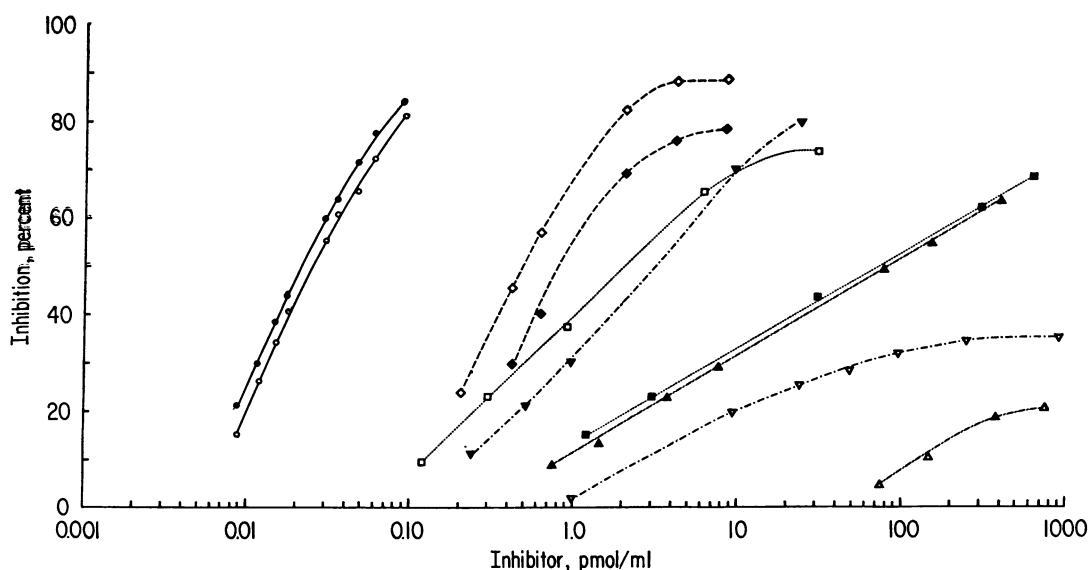


FIGURE 2 Inhibition of binding by G6A (solid symbols) and CK513 (open symbols) antisera of ^{125}I -labeled CT 1-32 by CT 1-32 (\bullet or \circ), H-11-32-NH₂ (\blacklozenge or \lozenge), H-17-32-NH₂ (\blacksquare or \square), H-11-28-OH (\blacktriangledown or \triangledown), and H-17-28-OH (\blacktriangle or \triangle). Note that similar concentrations of CT 1-32 produced equal inhibition of binding to both antisera and that higher concentrations of H-11-32-NH₂ were required to inhibit binding to both antisera compared to CT 1-32. For comparable inhibition of binding to CK513 antiserum, far greater concentrations of H-17-28-OH than H-17-32-NH₂ were required, but equal concentrations of these fragments produced equal inhibition of binding to G6A antiserum.

as the percentage decrease in the B/F (bound to free) ratio from the "trace" B/F ratio for tubes containing comparable amounts of the column buffer and protein. At these dilutions there was no nonspecific effect on the immune reaction by the buffer or protein present. The absolute concentrations of iCT plotted for each effluent fraction represent the mean of measurements in at least two dilutions in each immunoassay.

Antiserum specificity. The sequence-specific antigenic recognition sites of the two antisera were determined by comparing the ability of CT 1-32 and synthetic peptide fragments containing portions of the hCT sequence to inhibit the binding of ^{125}I -labeled CT 1-32 to each antiserum. Each antiserum was added to the immunoassay so that its final concentration was the same as in the immunoassays of plasma and column effluents (G6A, 1:75,000; CK513, 1:2,000) and so that each antiserum bound approximately 40% of the ^{125}I -labeled CT 1-32 added, giving B/F ratios of 0.8 in assay tubes containing no unlabeled calcitonin or peptide fragment. Antigen additions consisted of increasing amounts of either unlabeled CT 1-32 or one of the following synthetic fragments of the hCT sequence: H-11-32-NH₂; H-17-32-NH₂; H-11-28-OH; and H-17-28-OH. The relative inhibition of binding of ^{125}I -labeled CT 1-32 to each antiserum produced by these antigens was measured by comparing the amount required to produce 50% inhibition of binding compared to tubes containing only ^{125}I -labeled CT 1-32 and antiserum.

RESULTS

Calcitonin measurement in whole plasma. In plasma samples from all 18 patients with MTC, the iCT concentration was higher by the immunoassay with G6A

antiserum than by the immunoassay with CK513 antiserum. In 16 patients the mean ratio, G6A value/CK513 value, was 2.41 (range 1.61–4.16) (Fig. 1). The two patients with generalized metastases (patients A and B) had very high plasma iCT concentrations, and the G6A/CK513 ratios were also high.^{*} In all patients with MTC, the iCT concentration was higher than in normal subjects. For comparative purposes, iCT was measured in 10 normal subjects in parallel assays with both antisera. With the G6A assay, the maximum in normal subjects was 0.16 ng/ml; in the patients with MTC, the iCT concentration range was 0.96–764.4 ng/ml. With the CK513 assay, the maximum was 0.09 ng/ml in normal subjects, and the iCT concentration range was 0.42–291.8 ng/ml in the patients with MTC.

Specificities of antisera. Fig. 2 shows the inhibition of binding of ^{125}I -labeled CT 1-32 tracer to antiserum, produced by the addition of CT 1-32 or its synthetic peptide fragments. The concentrations required for 50% inhibition of binding, as derived from the dose-response curves, are shown in Table I. Both antisera had equal binding affinity for CT 1-32 (50% inhibition of binding occurred at the same concentration). The concentrations

^{*} Patient A: iCT by G6A, 764.4 ng/ml; iCT by CK513, 291.8 ng/ml; ratio, 2.62. Patient B: iCT by G6A, 305.3 ng/ml; iCT by CK513, 111.6 ng/ml; ratio, 2.74.

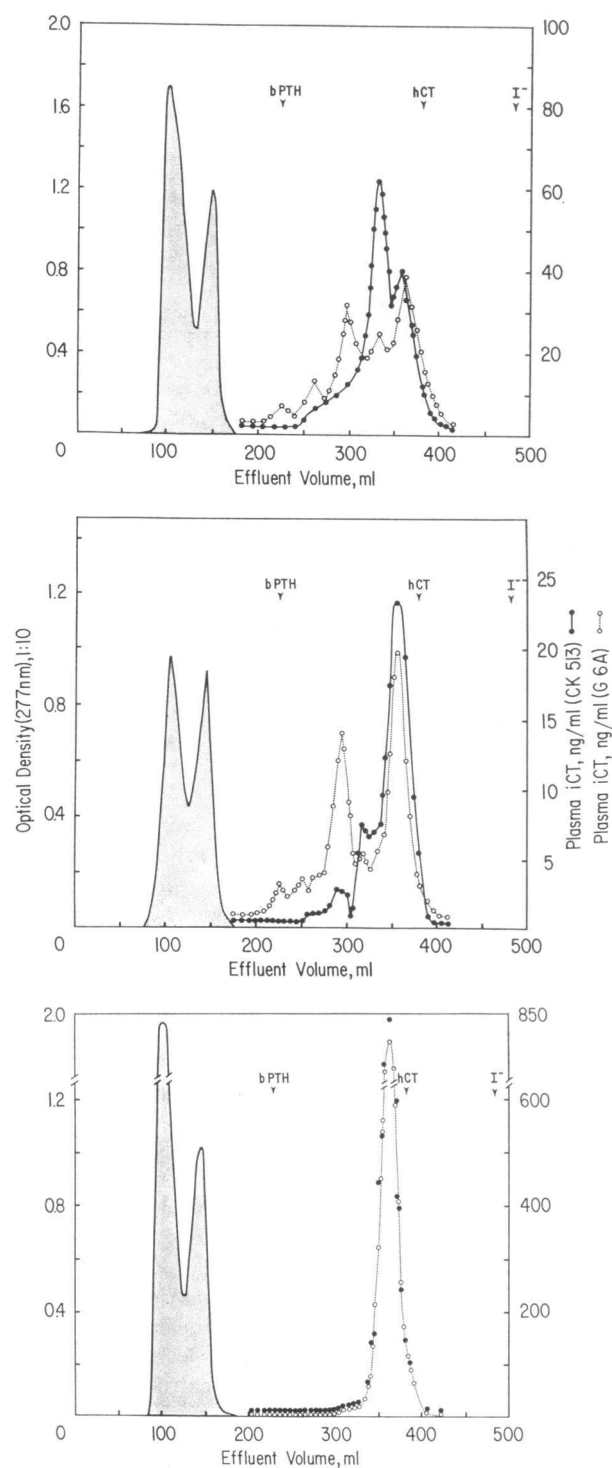


FIGURE 3 Gel filtration of plasma samples from patients with MTC (top, patient A; middle, patient B) and of pooled plasma from athyreotic patients to which CT 1-32 had been added (bottom, plasma C). Bio-Gel P-150 column (2.5 by 80 cm), 0.2 M ammonium acetate (pH 4.6); column

TABLE I
Relative Inhibition of Binding: Inhibitor Concentrations
Required for 50% Inhibition

Inhibitor	Concentration required*	
	Antiserum G6A	Antiserum CK513
	<i>pmol/ml</i>	
CT 1-32	0.022	0.025
H-11-32-NH ₂	0.80	0.49
H-17-32-NH ₂	74.0	2.1
H-11-28-OH	3.3	>1,000
H-17-28-OH	81.0	>1,000

* At constant final antiserum dilution of 1:75,000 (G6A) or 1:2,000 (CK513).

of the H-11-32-NH₂ fragment required to produce 50% inhibition of tracer binding were 36-fold (G6A antiserum) and 20-fold (CK513 antiserum) greater than those of the intact molecule. This provides indirect evidence that both antisera had binding affinity for the missing NH₂-terminal fragment, and that G6A antiserum had a higher affinity than CK513 antiserum.

A major difference between the antisera was the apparent greater specificity of CK513 antiserum for the sequence region 29-32. With this antiserum, there was a marked loss of immunoreactivity when this sequence was not present (compare curves produced by H-11-28-OH and H-11-32-NH₂ and by H-17-28-OH and H-17-32-NH₂ with each antiserum). With antiserum G6A, the loss of immunoreactivity was much less. Additional evidence that the sequence 29-32 is not a major immunologic determinant for antiserum G6A is shown by the superimposition of inhibition curves produced by fragments H-17-32-NH₂ and H-17-28-OH in the assay using this antiserum.

Finally, both antisera had specificity for a portion of the sequence that includes the region 11-17, but the limited quantities of available peptide fragments have prevented definitive quantitation of individual antiserum recognition of this region of the molecule.

Gel filtration studies. Elution patterns from gel filtration of plasma from patients A and B and of pooled plasma from thyroidectomized patients to which CT 1-32 had been added (plasma C) are compared in Fig. 3. The labeled markers eluted consistently: the K_d of [¹²⁵I]bPTH was 0.32, 0.31, and 0.32 for columns A, B, and C, respectively; the K_d of [¹²⁵I]CT 1-32 was 0.73, 0.73, and 0.74, respectively. [¹²⁵I]CT 1-32 con-

sisted of three immunoreactive peaks in plasma from patients A and B; CK513 antiserum defined two and three immunoreactive peaks, respectively. In plasma C (bottom), both antisera defined only one immunoreactive peak.

TABLE II
Recoveries of iCT from Gel Filtration Columns

Column	With G6A antiserum	With CK513 antiserum
Patient A		
Added, ng*	7,262	2,772
Recovered, ng	3,497 (48%)	3,309 (119%)
Patient B		
Added, ng*	2,332	854
Recovered, ng	1,253 (54%)	978 (115%)

* Calculated from milliliters of plasma added to column times iCT concentration in plasma.

tently eluted at least 16 ml after the last immunoreactive peak (monomer) in each of the gel filtration studies.

G6A antiserum recognized five iCT components in the plasma of MTC patients A and B. Peak one was coeluted with the [¹²⁵I]bPTH marker (Kd = 0.32 and 0.32, respectively). The remaining peaks were eluted later with Kd values as follows: peak two, 0.41 and 0.40; peak three, 0.51 and 0.50; peak four, 0.60 and 0.56; and peak five, 0.67 and 0.67. In contrast, G6A antiserum recognized only one iCT component in the athyreotic plasma to which hCT had been added (plasma C). The elution position of this immunoreactive peak (Kd = 0.69) corresponded to that of peak five of the plasma from the MTC patients. The immunoreactive material in peak four, from the gel filtration study illustrated at the top of Fig. 3, was rechromatographed in buffer with and without mercaptoethanol and was then measured with G6A antiserum. Mercaptoethanol did not increase the elution volume of peak four material to a value corresponding to that of CT 1-32. However, the Kd of peak four material and ¹²⁵I-labeled CT 1-32 dimer were identical.

CK513 antiserum recognized only three of the five iCT components recognized by antiserum G6A. In plasma from patient A, it recognized immunoreactive

peaks four and five (Kd values of 0.60 and 0.67, respectively). Although there was a shoulder of immunoreactivity beginning at 250 ml and preceding peak four, no definitive immunoreactive peak was detected. In plasma from patient B, it recognized peaks three, four, and five (Kd values of 0.50, 0.56, and 0.67, respectively). As was the case with G6A antiserum, CK513 antiserum recognized only one iCT component in plasma C, and this component also corresponded to peak five of the plasma from the MTC patients. No immunoreactivity was detected, by either assay, in the P-150 column effluents between 356 ml (peak five) and 600 ml (after salt peak). Preliminary studies on fractions corresponding to the column profile region between 75 and 175 ml (high molecular weight proteins) have shown the presence of immunoreactivity, similar to that of iCT, in the immunoassay with G6A antiserum.

Recoveries of the [¹²⁵I]CT 1-32 markers added to the P-150 columns ranged between 92 and 100%. The recovery of the iCT added to each column could be estimated quantitatively (Table II) because the slopes of the immunodilution curves of whole plasma, each component (peaks one through five) of the gel filtration studies, and CT 1-32 were parallel. Recoveries of the iCT contained in the gel filtration fractions of plasma from patient B are shown in Table III. There was complete recovery of the CT 1-32 immunoreactivity in plasma C by both G6A (100%) and CK513 (100%) antisera.

DISCUSSION

Monomer and dimer forms of hCT with similar biologic activity have been extracted from MTC by the Basel and London groups (23, 24). They found that the amounts of each form in the tumors were variable. We believe that peak five in our gel filtration studies is monomer because its Kd value was similar to that of the synthetic calcitonin monomer added to athyreotic

TABLE III
Recoveries of iCT in Gel Filtration Fractions of Plasma from Patient B

Fraction	Location	iCT	
		With G6A antiserum	With CK513 antiserum
<i>ml of effluent</i>		<i>ng</i>	<i>ng</i>
179-213	Before peak 1	≤ 40.3	≤ 34.9
213-242	Peak 1	66.8	≤ 28.5
242-258	Peak 2	48.4	≤ 16.7
258-283	Shoulder 3	87.8	30.2
283-314	Peak 3	307.2	71.8
Subtotal iCT		550.5	182.1
314-334	Peak 4	90.2	116.3
334-419	Peak 5	612.1	679.9
Total		1,252.8	978.3

plasma. The significance of the forms of iCT with apparent molecular weights larger than monomer must await further studies. They may be biosynthetic precursors of the monomer, similar to those found by Roos, Okano, and Defetos (25) in their *in vitro* work with trout ultimobranchial tissue.

Heterogeneity of circulating iCT has also been reported, in an abstract, by Singer and Habener (26); however, they found four rather than five peaks of immunoreactivity. The discrepancy may be explained by differences in the resolution of iCT components (they used Bio-Gel P-10 and we used Bio-Gel P-150) or by our use of two antisera, which facilitated differentiation of peak four from peaks three and five. One of their peaks also was coeluted with labeled calcitonin dimer, and there was no change in its chromatographic mobility after incubation in mercaptoethanol.

The immunoreactivity profiles from the gel filtrations show that the relative amounts of the different iCT forms, particularly monomer and dimer, may be variable, as was true for tumor extracts (23, 24). In our study, the ratio, peak four/peak five, was greater for patient A than for patient B. We do not know whether this variability reflects differences in secretion by the medullary tumors. However, it appears that the antiserum with COOH-terminal specificity, CK513, recognized the immunoreactive material of peak four better than did the NH₂-terminal antiserum in both gel filtration studies. If this peak four material is formed by means of disulfide bridges in the NH₂-terminal region, the resulting dimer may have steric changes such that the COOH-terminal sites would become the ones relatively more available for reaction with an antiserum having COOH-terminal affinity.

iCT was not detected in column effluents between the elution volume of monomer (356–364 ml) and well after the salt peak (600 ml) in assays utilizing either antiserum. Although both antisera have recognition sites for regions of the calcitonin sequence shorter than monomer, it is possible that some fragments of the calcitonin monomer lacked the immunologic determinants required for recognition by our antisera and were not detected. The existence of such fragments has been suggested by Baylin, Hsu, and Foster (27). They have shown that fragmentation of ¹²⁵I-labeled CT 1-32 added to human plasma occurs during *in vitro* incubation and that the rate of fragmentation parallels the rate of loss of endogenous iCT in the same sample. However, fragmentation need not occur before excretion. In studies (28, 29) of urine of patients with MTC in Tashjian's laboratory, a single peak of calcitonin-like material was eluted from Sephadex G-75 in the same position as ¹²⁵I-labeled CT 1-32, and this biologically active material was described as "similar to if not the same as"

hCT. These authors explained the high ratio of immunologic to biologic activities of this material on the basis of a subtle change in structure that made the material less active biologically.

In our immunoassays, G6A antiserum gave higher iCT values in plasma of MTC patients, gave similar values for peaks four and five in the gel filtration studies, and identified more immunoreactive peaks compared to CK513 antiserum. We initially suspected that the additional iCT detected by the G6A antiserum in peaks one, two, and three might explain the difference in total iCT values, but this is not entirely correct. As shown in Table III, recovery of iCT in column effluents up to peak three inclusive was three times greater by the G6A assay than by the CK513 assay. But for total iCT, the ratio, G6A/CK513, was only 1.28 in column effluents compared to 2.74 in the plasma that had been added to the column. The results for patient A were similar. Thus, although recognition of peaks one, two, and three by G6A antiserum partially explains the higher plasma values for iCT with this antiserum, it is not the entire answer.

By the G6A assay, 48 and 54% of the iCT added to gel filtration columns in plasma from patients A and B was recovered. In contrast, recoveries were virtually complete for the [¹²⁵I]CT 1-32 markers, for the heterogeneous iCT in plasma from both MTC patients measured with the CK513 assay, and for iCT monomer added to athyreotic plasma (plasma C) and measured in both assays. Two recent findings offer possible explanations for this apparent discrepancy. Rodriguez (30) and Jackson and Rodriguez (31) have discovered distinct calcitonin-binding β -globulins with mol wt ranging between 26,000 and 84,000 in bovine and porcine plasma. Our preliminary radioimmunoassays with G6A antiserum in column effluent fractions between 75 and 175 ml suggest the presence of iCT because aliquots of these fractions produce more depression of the B/F ratio than would be predicted from their protein concentration alone; however, more work needs to be done to be absolutely certain that these observations are not artifactual.

The two major results of our studies are (a) that there is immunochemical heterogeneity of calcitonin in human plasma and (b) that the absolute iCT concentration measured by radioimmunoassay depends on recognition of these distinct molecular species as well as on the specific binding affinities of the antiserum used to detect them. These observations suggest that some disagreement between laboratories about values for iCT in the same plasma samples from patients with MTC may be expected when different antisera are used in immunoassays. The data also suggest one possible explanation for the variations among iCT values in nor-

mal subjects reported by different laboratories. Our results also reinforce the concern, originally expressed by Moukhtar, Jullienne, Tharaud, Taboulet, and Milhaud (32), that concentration of plasma iCT by means of affinity chromatography (32, 33) or immunoextraction (34) before measurement also may lead to variable results. Concentration of circulating iCT species by either method probably depends on the specificity of the antisera used in such concentration methods. If an antiserum like CK513 were used (which failed to recognize three of the circulating iCT species we have demonstrated), not all of the iCT in a plasma sample would be concentrated and the total iCT concentration would be underestimated.

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