

## Immunoreactive Forms of Circulating Parathyroid Hormone in Primary and Ectopic Hyperparathyroidism

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### Research Article

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# Immunoreactive Forms of Circulating Parathyroid Hormone in Primary and Ectopic Hyperparathyroidism

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**ABSTRACT** The immunoreactive forms of parathyroid hormone (iPTH) in the plasma of six patients with primary, adenomatous hyperparathyroidism and six patients with ectopic hyperparathyroidism due to nonparathyroid cancer were compared by using gel filtration on columns of Bio-Gel P-150 and radioimmunoassay of iPTH in eluted fractions after concentration. We found much less ( $p < 0.001$ ) small (mol wt  $< 9,500$ ) COOH-terminal fragments of iPTH in plasma samples from ectopic hyperparathyroid patients ( $0.52 \pm 0.13$  ng eq/ml) than in samples from primary hyperparathyroid patients ( $3.70 \pm 1.15$  ng eq/ml). The quantity of iPTH eluting with or before native bovine PTH [1-84] was the same in both syndromes (ectopic hyperparathyroidism,  $0.82 \pm 0.22$  ng eq/ml; primary hyperparathyroidism,  $0.73 \pm 0.09$  ng eq/ml), and these values correlated positively with plasma calcium concentration (ectopic hyperparathyroidism,  $r = 0.908$ ; primary hyperparathyroidism,  $r = 0.919$ ). In both syndromes, plasma samples had an iPTH component that eluted well before PTH [1-84] (mol wt 9,500), but this component was present in much larger quantities in three patients with ectopic hyperparathyroidism. We conclude that (a) the decreased quantity of biologically inactive COOH-terminal fragments of iPTH circulating in ectopic hyperparathyroidism accounts for the previously reported relatively lower total serum iPTH values in this syndrome as compared with primary hyperparathyroidism (Riggs et al. 1971. *J. Clin. Invest.* 50: 2079); (b) there appears to be sufficient iPTH with presumed biologic activity to account for the hypercalcemia in both syndromes; (c) a large PTH component, not previously recognized in plasma, is present in both ectopic and primary hyperparathyroidism and may exist as the predominant immunoreactive form of the hormone in some patients with ectopic hyperparathyroidism.

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## INTRODUCTION

Ectopic hyperparathyroidism (HPT)<sup>1</sup> results from the synthesis and secretion of parathyroid hormone (PTH) by a nonparathyroid cancer (1-3); the syndrome simulates the biochemical features of primary HPT (4). In 1971, Roof, Carpenter, Fink, and Gordan (5) and Riggs, Arnaud, Reynolds, and Smith (6) observed that the serum immunoreactive PTH (iPTH) in ectopic HPT differed immunologically from that in primary HPT. We further observed (6) that most patients with ectopic HPT could be differentiated from patients with primary HPT on the basis of a lower iPTH value for a given serum calcium concentration, from which we hypothesized that this difference might be due to differences in the relative amounts of the circulating forms of iPTH.

The present study examines some of the physical and immunochemical properties of circulating iPTH in ectopic and primary HPT.

## METHODS

*Patients and biochemical studies.* Plasma samples from six hypercalcemic patients with cancer (hypernephroma in four, bronchogenic carcinoma in one, and adenocarcinoma of the colon in one), six patients with surgically proved primary HPT, and two patients with surgical hypoparathyroidism were studied; except for one patient with a solitary rib metastasis, there were no clinically apparent bone metastases. These 6 patients were from a larger group of 103 hypercalcemic patients with cancer who had abnormal plasma iPTH values; they were selected so that their plasma iPTH values were representative of the larger group.

Serum calcium was measured by atomic absorption flame spectrophotometry; serum creatinine was assessed by an automated version of the Jaffe reaction (7); and serum phosphorus was measured by an automated version of the Fiske and Subbarow technique (8).

<sup>1</sup> *Abbreviations used in this paper:* bPTH, bovine parathyroid hormone; HPT, hyperparathyroidism; iPTH, immunoreactive forms of parathyroid hormone; proPTH, precursor form of parathyroid hormone; PTH, parathyroid hormone.

**Gel filtration and concentration procedures.** Heparinized plasma was frozen within 1 h of collection and stored at  $-20^{\circ}\text{C}$ ; 10 ml of thawed sample was centrifuged at  $4^{\circ}\text{C}$  for 20 min at 28,000 *g*. For gel filtration,  $2.5 \times 100$ -cm columns were packed with Bio-Gel P-150 (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif.). Columns were calibrated with  $^{125}\text{I}$ -labeled human serum albumin,  $^{125}\text{I}$ -labeled luteinizing hormone, and  $^{125}\text{I}$ -labeled synthetic human PTH [1–34] (Beckman Instruments, Inc., Bioproducts Div., Palo Alto, Calif.). Immediately before the sample was applied to the column, highly purified  $^{125}\text{I}$ -labeled PTH, extracted from bovine parathyroid glands (bPTH [1–84]; 10,000 cpm), was added as a molecular marker. The elution positions of  $^{125}\text{I}$ -labeled bPTH [1–84] added in buffer and added in plasma were identical. The buffer was 0.2 M ammonium acetate, pH 4.6. All chromatographic procedures were carried out at  $4^{\circ}\text{C}$ . [ $^{125}\text{I}$ ]iodide was used to determine the elution position of the salt volume. The void volume was marked by the elution position of the largest plasma proteins. Effluent was collected in 1-ml fractions.

After measurement of radioactivity in a gamma ray scintillation counter equipped with a pulse-height analyzer to assess the location of the [ $^{125}\text{I}$ ]bPTH [1–84] and  $^{125}\text{I}$  markers and subsequent determination of optical density, the fractions were combined into pools beginning with those fractions having an absorbance of less than 0.15 at 277 nm (the pools were 5 ml when the iPTH value was greater than 2 ng eq/ml and 10 ml when the iPTH value was less than this). The fractions were maintained at  $4^{\circ}\text{C}$  during all procedures, except for the 1 h required for measurement of radioactivity. The pools were immediately shell-frozen, lyophilized for 24 h, resuspended in 5 ml of 0.01 M acetic acid, relyophilized for 48 h, and finally resuspended in 1 ml of 0.01 M acetic acid for radioimmunoassay.

In our hands, iPTH values determined in serum and plasma of the same individual are always identical. Plasma was used because more plasma than serum could be obtained from the same amount of blood. To ensure that heparin did not affect the elution position of PTH, preliminary experiments were done in which [ $^{125}\text{I}$ ]bPTH was added to samples of serum, plasma, and serum containing 300 USP U of sodium heparin (Organon Inc., West Orange, N. J.). The samples were gel filtered on P-150, and the radioactivity of each fraction was measured. The elution positions of the added [ $^{125}\text{I}$ ]bPTH were identical in all three.

**Radioimmunoassay.** iPTH in plasma samples and in effluent pools was measured in multiple dilutions by the radioimmunoassay system described by Arnaud, Tsao, and Littledike (9). This system uses a guinea pig anti-porcine PTH (GP 1M, 1:50,000 final dilution), [ $^{125}\text{I}$ ]bPTH, and dextran-coated charcoal to separate antibody-bound from “free” [ $^{125}\text{I}$ ]bPTH; it measures iPTH in greater than 95% of normal adult human sera (normal range, undetectable to 1.0 ng eq/ml) and detects as little as 10 pg of highly purified human PTH [1–84]. At the dilution used in these studies, GP 1M antiserum does not react with either synthetic bPTH (Beckman Instruments, Inc.) or human  $\text{NH}_2$ -terminal PTH [1–34] (10) and therefore is considered to be specific for the COOH-terminal region of PTH [1–84]. In the present studies, values for iPTH were expressed in terms of highly purified human PTH [1–84] (11).

Samples of column effluent before the void volume were concentrated by lyophilization and reconstituted in 0.01 M acetic acid in the same way as other fractions in each

experiment. These samples were included in the assays to assess possible nonspecific effects of residual ammonium acetate on the immune reaction between our antiserum and [ $^{125}\text{I}$ ]bPTH [1–84]. In all of the experiments reported, there was no significant difference between the ratios of antibody-bound and free [ $^{125}\text{I}$ ]bPTH in incubations containing “blank” column effluent and in those containing an equal quantity of assay diluent (9).

To exclude potential nonspecific effects of protein and salt on the immune reaction, we ignored all assay results on fractions eluting with the plasma proteins (volumes between 105 and 180 ml) and the salt peak (volume  $> 300$  ml). Therefore, our studies assessed the immunoreactive components eluting at volumes between 180 and 300 ml.

## RESULTS

For ectopic HPT, means ( $\pm$ SE) for serum calcium, phosphorus, and creatinine, respectively, were  $12.7 \pm 0.5$  mg/dl,  $3.0 \pm 0.2$  mg/dl, and  $1.2 \pm 0.2$  mg/dl. For primary HPT, means ( $\pm$ SE) for serum calcium, phosphorus, and creatinine, respectively, were  $13.0 \pm 0.5$  mg/dl,  $2.0 \pm 0.2$  mg/dl, and  $1.0 \pm 0.2$  mg/dl. The serum phosphorus values were higher ( $P < 0.01$ ) in the patients with ectopic HPT; we have no explanation for this. Mean serum calcium and creatinine values did not differ significantly.

No iPTH was detected in any fractions from the gel filtration of plasma from the two hypoparathyroid patients, indicating the absence of substances that nonspecifically influence the radioimmunoassay in the effluent volumes we studied (180–300 ml). The characteristic elution patterns of iPTH in plasma from patients with ectopic and primary HPT are compared in Figs. 1 and 2. Three major immunoreactive components were consistently recognized: peak I eluting ( $K_d = 0.28$ )<sup>a</sup> well before the added [ $^{125}\text{I}$ ]bPTH marker; peak II eluting ( $K_d = 0.33$ ) with or just before the marker; and peak III eluting ( $K_d = 0.46$ ) after the marker. Molecular weights were estimated by noting their elution position relative to the positions of the radioiodine-labeled molecular markers. It was not possible to run these columns in a denaturing agent such as urea or guanidine, because these substances interfere significantly with the immune response. Consequently, we consider that the following estimates of molecular weights are gross approximations: peak I,  $> 25,000$ ; peak II, 9,500–11,000; and peak III,  $< 9,500$ . In two patients with primary HPT and in one patient with ectopic HPT, there was an additional peak (peak IV) that eluted ( $K_d = 0.53$ ) between peak III and the salt peak (Fig. 2).

The amounts of iPTH, estimated by integration, in these peaks in ectopic and primary HPT are given in Table I. All patients with primary HPT had small amounts of iPTH in peak I. Three patients with ectopic

<sup>a</sup>  $K_d$  is defined as: (elution volume of iPTH peak – void volume)/(elution volume of salt peak – void volume).

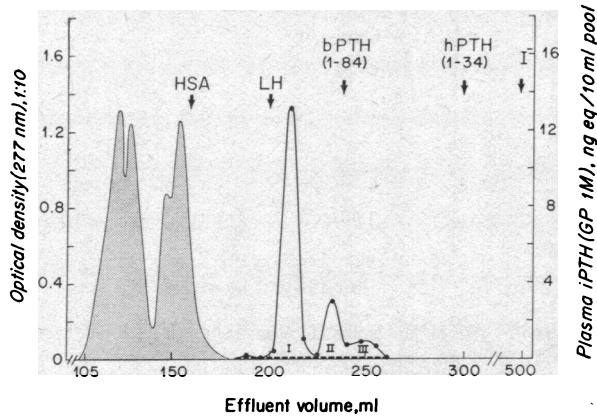


FIGURE 1 Gel filtration of 10-ml samples of plasma from patient (E4) with ectopic HPT due to hypernephroma (solid line) and from patient with postsurgical hypoparathyroidism (broken line). Bio-Gel P-150 column (2.5 × 100 cm), 0.2 M ammonium acetate (pH 4.6); calibrating substances were human serum albumin (HSA), luteinizing hormone (LH), synthetic human PTH (hPTH) [1-34] labeled with <sup>125</sup>I, bPTH [1-84] labeled with <sup>125</sup>I, iodide as <sup>131</sup>I. Optical density readings of 1-ml effluent fractions are represented by shaded area. Note that there are three clearly defined immunoreactive peaks (I, II, and III).

HPT had much greater amounts of iPTH in peak I, and one had a similar amount compared to primary HPT; in two patients with ectopic HPT, peak I could not be

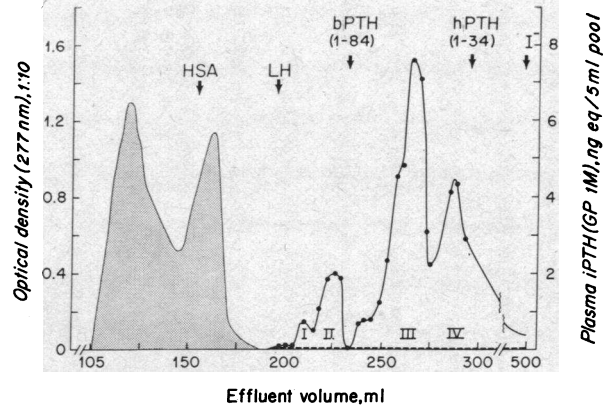


FIGURE 2 Gel filtration of 10-ml samples of plasma from patient with primary HPT (solid line) and from patient with postsurgical hypoparathyroidism (broken line). Methods as in Fig. 1. Note that peak I is small and that peaks III and IV account for most of total iPTH.

detected. We speculate that this may be due to the necessity of using 10-ml, rather than 5-ml, elution pools because of the low iPTH values in these patients and the resultant inability to resolve small amounts of iPTH in peak I from peak II. In both ectopic and primary HPT, easily detectable amounts of iPTH were present in peak II, but there was a slightly greater amount ( $P < 0.05$ ) in primary HPT (mean ± SE,  $0.55 \pm 0.09$  ng eq/ml)

TABLE I  
Amounts of Various Forms of Circulating iPTH

Patient	Recovery %	Serum calcium mg/dl	Unfractionated plasma	iPTH in plasma			
				Peaks			
				I	II	I + II	III + IV
				ng eq/ml			
<b>Ectopic HPT</b>							
E1	98	11.4	0.8	—	0.27	0.27	0.51
E2	109	11.5	0.6	—	0.35	0.35	0.31
E3	87	12.0	1.1	0.18	0.26	0.44	0.53
E4	125	13.5	1.4	1.27	0.36	1.63	0.15
E5	123	13.7	1.7	0.62	0.38	1.00	1.10
E6	124	13.8	1.4	0.76	0.48	1.24	0.50
Mean ± SE	111 ± 7	12.7 ± 0.5	1.2 ± 0.2	0.47 ± 0.21	0.35 ± 0.03	0.82 ± 0.23	0.51 ± 0.13
<b>Primary HPT</b>							
P1	59	11.9	4.1	0.21	0.24	0.45	1.96
P2	60	12.3	5.0	0.12	0.66	0.78	2.19
P3	63	12.5	4.2	0.22	0.40	0.62	2.01
P4	113	12.6	2.3	0.18	0.46	0.64	1.97
P5	61	14.0	16.0	0.10	0.77	0.87	8.83
P6	63	14.9	10.0	0.26	0.77	1.03	5.27
Mean ± SE	70 ± 9	13.0 ± 0.5	6.9 ± 2.1	0.18 ± 0.03	0.55 ± 0.09	0.73 ± 0.08	3.70 ± 1.15
P*	<0.01	NS	<0.05	NS	NS	NS	<0.05

\* For difference between means of two groups.

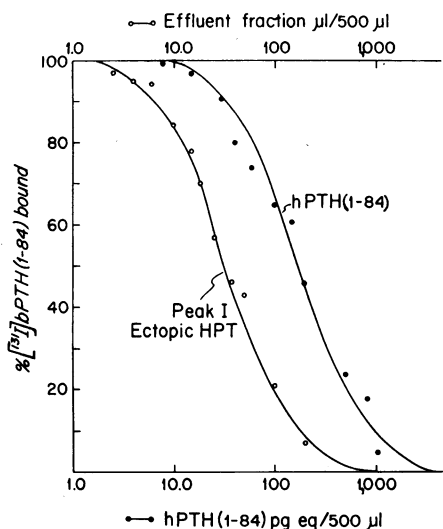


FIGURE 3 Immunodilutional curves of peak I obtained by gel filtration from patient with ectopic HPT due to hypernephroma (open circles) and similarly constructed curve from highly purified gland-extracted human PTH (hPTH) [1-84] (solid circles). Note that the two curves are superimposable.

than in ectopic HPT ( $0.35 \pm 0.03$  ng eq/ml). However, there was no significant difference in the amount of iPTH eluting with or before the bPTH [1-84] marker, i.e. in peak I plus peak II, in ectopic HPT ( $0.82 \pm 0.22$  ng eq/ml) and in primary HPT ( $0.73 \pm 0.09$  ng eq/ml).

Peak III was always present in both syndromes, but there was a much larger amount ( $P < 0.001$ ) in primary HPT ( $3.71 \pm 1.15$  ng eq/ml) than in ectopic HPT ( $0.52 \pm 0.13$  ng eq/ml).

Recovery of iPTH after filtration and concentration ranged from 87 to 125% in ectopic HPT and from 58 to 113% in primary HPT. The lower recoveries in primary HPT are probably due to the significant amounts of iPTH that trailed into the salt peak and that could not be measured accurately (see Methods section, Radioimmunoassay).

In patient E4 (elution pattern shown in Fig. 1), there was sufficient immunoreactivity in peak I so that an immunodilutional curve could be constructed. This curve was superimposable throughout its entire length on the curve obtained from gland-extracted homogeneous human PTH [1-84] (Fig. 3).

Significant positive correlations with serum calcium concentration were found for total plasma iPTH, peak I, and peaks I plus II in ectopic HPT, for peaks I plus II in primary HPT (Fig. 4), and for peak II and peaks I plus II when all values were merged (Table II).

## DISCUSSION

Nonmetastatic cancer (with presumed ectopic hormone synthesis) is the third most common cause of hyper-

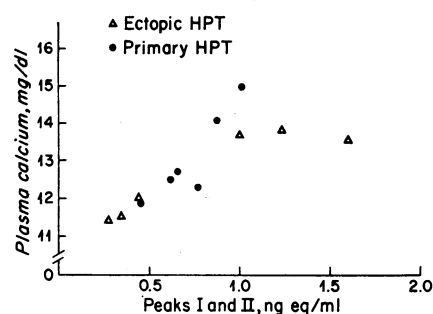


FIGURE 4 iPTH in peaks I plus II as function of serum calcium concentration in ectopic HPT (open triangles) and in primary HPT (solid circles). In both ectopic and primary HPT, the positive correlation is significant (Table II).

calcemia, ranking in frequency behind metastatic cancer and only slightly behind primary HPT (4). Recently, Powell, Singer, Murray, Minkin, and Potts (12) suggested that, in some hypercalcemic hypophosphatemic patients with cancer, the syndrome may be due to ectopic synthesis of a humoral substance other than PTH. However, we have found that 103 of 108 (95%) unselected hypercalcemic patients with cancer had plasma iPTH values (with our standard COOH-terminal-specific antiserum, GP 1M) that were considered to be abnormal (13). The findings that we are reporting here are relevant only to the large majority of hypercalcemic patients whose plasma iPTH values are abnormal and not to the small minority of patients in whom other mechanisms may be operative.

The present study was stimulated by our observation (6) that plasma iPTH was lower for a given serum calcium value in patients with ectopic HPT than in patients with primary HPT. This phenomenon suggested to us that there might be differences in the relative quantity of different forms of iPTH in primary and ectopic HPT. Berson and Yalow (14) were the first to suggest that serum iPTH is heterogeneous. During the last 2 yr, workers in several laboratories (15-18) have demonstrated several components of iPTH

TABLE II  
Correlation Coefficients ( $r$ ) for Relationship of Serum Calcium to Plasma iPTH

	$n$	Total iPTH	$r$			
			Peaks			
			I	II	I + II	III + IV
Ectopic HPT	6	0.927*	0.868*	0.764	0.908*	0.284
Primary HPT	6	0.758	0.159	0.806	0.919*	0.771
Merged values	12	0.557	0.481	0.691*	0.796†	0.541

\*  $P < 0.05$ .

†  $P < 0.01$ .

in fractionated sera from patients with primary HPT—a proportionally small amount of intact native PTH [1–84] and a proportionally large amount of one or more COOH-terminal fragments of the intact molecule. The circulating COOH-terminal fragments fail to react with NH<sub>2</sub>-terminal-specific antisera (16, 17) and do not stimulate renal cortical adenylate cyclase activity (19); therefore, they appear to lack the NH<sub>2</sub>-terminal amino acid sequence that is necessary for biologic activity. There is disagreement (19, 20) as to whether NH<sub>2</sub>-terminal fragments also circulate.

The low total concentration of iPTH in the plasma of patients with ectopic HPT was a major obstacle in quantifying the circulating forms of iPTH by gel filtration. In separate studies, we found that various extraction procedures applied to plasma may extract the different circulating species of iPTH nonuniformly. Our experience with the concentration of iPTH from whole plasma by membrane filtration has been similar to that described by Canterbury and Reiss (15), in that large losses of iPTH occurred, and we were concerned that these losses also might be nonuniform. Our compromise was to use gel filtration of whole plasma and then concentrate pools of eluate after fractionation had been achieved. Unfortunately, there was too little iPTH in these pools from patients with ectopic HPT to permit study with more than one radioimmunoassay system.

We elected to assay with antiserum GP 1M because our preliminary studies showed that it measured iPTH in unfractionated sera of patients with ectopic HPT better than did a sensitive NH<sub>2</sub>-terminal-specific antiserum. Also, because this antiserum is COOH-terminal specific, it would be expected to react with circulating precursor molecules (21) and COOH-terminal fragments as well as native PTH [1–84]. However, it would not be expected to react with circulating NH<sub>2</sub>-terminal fragments.

Large-scale fractionation of whole plasma by gel filtration on columns of Bio-Gel P-150 resolved three major components of circulating iPTH in both ectopic and primary HPT. However, the iPTH elution profiles differed quantitatively between the two syndromes. The most striking difference was that the quantity of circulating COOH-terminal immunoreactive equivalents with mol wt of < 9,500 was seven times greater in primary HPT than in ectopic HPT. We believe that the decreased quantity of circulating biologically inactive COOH-terminal fragments accounts for our previous observation (6) that, for a given serum calcium value, serum iPTH is relatively lower in ectopic HPT than in primary HPT.

The high specificity of GP 1M antiserum used in this study for determinants in the COOH-terminal region of PTH was crucial in demonstrating this phenomenon.

Differences in antiserum specificity probably account for the variations among laboratories, with observations ranging from little difference to a large overlap in serum iPTH values in ectopic primary HPT (22, 23). It appears that radioimmunoassays that are able to detect low molecular weight COOH-terminal fragments are probably necessary for immunologic differentiation of the two syndromes.

The reason for the decreased quantity of COOH-terminal fragments in ectopic, as compared to primary, HPT is not clear. The explanation most compatible with our data is that cancer cells lack the enzyme required for cleavage of PTH to COOH-terminal fragments. This mechanism presupposes that at least some degree of cleavage of PTH occurs within parathyroid tissue, as was previously suggested by *in vitro* studies of parathyroid tissue cultures by Arnaud, Tsao, and Oldham (24) and Sherwood, Rodman, and Lundberg (25). An alternative explanation is that cleavage occurs in the peripheral tissues but is defective in ectopic HPT because the form of iPTH secreted by cancer cells may be less susceptible to the cleavage enzymes. Although Habener, Powell, Murray, Mayer, and Potts (26) found that the iPTH in blood obtained during parathyroid vein catheterization was composed largely of intact hormone, the observations of Silverman and Yalow (18) are more compatible with glandular cleavage producing a small amount of COOH-terminal fragments that then accumulate in the circulation because they have a much slower metabolic clearance rate than does PTH [1–84].

In three of our six patients with ectopic HPT, there was an increased amount of a previously unrecognized circulating species of iPTH (peak I) that eluted well before native PTH [1–84]. This species was also present (although in smaller amounts) in all patients with primary HPT. The identity of this component is unknown at the present time. It may represent an aggregate or PTH bound to a high molecular weight protein; however, we believe that this is unlikely because it was present in large quantities only in ectopic HPT, and all plasma samples were processed identically during fractionation procedures. We speculate that this component may be a large molecular weight precursor form of the native PTH [1–84] molecule. Silverman and Yalow (18) identified a large iPTH component in extracts of parathyroid glandular tissue chromatographed on Sephadex G-100. However, it is unlikely that their component, which eluted well before the [<sup>125</sup>I]-albumin marker, is identical to the iPTH in our peak I, which eluted well after the [<sup>125</sup>I]-albumin marker.

Based on its elution position, peak I appears to have a much larger molecular weight than does proPTH, the immediate precursor of PTH [1–84] described by

Cohn, MacGregor, Chu, Kimmel, and Hamilton (27) and by Habener, Kemper, Potts, and Rich (28). These workers have respectively estimated the mol wt of proPTH to be 12,500 or 10,500. We were unable to detect a circulating species of iPTH with a mol wt approximating that of proPTH in either ectopic or primary HPT. However, proPTH would be expected to elute with and immediately before PTH [1-84] and would probably not be resolved from it in our method, which required concentration of 5- or 10-ml pools of eluted fractions. Consequently, we are unable to determine whether proPTH circulates in either ectopic or primary HPT:

The quantity of iPTH eluting with or before the PTH [1-84] marker (peaks I plus II) was the same in both syndromes. This probably is the reason that the degree of hypercalcemia is similar in both syndromes. Peak II represents native PTH [1-84] and is known to be biologically active. We have not yet been able to test the biologic activity of peak I, but this component presumably represents a molecular species of iPTH that includes the entire 84 amino acids and, therefore, has at least potential biologic activity.

iPTH values in peaks I plus II correlated positively with serum calcium concentration in both syndromes. In ectopic HPT (with increased iPTH in peak I in three of six patients), the correlation was better for peak I than for peak II, the native hormone. These observations support the idea that peak I may possess biologic activity.

#### ACKNOWLEDGMENTS

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