RADIOIMMUNOASSAY OF PARATHYROID HORMONE: PERIPHERAL PLASMA IMMUNOREACTIVE PARATHYROID HORMONE RESPONSE TO ETHYLENEDIAMINETETRAACETATE

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Plasma immunoreactive parathyroid hormone (IPTH) concentration was measured by radioimmunoassay according to the modified procedures of Arnaud and coworkers. Thirteen healthy control subjects and 20 patients with operatively proved primary hyperparathyroidism were studied. Each was given an infusion of ethylenediaminetetraacetate (EDTA) to produce a standard hypocalcemic insult. The parameters examined were the basal IPTH value, the peak post-EDTA IPTH value, the difference between these two values (absolute rise), and the ratio of these two values (EDTA-Index). All parameters were found to be significantly different between control and disease groups. Eleven of these 16 were studied postoperatively. In the nine thought to be "cured," the postoperative EDTA studies revealed significant change toward normality. It is concluded that (A) parathyroid tissue in patients with primary hyperparathyroidism is under at least positive feedback control; (B) the hyperfunctioning parathyroid tissue in primary hyperparathyroidism patients is the hyperresponsive tissue; and (C) using our antiserum, the parameters derived from the EDTA study have served to separate normal subjects from patients with primary hyperparathyroidism better than the basal plasma IPTH concentration alone.

Since the introduction of the radioimmunoassay of parathyroid hormone (PTH) by Berson, et al (*1*), many investigators have demonstrated elevation of serum immunoreactive PTH (IPTH) concentration in patients with proven hyperparathyroidism. Such elevation, although a common finding, has been by no means invariable (*2–5*). Unfortunately, we have encountered patients with proven hyperparathyroidism in whom the serum IPTH concentration (with our antiserum) was not only within normal limits but also appropriate for the particular serum calcium concentration simultaneously measured. Whereas such experience may reflect in part the properties of our own antiserum, other investigators have also uncovered overlap in results between normal and abnormal subjects. We decided to look for a dynamic test to assess parathyroid functional status and to try to establish a test which, in our hands, is precise, readily reproducible, and highly discriminatory for the diagnosis of parathyroid disease. This should also help us in the recognition of normocalcemic hyperparathyroidism.

In view of the known fluctuations in the rate of secretion of growth hormone (*6*), catecholamines (*7*), gonadotropins (*8*), and possibly also of PTH (our own observation), we thought that random, isolated blood sampling should maximize the error in the recognition of parathyroid hyperfunction whereas multiple sampling under controlled, dynamic conditions of stimulation and/or suppression might minimize the error. Furthermore, reasoning from experience with the adrenocorticotropic hormone stimulation of hyperfunctioning adrenal tissue (*9*), we thought that stimulation of hyperfunctioning parathyroid tissue in a standard manner might produce

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a paradoxical hyperresponsiveness. EDTA-induced hypocalcemia was used to stimulate PTH output. In this presentation, the effects of EDTA infusion on plasma IPTH and calcium concentrations in (A) healthy control subjects and (B) patients with documented, surgically proven primary parathyroid hyperfunction before and after surgical removal of parathyroid adenoma are reported and compared.

MATERIALS AND METHODS

Subjects. The subjects studied in this report were 13 healthy volunteers and 20 patients with hyperparathyroidism. These assessments were made by clinical and laboratory data other than the plasma IPTH content. Each of the volunteers had normal serum values for calcium, phosphorus, alkaline phosphatase, albumin, total protein, and liver enzymes by the multichannel analyzer technique. Each of the patients had typical primary hyperparathyroidism by all of the usual standards and each was subjected to neck operation and found to have one or more abnormal parathyroid glands (using conventional light microscopy) which were removed. The so-called normal glands were often biopsied.

Assay procedures for parathyroid hormone. Parathyroid hormone concentration in plasma was determined by the radioimmunoassay technique of Arnaud, et al (*4*) with the following modification. The standard diluent contained 2% (by volume) "hypo-PTH" plasma (plasma from patients with known postoperative hypoparathyroidism) and 250 units/ml trasylol (FBA Pharmaceutical Inc., New York) in barbital buffer, pH 8.6, ionic strength 0.02. The incubation volume before addition of ^{131}I-bovine PTH (bPTH) was 1 ml, which contained up to 0.6 ml of sample or standard. Incubation was carried out at 4°C for 2 days. A 0.2-ml aliquot of ^{131}I-bPTH was then added and the incubation was continued for another 2 days at 4°C. The quantity of ^{131}I-bPTH used was such that about 50% of it could be bound to antibody in the absence of added PTH. A series of control incubations were carried out to correct for nonspecific effects of plasma on antibody binding and incubation damage to ^{131}I-bPTH and plasma IPTH concentration was calculated as described by Arnaud, et al (*4*).

A guinea pig antiserum used in this assay (1:10,000 final dilution) was prepared against partially purified bPTH. Highly purified radioimmunoassay grade bPTH (both bPTH preparations were purchased from Wilson Laboratory, Chicago, Ill.) was used for iodination with Na^{131}I according to the method of Yalow and Berson (*10*). A cellulose column alone was used originally for purification of ^{131}I-labeled bPTH but in later experiments the column-purified ^{131}I-bPTH was further purified by absorption to microfine precipitated silica (Quso 32, Philadelphia Quartz Company, Philadelphia, Pa.) (*10*). Pooled "hyper-PTH" plasma obtained from the peripheral blood of hemodialysis patients was used as standard and IPTH concentration in the unknown sample was expressed as microliter-equivalent (to standard plasma) per milliliter of sample plasma (μl Eq/ml). All samples were assayed in duplicate and, wherever possible, in multiple dilutions to ensure immunological identity of IPTH assayed.

Infusions of disodium ethylenediaminetetraacetate. EDTA infusion was carried out according to the method of King, et al (*11*) with slight modification.

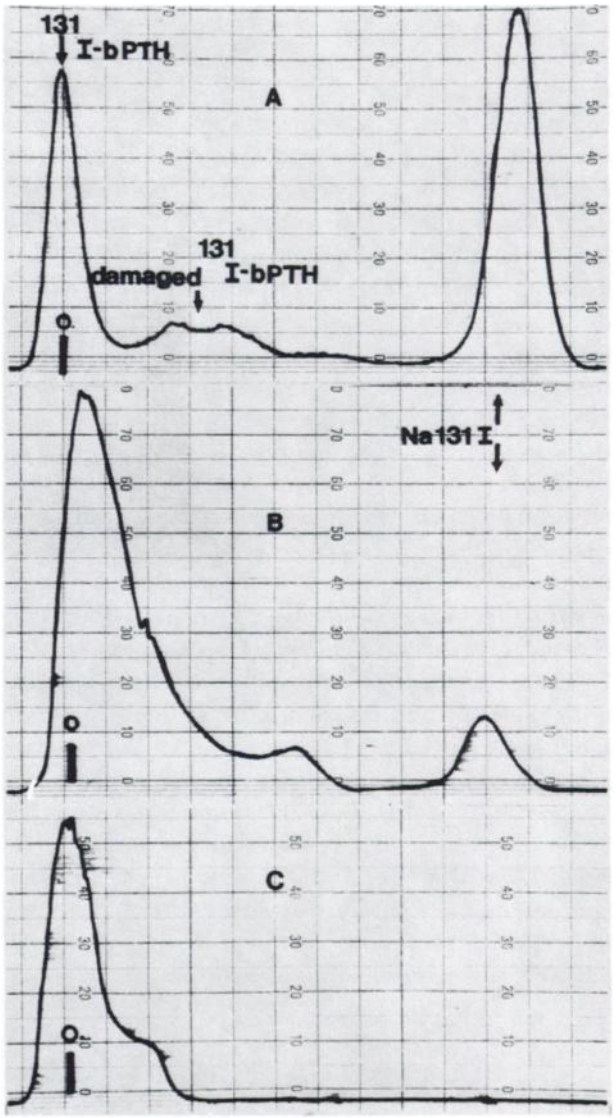

FIG. 1. Radioactive tracings of chromatoelectrophoretograms of ^{131}I-bPTH before (A) and after consecutive purification by cellulose column (B) and silica (C).

Fifty milligrams per kilogram of body weight, but no more than 3000 mg/infusion, were given intravenously in 500 ml of normal saline containing 15–20 ml of 2% lidocaine. Lidocaine in saline was infused without EDTA for several minutes initially. EDTA was then added to the infusion bottle and the entire volume was infused at a constant rate over a period of 60 min. Venous blood sampling was done (without tourniquet stasis) in the opposite arm at various times (0, ½, 1, 2 and, in some patients, 3, 4, and 6 hr after initiation of EDTA administration) with heparin in the collection tubes. EDTA infusion was always performed between 8 and 10 am following an overnight fast. At this time of day, blood calcium and IPTH concentrations are thought to be at a low ebb (*4,12,13*). The overnight fast eliminated any acute effect of dietary calcium on serum calcium and IPTH levels. Postoperative EDTA infusion was performed 6–170 days after the surgery.

All samples of plasma were analyzed for calcium and IPTH. The EDTA titration method (*14*) was used for determination of calcium concentration in plasma samples obtained during EDTA infusion studies and the atomic absorption technique was used for all other calcium determinations (normal range 9.0–10.5 mg/100 ml).

RESULTS

Radioimmunoassay. As shown in Fig. 1, purification through a cellulose column alone could not completely remove unreacted $Na^{131}I$ and damaged ^{131}I products. However, when the ^{131}I-bPTH was further purified through Quso 32, an ^{131}I-bPTH preparation almost completely free from the aforementioned contaminants could be obtained. Specific radioactivity of the final product was 150–300 μCi-μg. Standard curves using this twice-purified ^{131}I-bPTH were more reproducible and linear than those using once-purified ^{181}I-bPTH. Approximately one-third of the data shown in Table 2 were obtained with the twice-purified ^{131}I-bPTH. Figure 2 shows a standard curve representative of those in our studies. A good linear correlation ($r = 0.956$, $p < 0.001$) was obtained with from 15 to 800 μl of standard plasma. With 800 μl of standard plasma, the binding of ^{131}I-bPTH to antibody was suppressed by about 70% whereas the suppression was only 5% with 15 μl. A drop of 5% from the antibody-bound ^{131}I-bPTH to free ^{131}I-bPTH (B/F) found in the absence of added PTH was statistically significant ($p < 0.05$).

The minimum detectable amount of PTH varied with the specific activity of each ^{131}I-bPTH preparation. This, in turn, depended on the quality of each batch of $Na^{131}I$ purchased from the manufacturers (Cambridge Nuclear, Union Carbide, or New Eng-

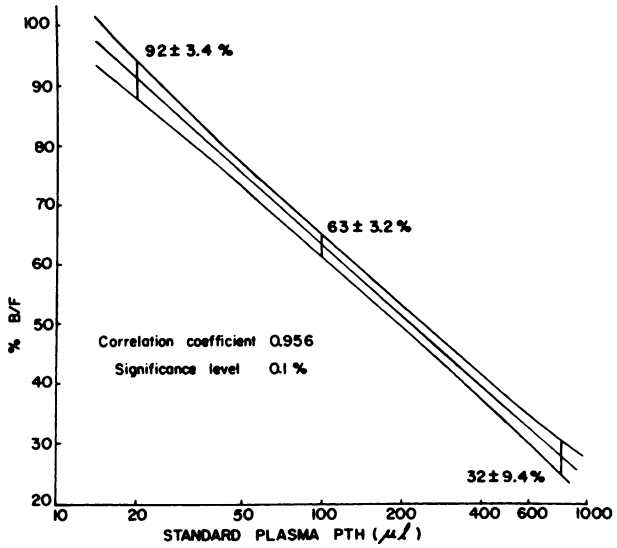

FIG. 2. Standard curve for radioimmunoassay of parathyroid hormone (semilogarithmic plot). Standard curve was constructed from assays of nine different amounts of standard plasma (15–800 μl range), each analyzed in sextuplicate (54 total analyses). Outer two curves represent 95% confidence interval estimate band for standard curve. Ratio of antibody-bound ^{131}I-bPTH to free ^{131}I-bPTH, (B/F), in absence of added standard plasma, was assumed to be 100%.

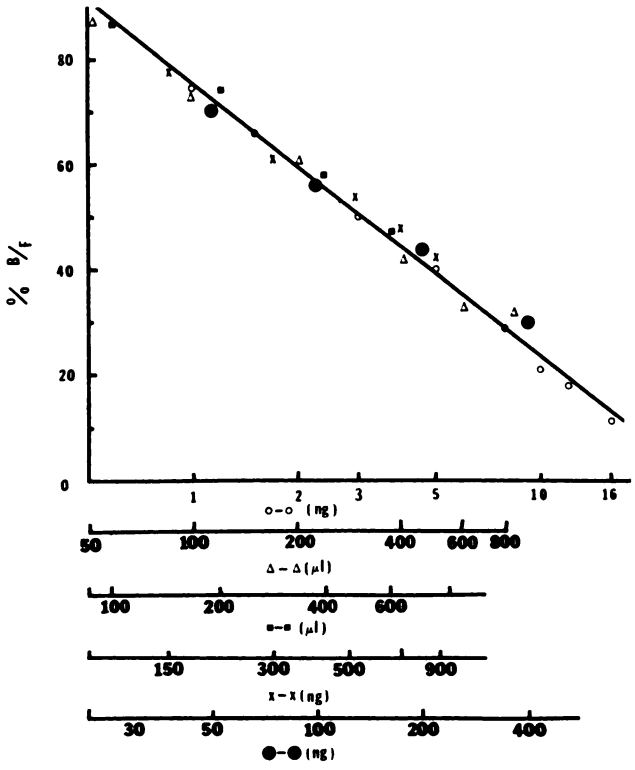

FIG. 3. Inhibition of antiserum binding of ^{131}I-bPTH by various parathyroid hormones in the radioimmunoassay system: (○) highly purified bPTH (Wilson Laboratories), (△) standard plasma from peripheral blood of hemodialysis patients, (□) thyroid-venous-plasma from primary hyperparathyroidism patient, (X) and (●) PTH preparation from culture media of human parathyroid adenoma tissue. Our guinea pig antiserum was used in studies marked by (○), (△), (□), and (X); Arnaud's chicken-14 antiserum was used in study marked (●).

land Nuclear). In general, the minimum detectable amount of IPTH was about 25 μl Eq/ml of plasma (a drop of 5% in B/F) and, at this level, the coefficient of variation was 9% for intra- and 14% for interassay comparisons. Reproducibility was better at higher PTH values. The sensitivity of our assay method allowed us to detect plasma IPTH in 89% of the normal population. Day-to-day fluctuations in the fasting basal IPTH value were seen in several individuals, both controls and patients. The reasons for this are not apparent. The median plasma IPTH value for 60 normal subjects studied was 40 μl Eq/ml and 99% of the normal population had a value less than 180 μl Eq/ml.

Our antiserum, in addition to the PTH present in the standard plasma, also recognizes (A) some fraction of the PTH obtained from the culture medium of human parathyroid adenoma (a generous gift from C. D. Arnaud), (B) PTH present in the inferior thyroid vein [known to contain only intact PTH (*15*)] of a patient with primary hyperparathyroidism, and (C) purified bPTH. This is shown in Fig. 3, in which the immunologic behavior of each of the aforementioned PTHs was studied in the radioimmunoassay system with ^{131}I-bPTH and our guinea pig antiserum or chicken-14 antiserum (also a gift from C. D. Arnaud). The inhibition curves for each IPTH could be superimposed upon one another by appropriate adjustment of the abscissas as shown. We also observed that human growth hormone, luteinizing hormone, and thyroid-stimulating hormone, at concentrations of 50 ng, 30 ng, and 60 μIU, respectively, per incubation tube, failed to inhibit the binding of ^{131}I-bPTH to antiserum.

EDTA infusion. In general, there were no accompanying problems or sequellae with intravenous EDTA. The most annoying reactions encountered were pain and burning along the vein used for infusion. These complaints were kept to a minimum by addition of lidocaine to the infusion solution. Other reactions to be expected with hypocalcemia such as circumoral or digital numbness and tingling, when present, were only transient, and then only in healthy control subjects. One such subject complained of marked numbness and weakness at the end of infusion and his serum calcium was found to be 6 mg/100 ml of serum. These symptoms cleared rapidly after intravenous injection of calcium glutonate. Neither hypocalcemia nor related symptoms were noted in any hyperparathyroid patient and no evidence of renal complication or of later thrombophlebitis at the site of EDTA infusion was found.

Time courses of plasma calcium and IPTH changes following EDTA infusion in healthy controls and a hyperparathyroid adenoma patient before and after surgical removal of adenoma are summarized in Fig. 4. A sharp decrease in plasma calcium could be seen at the earliest sampling time after the start of EDTA infusion. Calcium concentration fell to its nadir at the end of the infusion and increased gradually thereafter. This fall was followed closely in time by a rise in plasma IPTH concentration in both healthy and diseased subjects. Several parameters were defined and examined: the basal IPTH value, the peak (post-EDTA) IPTH value, the absolute rise in IPTH (basal to peak), and the ratio of peak-to-basal IPTH values (EDTA-Index). Table 1 shows the mean values for each of these parameters for 13 healthy volunteers and 20 patients with surgically proven primary hyperparathyroidism together with the accompanying calcium values. In the control group, the mean fall in plasma calcium was 2.7 mg/100 ml. The mean basal and mean peak IPTH values were 66 and 87 μl Eq/ml, respectively; the latter value was found at the end of the infusion coincident with the lowest calcium value. These pre- and post-EDTA mean values were significantly different from each other (paired Student's t-tests) with $p < 0.001$ (*16*). The mean of the individual increases was 42% of the individual basal value, a

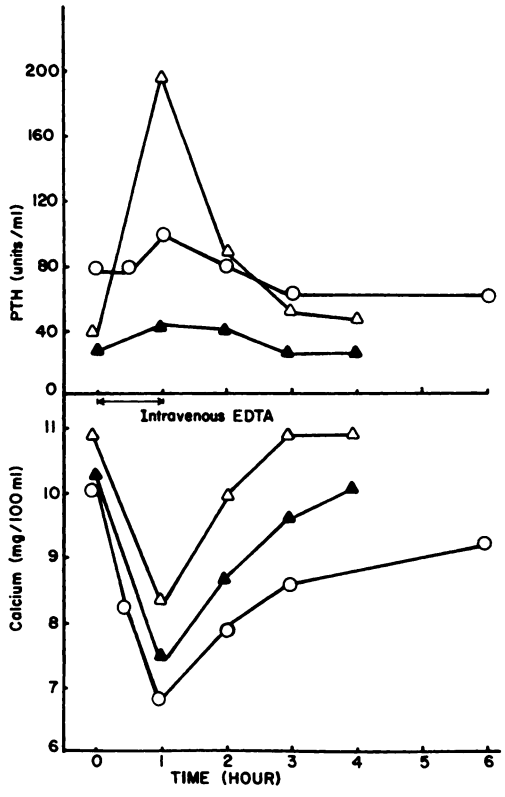

FIG. 4. Time course of plasma calcium and IPTH concentrations in response to infusion of EDTA. Mean values of eight control subjects (○); values for one specific patient with primary hyperparathyroidism: before (△) and after (△) surgery.

mean EDTA index of 1.42. In the adenoma group, the mean fall in plasma calcium before surgery was also 2.7 mg/100 ml with the peak IPTH level appearing, as above, at the end of infusion. The mean basal and mean peak IPTH values were 257 and 613 μl Eq/ml, respectively. These pre- and post-EDTA mean values were significantly different from each other (paired Student's t-test) with $p < 0.001$. The mean of the individual pre- and post-EDTA IPTH difference was 356 μl Eq/ml, and the mean EDTA-index was 2.59 as compared with 21 and 1.42, respectively, for the control group. The IPTH response to EDTA infusion as determined by mean values for the peak post-EDTA IPTH, the absolute rise in IPTH, and the EDTA index was significantly greater in the patient group than in the control group with $p < 0.002$ (standard Student's t-tests). These studies suggest that parathyroid tissue in hyperparathyroid patients is hyperresponsive to EDTA infusion stimulation.

Of 20 hyperparathyroid patients studied, 10 could be diagnosed as hyperparathyroid from the elevated basal plasma IPTH concentration alone. In the other ten patients, the basal IPTH concentration was within the normal range. In eight of these latter ten patients, the absolute rise (basal to peak) in IPTH due to EDTA was higher than the mean for normal individuals by two standard deviations. Six of these eight patients also demonstrated an elevated peak post-EDTA IPTH value and EDTA-Index. Only one patient looked normal by all parameters.

EDTA infusion was also carried out postoperatively in nine patients. The results are summarized in Table 2. After neck operation, the values for all parameters fell except for the EDTA-index in one patient. The postoperative mean value for each parameter was significantly different from the preoperative mean value (paired Student's t-test). In the patient shown in Fig. 4 with initial basal IPTH values in the normal range, there was no significant fall in this parameter after surgery. However, IPTH response to EDTA (in terms of the peak IPTH value, the absolute rise in IPTH, and the EDTA-Index) showed a distinct decrease. These results of postoperative studies strongly suggest that the resected abnormal rather than the remaining normal parathyroid tissue was the tissue hyperresponding to EDTA-induced hypocalcemia.

The assumption in all EDTA testing has been that EDTA-induced hypocalcemia is the factor stimulating the PTH response (measured by calcium and IPTH change). We evaluated this assumption. One subject was infused with EDTA preloaded with calcium; his serum content fell from 9.9 mg to 9.2 mg/100 ml plasma during the test as his plasma IPTH rose 11%. Another subject was merely placed at rest under the usual test conditions and exposed to venopuncture at the usual times; his calcium value fell from 9.0 to 8.2 mg/100 ml of plasma whereas his IPTH remained essentially unchanged. These results indicate that factors other than EDTA may be operative during our studies and probably caused a fraction of the fall in calcium concentration noted; however, IPTH concentration should not have been altered significantly by that calcium change alone.

TABLE 1. IPTH RESPONSE TO EDTA

Parameters	Normal volunteers	Primary hyperparathyroid	p‡
Basal IPTH (μl Eq/ml)	66 ± 13* (20–130)†	257 ± 60* (37–930)	<0.002
Peak IPTH (μl Eq/ml)	87 ± 14 (25–175)	613 ± 125 (143–1900)	<0.002
Peak-basal IPHT (μl Eq/ml)	21 ± 5 (5–40)	356 ± 79 (26–1270)	<0.001
Peak/basal IPTH	1.42 ± 0.08 (1.06–1.81)	2.59 ± 0.25 (1.34–6.64)	<0.001
Basal Ca (mg/100 ml)	9.8 ± 0.1	11.9 ± 0.4	—
Nadir Ca (mg/100 ml)	7.1 ± 0.2	9.2 ± 0.3	—

* Mean ±1 s.e. of the mean from 13 volunteers and 20 patients with primary hyperparathyroidism.
† Numbers in parentheses indicate the range.
‡ Significant level (Student's t-test).

TABLE 2. PREOPERATIVE AND POSTOPERATIVE IPTH RESPONSE TO EDTA

Parameters	Pre-operative	Post-operative	p†
Basal IPTH (μl Eq/ml)	301 ± 95*	81 ± 19*	<0.05
Peak IPTH (μl Eq/ml)	803 ± 230	114 ± 24	<0.02
Peak-basal IPTH (μl Eq/ml)	502 ± 113	33 ± 11	<0.02
Peak/basal IPTH	2.90 ± 0.38	1.43 ± 0.12	<0.001
Basal Ca (mg/100 ml)	11.9 ± 0.4	10.0 ± 0.5	—
Nadir Ca (mg/100 ml)	8.9 ± 0.5	7.7 ± 0.1	—

* Mean ±1 s.e. of the mean from nine patients with primary hyperparathyroidism.
† Significant level (paired Student's t-test).

DISCUSSION

To date only two groups of investigators have reported measurable IPTH values in all normal

serum samples analyzed (*3,5*). Presumably these investigators used an antibody recognizing the carboxy terminal group of the PTH molecule and probably recognizing carboxy terminal fragments with a half-life longer than the intact PTH molecule (*17*). Despite the limitation of the very rapid plasma clearance of the antigen which we recognize with our antibody, 89% of the normal subjects, whose sera we analyzed had detectable PTH levels. This sensitivity was achieved only after having adopted certain modifications in the original methodology of the assay. The results shown in Fig. 2 indicate continued linearity with as much as 800 μl of standard plasma.

The results shown in Fig. 3 indicate that IPTH measured by our guinea pig antiserum is immunologically similar to (A) that species of PTH measured by Arnaud's chicken-14 antiserum which he has shown to recognize the 9500-molecular weight component (*18*) as well as to (B) human thyroid-venous-plasma IPTH which represents only intact polypeptide (*15*). These results suggest that our antiserum measures predominately an intact PTH molecule and presumably recognizes the N-terminal portion of a PTH molecule as reported recently by Arnaud (*18*). We have no data to evaluate the possibility that our antiserum also recognized smaller fragments such as the 4000-MW biologically active PTH fragment in hyperparathyroid serum recently reported by Canterbury, et al (*19*). This propensity to measure the 9500-MW component of peripheral plasma IPTH would seem to be particularly useful for the EDTA infusion challenge used in the present investigation because sudden hypocalcemic stimulation of parathyroid tissue would result in at least a transiently increased peripheral concentration of the intact hormone due probably to acute secretory change in parathyroid gland (*18*). We recognize that the ideal hyper-PTH standard for this assay using our antiserum should be plasma from a patient with primary hyperparathyroidism (which would minimize the heterogeneity of the potential antigens recognized by the antiserum) and, ideally, plasma obtained after acute stimulation (since our antiserum identifies intact molecule best). Unfortunately, this is not feasible; the only hyper-PTH blood available to us in quantities sufficient to satisfy our needs is pooled specimens drawn from patients with chronic renal insufficiency at the time of hemodialysis.

The EDTA study results obtained preoperatively suggest that patients with primary hyperparathyroidism not only respond to a hypocalcemic insult, they hyperrespond when compared with healthy controls. These findings are similar to those recorded in patients with adrenal hyperplasia (*9*). The postoperative EDTA results suggest that indeed the abnormal (operatively removed) tissue was the tissue hyperresponding.

The use of an EDTA infusion to induce hypocalcemia and thereby stimulate PTH secretion has been extensively studied in man and other animals. Care, et al demonstrated hyperresponsiveness of IPTH output in hypocalcemia-adapted cows compared with normal cows (*20*). Their conclusion was that the parathyroids in the adapted cows, being chronically hyperfunctional, had become hyperresponsive. Potts, et al, in another study, showed a significant but not necessarily abnormal response in the IPTH concentration (following EDTA infusion) of a group of patients with primary hyperparathyroidism (*21*); they did not comment on what parathyroid tissue (abnormal or normal) was responding. These reports together with our EDTA-IPTH results only demonstrate presence of the stimulation (hypocalcemia-related) arm of the feedback mechanisms in adenoma patients and are more consistent with the conclusion that hyperfunctioning parathyroid tissue is, at the very most, incompletely autonomous and possibly not autonomous at all. Some authors report that calcium infusion inhibits PTH secretion in adenomatous hyperparathyroidism (*21*) whereas others find no inhibition (*22*); still others report calcium infusion suppression of PTH only in *some* hyperparathyroid patients, especially those with glandular hyperplasia (*23*).

The potential of dynamic testing of parathyroid function for differential diagnostic use is obviously worthy of further evaluation.

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