Heterogeneity of Parathyroid Hormone

CLINICAL AND PHYSIOLOGIC IMPLICATIONS

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ABSTRACT When immunoreactive human parathyroid hormone (hPTH), extracted by three different solvents (20% acetone in 1% acetic acid, 8 M urea, or normal saline) from parathyroid glandular tissue was subjected to Sephadex G-100 gel filtration and immunoassay using two different antisera (273 and C-329), four distinct fractions were observed. The first (I), a void volume peak, was detected by both antisera with similar immunoreactivity, as was a second (II), which had the elution and sedimentation properties of highly purified bovine parathyroid hormone (bPTH); a third (III) eluted between [^{125}I]growth hormone and [^{125}I]insulin, sedimented with the velocity of a molecule of approximately 6,000 mol wt, and was detected primarily by antiserum 273; a final fraction (IV), detected primarily by C-329, eluted just prior to [^{125}I]insulin. The elution profiles of the acetone-acetic acid and 8 M urea extracts were similar and contained fraction II as their major component. In saline extracts, however, fraction III predominated.

Three fractions, having gel filtration and immunologic characteristics similar to fractions II, III, and IV, respectively, of saline glandular extracts, were detected in the plasma of patients with both primary (adenomatous or carcinomatous) and secondary hyperparathyroidism. The predominant component in every plasma was the intermediate fraction that, like III, was detected primarily by antiserum 273, while the least abundant form was consistently the final fraction, detected primarily by antiserum C-329. The first fraction, like II, was detected with about equal potency by both antisera and had an elution volume on Sephadex corresponding to that of intact bPTH. It bore a reciprocal relationship to serum calcium and disappeared from the plasma of a uremic patient during calcium infusion or following parathyroidectomy with a half-time of no more than 20 min. This component therefore probably represents biologically active hormone. The intermediate and final fractions had turnover times in the plasma of a uremic patient more than 100 times greater than the active form, remained elevated even in the presence of post-parathyroidectomy hypoparathyroidism in this patient and were presumed to be biologically inactive. The ratio of biologically inactive fragments to the active form was greater in secondary hyperparathyroidism. The evidence presented favors a glandular origin for the fragments.

Comparison of hormonal assays with the two antisera reveals a striking advantage in the preoperative diagnosis of primary hyperparathyroidism with antiserum 273 that is due to the enhanced sensitivity occasioned by its detection of a biologically inactive as well as the biologically active hormonal form.

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INTRODUCTION

In 1968, studies from our laboratory (1) established that there were striking immunochemical differences between the parathyroid hormone (PTH) [1] found in the circulation and that present in most glandular extracts. The studies revealed that there appeared to be at least two components of immunoreactive PTH in plasma which were demonstrable by differences in their reactivity with two distinct antisera. These components disappeared at different rates following parathyroidectomy and had different relative distributions in patients with primary and secondary hyperparathyroidism.

The heterogeneity of plasma PTH has been widely confirmed but subsequent investigations have yielded conflicting results. Arnaud et al. (2, 3) concluded from in vitro work that the predominant secreted spe-

[1] *Abbreviations used in this paper:* hGH, human growth hormone; PTH, parathyroid hormone; bPTH, bovine PTH; hPTH, human PTH.

cies is between 5,000 and 7,000 mol wt, is immunochemically distinct from the 9,500 mol wt form of PTH present in parathyroid tissue, and is cleaved from this larger, glandular hormone within the parathyroid gland itself. Sherwood et al. (4) reached similar conclusions, estimating the molecular weight of the secreted form to be about 7,000. In contrast, Habener et al. (5) reported from in vivo studies that the secreted form of human PTH (hPTH) has a molecular weight of about 9,500, is identical in size and immunochemical behavior to the hormone extractable from glandular tissue, and that cleavage of the secreted species into one or more immunologically heterogeneous fragments occurs peripherally, after its release from the gland. Canterbury and Reiss (6) have demonstrated in pooled, hyperparathyroid sera at least three fractions (Peaks I, II, and III) with estimated molecular weights of 9,500, 7,000–7,500, and 4,500–5,000, respectively, and suggested that unfractionated serum was immunologically similar to peak I and different from peaks II and III.

Data on the number and nature of the components of immunoreactive PTH in glandular tissue are also discrepant. When parathyroid glandular extracts have been subjected to gel filtration some investigators (4) have reported that the eluted immunoreactive PTH emerged as a single peak, while others have observed size heterogeneity (2, 7). More recently, use of the pulse-chase technique has established the existence of a prohormonal precursor with a molecular weight of about 12,000 both for bovine (8, 9) and for human (10) PTH.

In this paper two antisera, 273 and C-329, having markedly different reactivities with the different hormonal forms of immunoreactive hPTH, are used to elucidate more definitively the nature of glandular and plasma immunoreactive hPTH and to give insight into the possible secretory rates of these forms from a consideration of their relative rates of turnover.

METHODS

Extraction of glandular hPTH. Human parathyroid glands and adenomas obtained at surgery were frozen immediately and stored at −20°C until extraction. In general, glandular tissue was divided into three portions of approximately equal weight and each portion then extracted with one of three different solvent systems (either 20% acetone in 1% acetic acid, 8 M urea, or normal saline). The tissue, weighing 75–400 mg, was minced, transferred to a Teflon tissue grinder kept in an ice bath, and extracted for 5 min in 1.5 ml of extracting solvent. The suspension was decanted into a test tube, centrifuged at 3,000 rpm and 5°C for 15 min, and the supernate removed and saved. The sediment was added to that remaining in the grinder and the extraction process repeated twice more. The supernates obtained with a given solvent were pooled and stored at −20°C until assayed.

Gel filtration of glandular extracts or plasma. Columns of Sephadex G-100, fine, 1 × 50 cm, were equilibrated immediately prior to use with eluant consisting of outdated bank plasma (previously tested for absence of immunoreactive hPTH and extent of damage to [^{125}I]bovine PTH (bPTH)) diluted 1:10 in 0.02 M barbital buffer, pH 8.5, containing 500 KIU Trasylol/ml. Markers including bromphenol blue tagged albumin, [^{131}I]albumin, [^{125}I]human growth hormone (hGH), [^{125}I]insulin, and Na^{131}I were added to samples of glandular extracts or plasma before application to columns. The peak of irradiation-damaged [^{125}I]hGH and [^{125}I]insulin, presumably bound to α_2-macroglobulin in plasma and termed "[^{125}I]macromolecule" in the figures, served as a marker for the void volume. The ^{125}I and ^{131}I activity of the column fractions was measured simultaneously in a dual-channel, gamma-well spectrometer, the total radioactivity of each indicator of molecular size being kept low enough so as not to interfere in the radioimmunoassay of the eluate.

Ultracentrifugation. Rough estimates of the molecular size of the predominant components of glandular extracts were also obtained by centrifugal analysis. Portions of Sephadex G-100 fractions of saline human parathyroid extracts or of freshly iodinated and purified [^{125}I]bPTH were added to bank plasma diluted with equal volumes of either normal saline or 0.02 M barbital buffer, pH 8.5, each containing 500 KIU Trasylol/ml. Similar dilutions of bank plasma were prepared containing [^{131}I]albumin, [^{125}I]hGH, or [^{125}I]insulin. Samples containing 0.5 ml were subjected to ultracentrifugation at 36,000 rpm for 16 h in a Spinco Model L fitted with a swinging bucket rotor (SW 50.1). After the centrifuge had come to rest without braking, four successive 100 μl aliquots were removed from each tube, working from the top down, for counting or radioimmunoassay of PTH, hGH, insulin, and albumin. The principles and methods involved have been described previously for insulin (11) and ACTH (12).

Radioimmunoassay. Highly purified bPTH prepared by CMC chromatography, generously supplied by Doctors G. T. Aurbach and J. T. Potts, Jr. in 1965, was iodinated with ^{125}I to specific activities of 100–150 μCi/μg using a modification of the method of Hunter and Greenwood (13), then purified on QUSO G-32 (14). Suitable dilutions of the same purified preparation of bPTH used for iodination or of an acetone-acetic acid extract of a human parathyroid adenoma were employed as standards in the radioimmune assay of bPTH or hPTH, respectively. Samples to be assayed were incubated in a final volume of 2.5 ml containing about 5,000 cpm of [^{125}I]bPTH and an appropriate dilution of guinea pig anti-bPTH antiserum. The standard diluent generally consisted of outdated bank plasma (previously tested for absence of immunoreactive PTH and extent of damage to tracer) diluted 1:10 in 0.02 M barbital buffer, pH 8.5, containing 1% nonimmune guinea pig serum and 500 KIU Trasylol/ml. Antisera 273 and C-329 at dilutions of 1:200,000 and 1:400,000, respectively, yielded B/F ratios of 0.9 to 1.0 in the absence of unlabeled hormone after incubations of 4–5 days at 5°C. Separation of antibody-bound from free ^{125}I-bPTH was effected by the addition of 50 mg talc (15).

RESULTS

Extracts of human and bovine glandular PTH

Sephadex gel filtration. The Sephadex G-100 gel filtration patterns of labeled and unlabeled purified bPTH are shown in Fig. 1. The peaks of immuno-

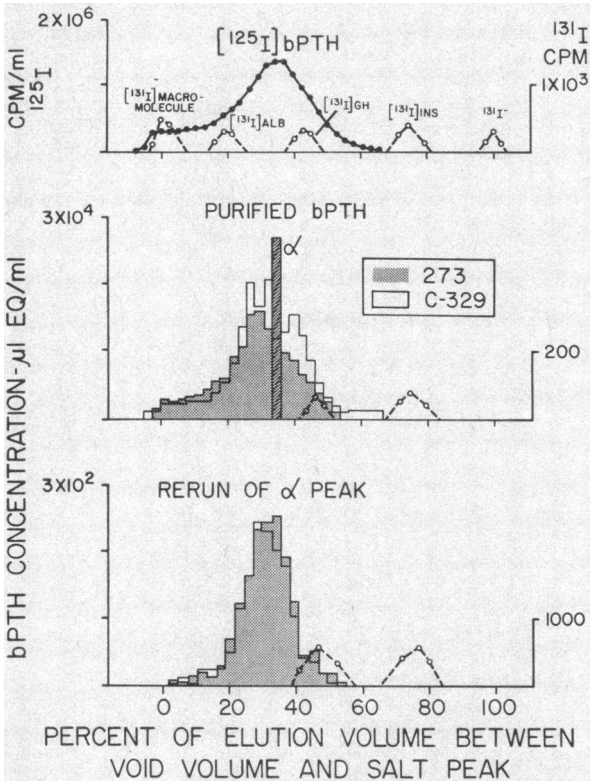

FIGURE 1 Sephadex G-100 gel fractionation of highly purified ^{125}I-labeled (top) and unlabeled (middle) bPTH and refractionation of peak α of immunoreactive bPTH (bottom). In this and subsequent fractionations of immunoreactive PTH, [^{131}I]albumin, [^{125}I]GH, [^{125}I]insulin, and Na^{131}I were added as tracers before the sample was applied to the column. In the fractionation of [^{125}I]bPTH, [^{131}I]GH, and [^{131}I]insulin were substituted for their ^{125}I-labeled counterparts. Concentration of immunoreactive hormone in all figures depicting gel filtration is given as microliter equivalents per milliliter of eluate on the ordinate to the left, and counting rates of tracers as counts per minute per milliliter of eluate on the ordinate to the right of the figures.

reactivity, as measured in antisera 273 and C-329, and of radioactivity coincided, corresponding to an elution volume somewhat less than that of labeled hGH. In three separate experiments, the volume of elution of labeled and unlabeled bPTH averaged 35%, and that of labeled hGH, 45%, of the total volume eluted between the void volume and the salt peak. A portion of the peak of unlabeled bPTH, as measured with both antisera, maintained its integrity on refractionation.

A single human parathyroid adenoma was extracted with 20% acetone in 1% acetic acid, 8 M urea, or physiologic saline, and aliquots of the three extracts, each containing 73 mg (wet weight) of adenoma, were fractionated by Sephadex G-100 gel filtration and assayed with both antisera (Fig. 2). The elution patterns of the acetone-acetic acid and 8 M urea extracts were similar and contained a dominant fraction that, like bPTH, eluted prior to [^{125}I]hGH. Equal volumes of this fraction produced the same percentage decrease in the B/F ratio in both antisera.

The elution pattern of the saline extract was strikingly different. Four components of immunoreactive hPTH were present (Fig. 2), and were denoted as follows: fraction I, as all immunoreactive hPTH eluted in the region of the void volume; fraction II, as all hPTH eluted between the [^{131}I]albumin and [^{125}I]-hGH tracers; fraction III, as all immunoreactivity detected between the apex of [^{125}I]hGH and the ascending limb of [^{125}I]insulin; and fraction IV as that immuno-

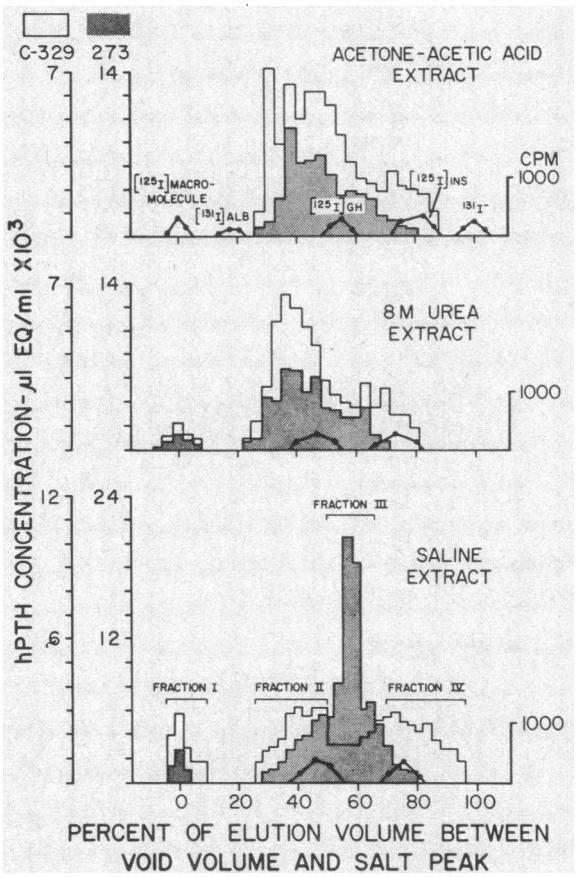

FIGURE 2 Sephadex G-100 gel fractionation of acetone-acetic acid (top), 8 M urea (middle), and saline (bottom) extracts of equal portions of a single human parathyroid adenoma. Note that in each fractionation the ordinate scale is twice as great for antiserum 273 as for antiserum C-329, so that a column fraction containing equal concentrations of hPTH in both antisera is represented by an hormonal peak in antiserum C-329 that is twice the vertical height of the same peak in antiserum 273.

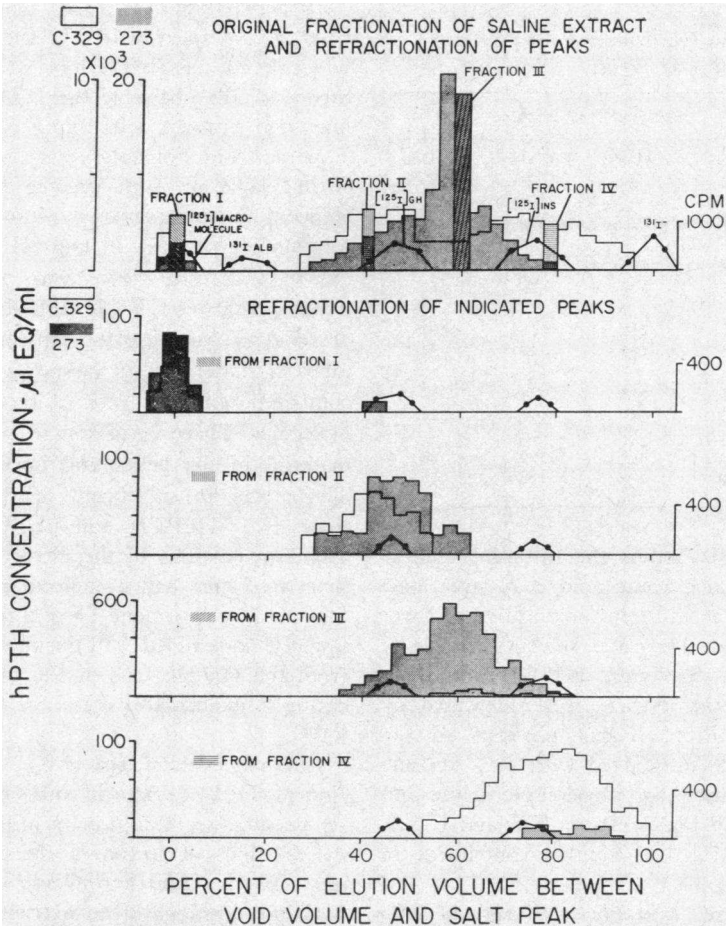

FIGURE 3 Replot (top) of the Sephadex G-100 gel fractionation of the saline extract of a human parathyroid adenoma shown in Fig. 2, and refractionation of aliquots of fractions I to IV. Note that in original fractionation the ordinate scale of antiserum *273* is twice that of antiserum C-*329*, but that in refractionations the two are the same.

reactive hPTH eluted between the leading edge of the [^{125}I]insulin tracer and the apex of the $^{131}I^-$ peak. Fraction I was not detected in the acetone-acetic acid extract and represented only a minor fraction in the 8 M urea and saline extracts. Although fraction II was also a peak of only secondary magnitude in the saline extract, it corresponded to the major fraction extracted by acetone-acetic acid and 8 M urea. Equal volumes of fraction I or fraction II obtained from the saline extract produced the same percentage fall in the B/F ratio in both antisera. The peak of fraction II was sharper when assayed with the C-*329* than with the *273* antiserum because of the merging of saline fractions II and III in the latter; fraction III, on the other hand, dominated the elution profile of the saline extracts when assayed with *273* but was less prominent when assayed with antiserum C-*329*. Fraction IV, detected in all extracts primarily with antiserum C-*329*, was present in highest concentration in the saline extract.

The relative concentrations of the various components depended both on the antiserum used for assay and on the method of extraction. For example, extraction with saline compared to the other solvents yielded five to seven times more of fraction III when assayed in *273*, while no significant increase in this fraction was detected by C-*329*. However, about twice the amount of both fractions I and IV, and one-half to two-thirds as much of fraction II per milligram of tissue were obtained from saline extracts regardless of which antiserum was used. Aliquots of each of the four fractions maintained their integrity when refractionated on Sephadex G-100 (Fig. 3), and similar elution profiles were observed in the saline extracts of several other specimens of both adenomatous and hyperplastic parathyroid glandular tissue (Table I).

TABLE I

Concentration of hPTH in Fractionated Saline Extracts of Parathyroid Tissue

	Hormone concentration			
	Fraction I	Fraction II	Fraction III	Fraction IV
	hPTH μl eq/mg tissue			
Extract A (adenoma)				
Antiserum 273	79	412	717	247
Antiserum 329	102	307	90	211
Extract B (adenoma)				
Antiserum 273	57	278	621	246
Antiserum 329	38	126	79	57
Extract C (hyperplasia)				
Antiserum 273	62	378	971	352
Antiserum 329	154	781	400	243
Extract D (adenoma)				
Antiserum 273	136	402	729	108
Antiserum 329	56	285	198	74

In order to determine whether the different elution patterns observed in acetone-acetic acid, 8 M urea, and saline extracts were due to different solubilities of their major components in the extraction solvents, fractions II and III, obtained by Sephadex gel filtration of a saline parathyroid adenoma extract, were identified by immunoassay and lyophilized. Equal portions of the lyophilized fractions were solubilized with 20% acetone in 1% acetic acid, 8 M urea, or physiologic saline and the supernates assayed in both antisera 273 and C-329. Both fractions II and III had similar solubilities in all three solvents.

The biologically active, synthetic, N-terminal 1–34 fragment of bPTH (MW 4109) eluted with [^{125}I]insulin on Sephadex G-100 gel filtration (Fig. 4) and was measurable only with C-329.

Ultracentrifugation. The elution of the presumed 9,500 mol wt form of bPTH and hPTH from Sephadex before hGH (~20,000 mol wt) is probably a consequence of the shape of the PTH molecule itself, since elongated proteins with large Stokes' radii are known to elute from Sephadex gels before globular proteins of the same or even greater molecular weight (16). Hence, an independent estimate of the molecular weights of fractions II and III from saline extracts of human glandular tissue was obtained from ultracentrifugal analysis (Table II). In this method the rate of removal from the top portion of the ultracentrifuge tube of materials of unknown molecular weight are compared with the rate of removal of known marker molecules. There is, of course, pile-up of the sedimented material in the pellet and lower portions of the tube. There was no significant difference in the concentrations of [^{125}I]bPTH and hPTH fraction II in corresponding portions of the ultracentrifuge tube indicating that the latter had a molecular weight about equal to that of intact hormone (~9,500) (17, 18). hPTH fraction III resembled [^{125}I]insulin in its rate of removal from the top portions of the ultracentrifuge tube, indicating a molecular weight for this component of about 6,000.

Immunochemical reactivity. The percentage inhibition of the B/F ratio in antiserum 273 as a function of its percentage inhibition in antiserum C-329 for identical dilutions of acetone-acetic acid, 8 M urea or saline extracts of a parathyroid adenoma is shown in Fig. 5. Multiple dilutions of an extract with identical immunoreactivity in both antisera would describe a 45° line passing through the origin. While this appeared to be the case for the acetone-acetic acid and 8 M urea ex-

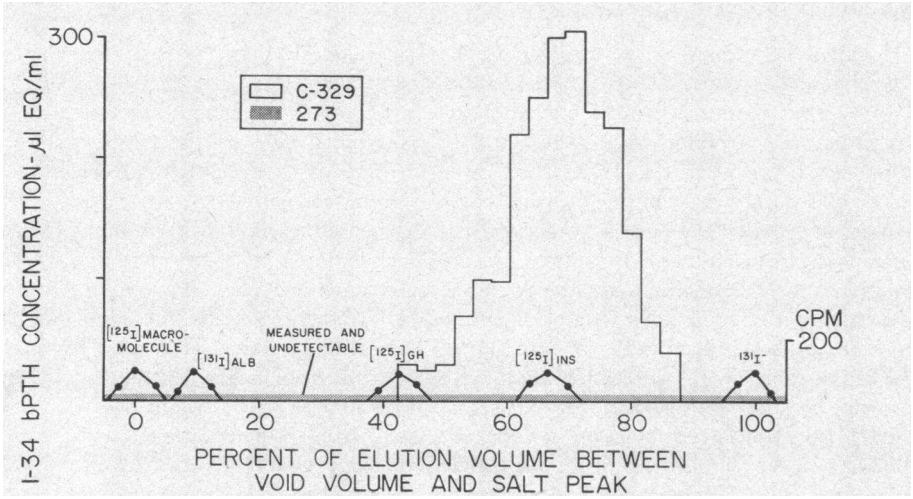

FIGURE 4 Sephadex G-100 gel fractionation of the biologically active, synthetic, N-terminal 1–34 fragment of bPTH.

TABLE II

*Concentration of Radioactivity or Immunoreactive PTH at Various Levels in Tube after Ultracentrifugation Expressed as Percent of Concentration in Mixture before Centrifugation**

	[^{131}I]-albumin‡	[^{125}I]-hGH§	[^{125}I]-insulin	[^{125}I]-bPTH¶	Fraction II**	Fraction III**
Top 0.1 ml	2.2±0.2	18±1	42	29±9	29±2	44±9
2nd 0.1 ml	2.7±0.3	34±1	68	55±1	58±2	62±4
3rd 0.1 ml	27±1	78±2	93	69±8	71±12	81±2

* The complement of the radioactivity or immunoreactivity originally added to each 0.5 ml sample is in the fourth 0.1 ml volume and the protein pellet at the bottom of each tube.
‡ Mean ±SEM 5 samples.
§ Mean ±SEM 2 samples.
‖ 1 sample.
¶ Mean ±SEM 3 samples.
** Mean ±SEM 2 independent determinations using antisera 273 and C-329.

tracts, the saline extract reacted much more strongly with antiserum 273 than with C-329.

To ascertain which of the components of the saline extract was responsible for its greater reactivity with antiserum 273, the percentage inhibition of the B/F ratio in both antisera caused by the unfractionated extract was compared with that produced by various volumes of fractions I through IV (Fig. 6). The effectiveness of both fractions I and II in inhibiting the binding of [^{125}I]bPTH to antibody was nearly equal in both antisera. Fraction III reacted more strongly with antiserum 273 than with C-329 and its curve nearly coincided with that of the unfractionated extract. Conversely, fraction IV reacted more strongly with antiserum C-329.

Superimposition of two fractions in a plot such as that shown in Fig. 6 does not necessarily imply that those fractions are immunologically identical. Rather, one must demonstrate parallelism of the "standard curves" relating the percentage inhibition of the B/F ratio to the concentration of the fractions. When the data from Fig. 6 were replotted in this way, fractions I and II yielded standard curves which were superimposable in both antisera (Fig. 7). Fraction III was clearly more immunoreactive than fraction IV in antiserum 273, while the reverse was true in antiserum C-329, and the standard curves for these fractions could not be superimposed on the curves for I and II.

The failure of 273 to detect the synthetic 1–34 N-terminal fragment of bPTH on Sephadex gel filtration (Fig. 4) was accounted for by the absence of measurable cross-reactivity in that antiserum, even with hormone concentrations as high as 100 ng/ml. However, concentrations as low as 20 pg/ml were measurable with C-329. In fact, with the latter antiserum the 1–34 fragment was nearly as potent on a molar basis as the

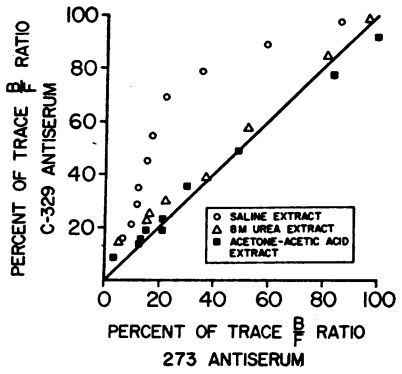

FIGURE 5 Inhibition of binding of [^{125}I]bPTH by unfractionated acetone-acetic acid, 8 M urea, and saline extracts of equal portions of a human parathyroid adenoma. Multiple dilutions of the extracts were assayed independently with antisera 273 and C-329. Trace B/F ratios (ratio in absence of unlabeled hormone) were approximately 0.8–0.9 for both antisera.

intact hormone, although the curves diverged at high hormone concentrations.

Plasma PTH

Sephadex gel filtration of human plasma. The elution patterns of plasma immunoreactive hPTH from Sephadex G-100 in patients with primary and secondary hyperparathyroidism are shown in Fig. 8. Three fractions, A, B, and C, having gel filtration characteristics corresponding to fractions II, III and IV, respectively, of saline glandular extracts, were detected using antisera 273 and C-329 in each case. In these plasmas no immunoreactivity was noted in the region which corresponded to glandular fraction I. Fraction A was measured by both antisera with approximately equal potency. Fraction B, detected with high sensitivity by 273, was quan-

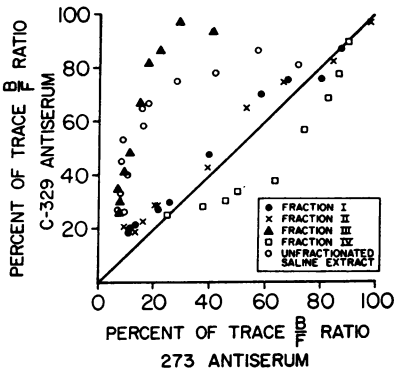

FIGURE 6 Inhibition of binding of [^{125}I]bPTH by an unfractionated saline extract of a human parathyroid adenoma and by fractions I through IV obtained from Sephadex G-100 gel filtration of the same extract. See legend to Fig. 5.

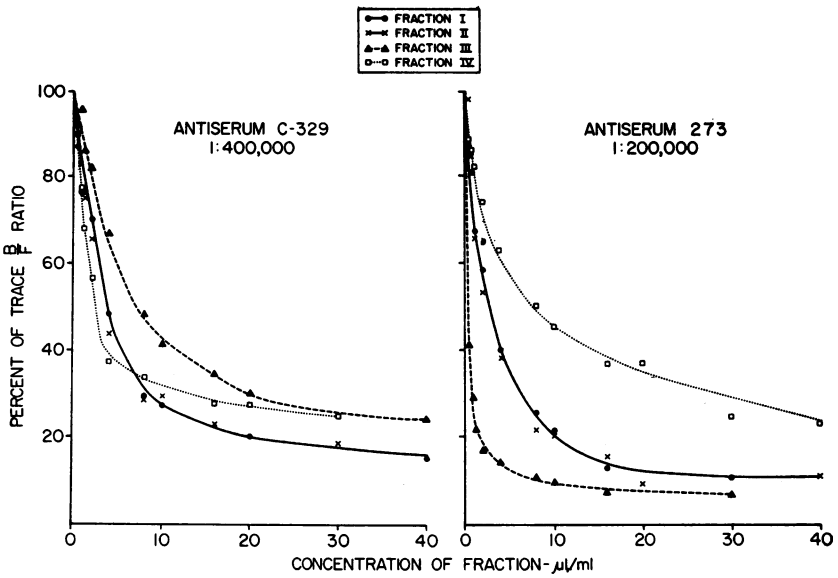

FIGURE 7 Inhibition of binding of [^{125}I]bPTH in antiserum C-329 (left) and antiserum 273 (right) by fractions I to IV obtained by Sephadex G-100 gel filtration of the saline extract shown in Fig. 5. Concentration of fractions are expressed as microliter fraction per milliliter incubation mixture.

titatively the predominant fraction of hPTH in each plasma sample. The immunoreactivity in the region of fraction C, as seen by this antiserum, probably represents spillover from the high concentration of the fraction B region. The relative concentration of B compared to the other fractions was greatest in uremic secondary hyperparathyroidism. Fraction C, as measured by C329, appeared as a distinct peak with almost 1/2 the immunochemical potency of A in secondary hyperparathyroidism. In primary hyperparathyroidism, fraction C is, at most, a very minor component.

The effect of an acute alteration of serum calcium on the various fractions of immunoreactive hPTH in the plasma of a patient with uremic secondary hyperparathyroidism is shown in Fig. 9. After 1 h of an infusion of calcium gluconate in 5% dextrose in water, when the serum calcium had risen from 9.9 mg/100 ml to 11.6 mg/100 ml, fraction A, which was measured primarily by C-329, fell to virtually undetectable levels. Since immunoreactivity corresponding to 10% of the peak value of A in the preinfusion specimen would have been readily detectable, the half-time for the disappearance of this fraction can be no longer than 20 min and may be considerably less. The concentration of fractions B and C remained practically unchanged, which could be due either to the failure of hypercalcemia to suppress their secretion or to turnover times for these fractions that were long compared to an hour. 1 h after the infusion was stopped, when the serum calcium had fallen from its maximum of 12.2 mg/100 ml to 10.2 mg/100 ml, fraction A reappeared, but no significant change was detected in B or C. 21 h after the infusion, when the serum calcium had fallen below its baseline value to 9.3 mg/100 ml, the concentrations of fractions A and B had risen above their preinfusion levels, while that of fraction C remained unchanged.

The elution patterns of plasma hPTH in a uremic patient (G1) were determined 6 wk prior to (Fig. 8, center), and 9 days after (Fig. 10) total parathyroidectomy. Postoperatively the patient was clinically hypoparathyroid, as manifested by the presence of positive Chvostek and Trousseau signs and by the large amount of calcium necessary to maintain his serum calcium at even 6.5 mg/100 ml (hemodialysis three times a week against a calcium concentration of 10.0 mg/100 ml and daily doses of 3 g of elemental calcium and 0.6 mg of dihydrotachysterol). From a comparison of the patterns shown in Fig. 8 and 10, it can be seen that at 9 days post-parathyroidectomy Fraction A had completely disappeared, fraction B, as seen primarily by antiserum 273, had decreased to about 3% of its preoperative level, and fraction C, as measured by C-329, appeared virtually undiminished.

In vivo turnover of 1–34 bPTH. Plasma fortified with synthetic, N-terminal 1–34 bPTH was administered intravenously to a dog and serial samples of peripheral blood were drawn from the opposite leg vein at intervals thereafter. Immunoassay of the plasma with antiserum C-329 revealed that the 134 bPTH fragment disappeared from the circulation with a half-time of

about 5 min (Table III). The apparent space of distribution at the first sampling (0.6 min) corresponded approximately to the plasma volume.

Unfractionated plasma hPTH. Samples of whole plasma from patients with suspected or surgically proven primary hyperparathyroidism were assayed for their hPTH content with both antisera 273 and C-329. Of 13 patients with surgically proven disease, 11 had values exceeding 97% of the controls when assayed with antiserum 273, but only 3 of the 13 patients had levels that exceeded 100% of the controls when assayed with antiserum C-329. Of 20 patients with suspected primary hyperparathyroidism, 19 had values which exceeded 97% of the controls when antiserum 273 was employed, while only 4 of these patients had elevated levels detected by antiserum C-329. In every case in which an elevated level of hPTH was measured by antiserum C-329, an elevated level of hPTH was also found with antiserum 273 (Fig. 11).

DISCUSSION

Earlier studies (1, 19) from our laboratory on the immunochemical heterogeneity of parathyroid hormone were consistent with the hypothesis that there were at least two forms of plasma hormone. It was postulated that one of these was turned over rapidly and reacted about

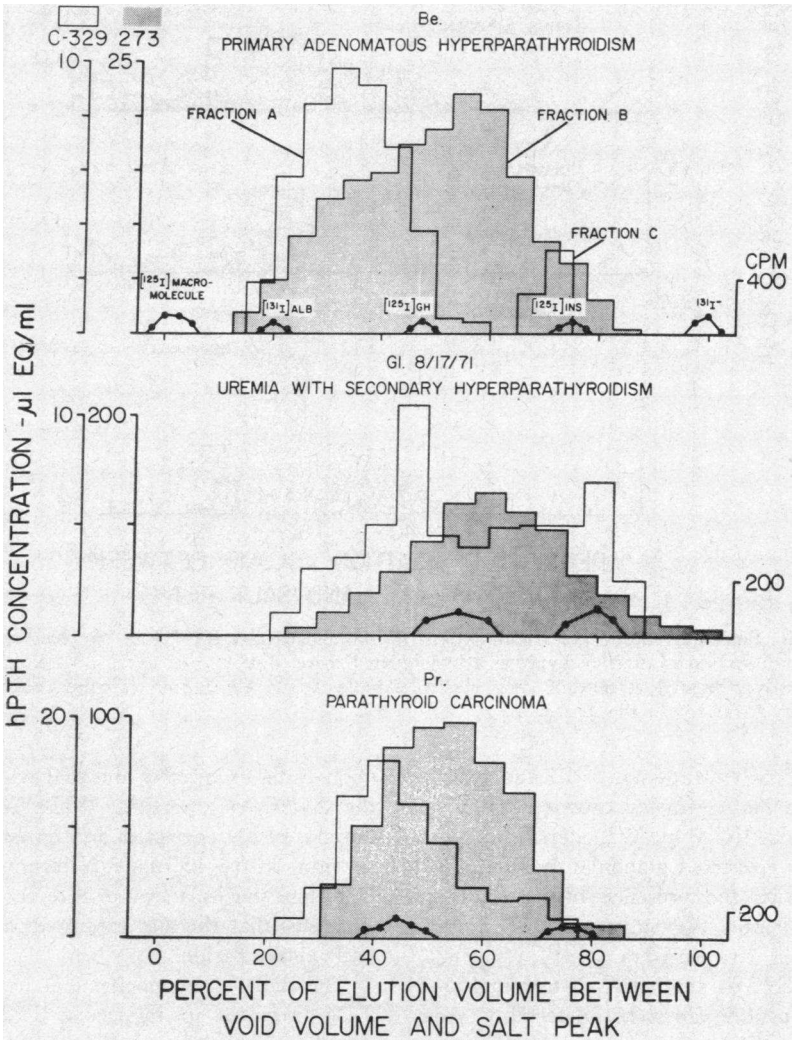

FIGURE 8 Sephadex G-100 gel fractionation of immunoreactive hPTH in plasma of patients with primary adenomatous, secondary uremic, and carcinomatous hyperparathyroidism. Volume of plasma applied to columns was 1 ml in primary and carcinomatous, and 3 ml in secondary hyperparathyroidism. Note variation in magnitude of ordinate scales of antisera 273 and C-329 in these fractionations.

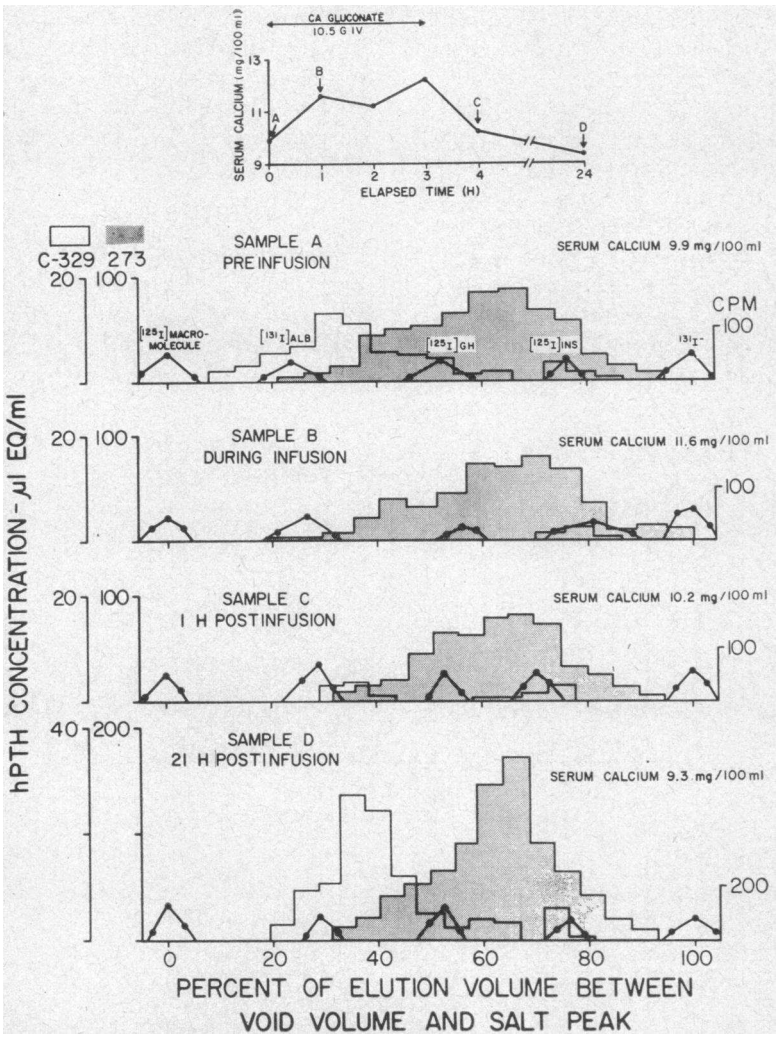

FIGURE 9 Sephadex G-100 gel fractionation of immunoreactive hPTH in plasma of a patient (De.) with secondary uremic hyperparathyroidism before, during, and after a calcium infusion test. The fractionated volume of each plasma sample was 3 ml. Serum calcium concentrations are shown at the top of the figure.

equally well with each of two antisera (273 and C-329), while the other form had a slower turnover rate, was detected with high sensitivity by 273, and differed immunochemically from extracted glandular hormone. The present report confirms the presence of these plasma fractions and characterizes the nature of the antigenic sites reacting with the two antisera.

The biologically active, synthetic, N-terminal 1–34 bovine parathyroid peptide does not detectably cross-react with antiserum 273 at concentrations 5,000 times as high as that which produces a significant inhibition of the binding of labeled bPTH to C-329. It would appear, therefore, that the antigenic site that reacts with 273 is contained within the C-terminal 35–84 portion of the intact molecule, and that which reacts with C-329, the N-terminal portion. While it is possible that the antigenic site detected by antiserum 273 includes a portion of the molecule N-terminal to residue 34, the failure of the 1–34 fragment to react with that antiserum indicates that the site cannot include the entire biologically active region.

The molar immunochemical potency of the synthetic 1–34 fragment in antiserum C-329 is almost the same as that of the intact hormone at low concentrations, while at high concentrations it is significantly weaker. This observation could be explained by extension of the N-terminal antigenic site reacting with C-329 beyond the 1–34 region in the intact hormone, alteration of the

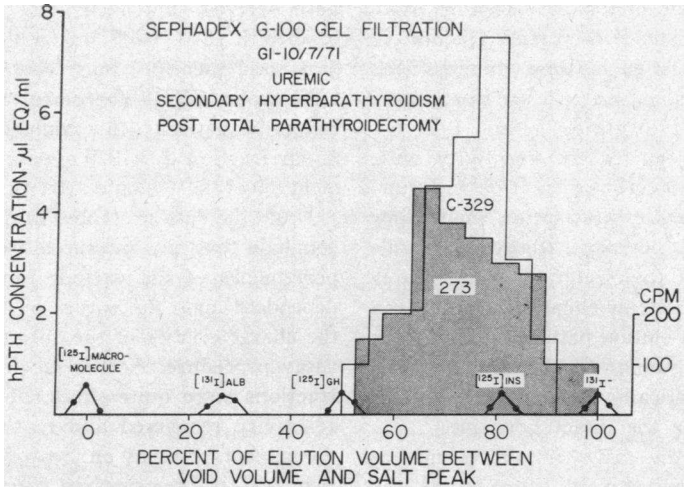

FIGURE 10 Sephadex G-100 gel fractionation of immunoreactive hPTH in plasma taken from a hypoparathyroid patient (Gl.) 9 days after total parathyroidectomy for uremic secondary hyperparathyroidism. Volume of plasma fractionated was 3 ml.

configuration of the site in the fragment compared to its shape in the intact hormone, or the existence of more than one reactive site in the region.

The question has been raised as to whether the lack of identity of human and bovine PTH might alter conclusions concerning the location of the antigenic site as seen by an individual antiserum. When an antiserum is generated in response to immunization with bPTH and [^{125}I]bPTH is employed in the assay, the determination as to whether the antibody-combining site(s) in that antiserum is directed toward the N-terminal or C-terminal segments of the intact hormone or some combination of these is best made with bovine peptide fragments. Weaker cross-reactivity might be found with human peptide fragments because of differences from the bovine hormone but this would not affect the evaluation of the *region* of the reactive site.

The presence of fractions B and C in the plasma of patient Gl, who was clinically hypoparathyroid 9 days after total parathyroidectomy, militates against either fraction having significant biological potency. Further-

TABLE III

Disappearance of N-terminal 1-34 bPTH from Dog Plasma after Intravenous Administration

Elapsed time	$\frac{\text{Fraction of dose remaining}}{\text{Liter plasma}} \times \text{Wt (kg)}$
min	
0.6	31.8
2.0	25.2
3.0	21.5
4.0	17.8
5.0	14.9
6.0	12.0
7.0	10.3
8.0	9.1
9.0	8.5
10.0	7.6
15.0	5.2
20.0	3.3

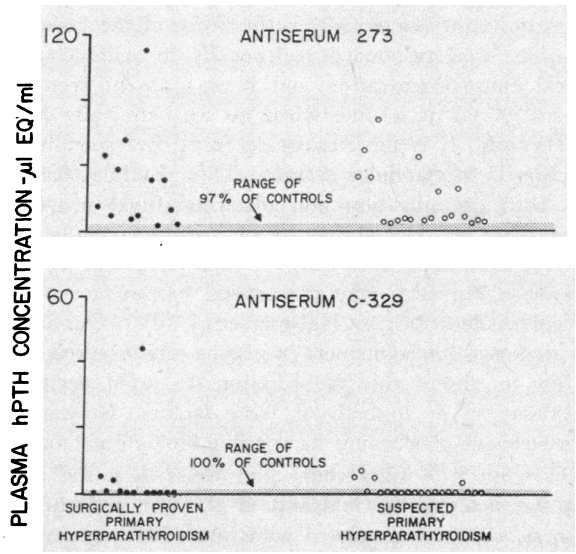

FIGURE 11 Immunoreactive hPTH concentration in plasma of patients with surgically proven or suspected primary hyperparathyroidism as measured by antisera 273 and C-329. Stippled areas show range of values for 97% and 100% of control subjects in antisera 273 and C-329, respectively. Each patient is represented by a pair of points, one in the upper and one in the lower frame, equidistant from the vertical axis.

more, the rapid disappearance of the 1–34 peptide from the circulation and the persistence of plasma fraction C indicate that, despite similarities in their immunologic and gel filtration characteristics, the two are not identical, and that the complete, biologically active, 1–34 N-terminal fragment is not required for reactivity with C-329. Since the molecular weight of fraction C is unknown, we have no quantitative information concerning its molar immunochemical potency. Nevertheless, although the concentration of this fragment in plasma is too low to permit direct immunochemical comparison with the intact hormone, its elution pattern on Sephadex gel filtration suggests it is similar to glandular fraction IV, a peptide immunochemically distinguishable from fraction II, presumed to be the intact hormone.

Plasma fraction B has the same elution volume as glandular fraction III on Sephadex gel filtration, as well as similar immunochemical properties; namely, strong reactivity with antiserum 273. The molecular weight of fraction III is estimated to be close to that of insulin (mol wt ∽6,000) from its behavior on ultracentrifugal analysis and from the observation that it elutes just before the synthetic, N-terminal 1–34 fragment (mol wt ∽4,100) on Sephadex gel filtration. It appears, therefore, to be a fragment representing about two-thirds of intact hPTH and corresponding primarily to its C-terminal portion.

Fraction A in plasma, which has the same elution volume on Sephadex gel filtration as does intact bPTH, appears to be the biologically active form of the hormone, since its level responded reciprocally to acute changes in calcium concentration and it was absent from the plasma of Gl at a time when he was clinically hypoparathyroid. It is the circulating form corresponding to fraction II of glandular extracts. This glandular fraction has both gel filtration and ultracentrifugal properties resembling bPTH and, like bPTH, reacts virtually identically with both antisera. Fraction I, found only in glandular extracts, may correspond to the biosynthetic precursor described by Habener et al. (10). Our inability to detect this component in plasma might be related to a low or absent rate of secretion, to rapid peripheral degradation, or to both of these factors. No estimate can be made concerning its possible biologic potency.

Fraction I of Canterbury and Reiss (6), the larger fraction detected by Goldsmith et al. (20), and our fraction A, all of which eluted with intact bPTH on gel filtration, are probably identical and correspond to intact (∽9,500 mol wt), biologically active hPTH. Our fractions B and C appear to be primarily C-terminal and N-terminal fragments, respectively, and have elution patterns similar to fractions II and III of Canterbury and Reiss (6). These workers did not determine from which regions of the intact hormone fractions II and III were derived. The 7,000 mol wt fraction observed by Goldsmith et al. (20) was reported to have biologic activity and therefore must contain intact the N-terminal 1-29 group (21). Therefore, in spite of the similarity in elution patterns, this component cannot be equivalent to our fraction B, which appears to be a C-terminal fragment devoid of biologic activity.

From the data presented in this report one is forced to conclude that any interpretation concerning the relative prominence of the various forms of glandular PTH is dependent upon the nature of the extracting solvent and the characteristics of the antiserum used in the immunoassay procedure. Acetone-acetic acid and 8 M urea extractions were quite similar, both in the total immunoreactivity recovered and in the general pattern of the components present on Sephadex gel filtration; i.e., the principal immunoreactive form was fraction II, which had similar immunoreactivity with both antisera. These data account for the earlier observations from this laboratory (1) of similar immunopotencies of different acetic-acid and urea extracts with antisera 273 and C-329.

Extraction of the same adenomas with saline resulted in an apparent increase in the total immunoreactivity measured with antiserum 273 and in marked differences in the patterns observed following Sephadex gel filtration. With antiserum C-329, the integrated immunoreactivity recovered was essentially the same as in the acetone-acetic acid and 8 M urea extracts, but peak II was significantly diminished and there was a well-defined peak in the IV region which accounted for much of the decrease in II. The greatest differences were seen with antiserum 273. Almost twice as much total immunoreactivity was extracted and virtually all of this increase could be accounted for by a striking peak in fraction III.

We have considered a number of possible explanations for the prominence of the fraction III peak in saline extracts. The first possibility considered was that of a greater inherent solubility of the different peaks in their respective solvents. However, re-extraction of lyophilized fractions II and III in the various solvents revealed solubilities so similar that this possibility was felt to be most unlikely. Another hypothesis was that there existed within the gland a pool of fraction III that was more accessible to saline than to the other solvents. Since all the tissues were finely ground and re-extracted three times, the possibility of a contained pool not exposed equally to all solvents was quite remote. Because assay with both antisera indicated that fraction II was diminished and the later fractions enhanced, the most likely explanation is that enzymatic conversion of fraction II to smaller molecular forms had occurred. The lesser conversion in the acetone-acetic acid or urea extracts could be accounted for by some inhibition of the action of the enzyme by the high concentrations of hydrogen ions or

urea present in those solvents. The possibility of such a conversion is consistent with the observations of Sherwood et al. (4), who reported the conversion of [^{131}I] bPTH to a lower molecular weight peptide after incubation at pH 7 in a homogenate of parathyroid gland but not in homogenates of other tissues. If our plasma fraction B and our glandular fraction III are the same, then the peptidase of Fischer, Oldham, Sizemore, and Arnand (22) cannot be involved since this enzyme is reported to generate biologically active hormonal fragments from intact hormone.

The increased immunochemical potency of the total extract and the increased prominence of the fraction III peak as seen by 273 could be explained on the basis of an unusually high sensitivity of that antiserum for the fraction III fragment, a sensitivity greater than would have been observed if that fragment were contained in an intact molecule. This hypothesis would predict that the fraction III fragment has an altered tertiary structure as compared to its configuration in the intact molecule. A likely explanation for the greater sensitivity to this altered form is that the immunizing antigen, which was of low purity, contained a fraction III-like component to which some guinea pigs, by chance, developed high potency and high sensitivity antisera which cross-react, but with lower sensitivity, with the same region in the intact molecule. It is interesting to note that, in our laboratory, enzymatic conversions of big gastrin to heptadecapeptide gastrin (23), big ACTH to 1-39 ACTH (24) and big, big insulin to the usual form of insulin (25) are quantitative, suggesting that the big, presumably precursor, species contains the usual small hormone within it in an unaltered immunochemical form. With the antisera available in our laboratory, fragments of the usual molecular forms of insulin, ACTH and gastrin cross-react quite poorly and therefore would not be detectable. However, it would be interesting for those laboratories having available antisera directed against fragments of, for instance, the 1-39 species of ACTH to determine whether controlled enzymatic degradation of this form can result in an apparent increase in total immunoreactivity.

In the earliest studies from our laboratory on the immunochemical heterogeneity of hPTH (1) we reached no definitive conclusion as to whether the circulating hormonal fragments arose from glandular secretion as such or from the peripheral conversion of the 9,500 mol wt intact hormone. Recently, several groups have considered this question and have arrived at different conclusions. Arnaud et al. (3) and Sherwood et al. (4) have concluded that the predominant secreted species is smaller than the 9,500 mol wt form considered to be intact PTH; Habener et al. (5) concluded that the intact form is secreted and that conversion occurs peripherally, a conclusion in which Canterbury and Reiss (6) concurred. Let us consider quantitatively the effect of the slower turnover rate of the fragments, as compared to that of the intact hormone, on the steady-state concentrations of the circulating parathyroid peptides and the ability to detect secreted hormonal fragments in the glandular effluent. In the present study the disappearance of plasma fraction A as measured by C-329 in a uremic subject, De, during a calcium infusion test set an upper limit to the half-time for removal of fraction A from his plasma of no more than 20 min. Since prior to the infusion the patient had been in a steady state it is likely that the disappearance rate was due to degradation and not to distribution into extravascular compartments. In another uremic patient, Gl, fraction B, as seen by antiserum 273, was reduced to 3% of its preoperative value 9 days after total parathyroidectomy, i.e. a half-time for disappearance of 2 days. Assuming that these relative rates are typical of uremic patients, the fractional disappearance rate for intact hormone (fraction A) is about 150 times that of fraction B. If the glandular secretion rates for both were the same then the steady state concentration of B would be 150 times that of A. Since the observed steady state concentration in patient Gl of B was only 10 times that of A, the secretion rate need be only 1/15 as great. Therefore, in the glandular effluent, B would have a concentration only 1/15 that of A, while its peripheral steady-state concentration would be 10 times greater than that of A. Similar but more extreme considerations hold for fraction C. Fraction C had hardly disappeared 9 days post-parathyroidectomy in paient Gl. Therefore its disappearance rate is less than 1/600 (20 min/9 × 24 × 60 min) that of A. Since the plasma level of C is about 1/2 that of A, its secretion rate need be no more than 1/1200 that of A to achieve this steady state concentration. From these considerations it is clear that the relative concentrations of intact hormone and hormonal fragments in the circulation do not reflect secretion rates unless quantitative estimates of turnover times are incorporated into the calculations. It is not surprising then that Habener et al. (5) found that the predominant fraction of the hormone present in parathyroid effluent blood was equal to or slightly larger than the approximately 84 amino acid hormone while the hormone in the peripheral circulation was principally in the form of smaller immunologically reactive fragments. However, since their data on fractionation of the hormone in the parathyroid effluent suggests the presence of the smaller circulating form with a concentration twice the concentration of that component in the periphery, their interpretation that the smaller circulating form arises from degradation of intact hPTH in the peripheral circulation is open to question.

Recently Canterbury and Reiss (6) demonstrated multiple immunoreactive forms of hPTH in pooled serum

and observed the very slow disappearance (following calcium suppression or parathyroidectomy) of immunoreactive fragments compared to intact hormone, but their interpretation of the source of the fragments was similar to that of Habener et al. (5). It is interesting that Arnaud et al. who in earlier papers (2, 3) concluded from in vitro work that the predominant secreted species is a fragment of PTH that had been cleaved from intact hormone in the gland, have concluded on the basis of more recent studies (20) that once in the plasma, 9,000 mol wt PTH is cleaved to 7,000 mol wt PTH leaving the latter as the major circulating form of PTH.

When due consideration is given to degradation rates, our own data and that cited from other laboratories lead us to conclude that the body of evidence is more consistent with the hypothesis of secretion of the various hormonal species from the gland rather than the hypothesis of peripheral conversion. Direct evidence for peripheral conversion could perhaps be obtained by administration of highly purified hPTH, sampling immediately and then at later time intervals, and then fractionating those plasma samples on Sephadex to determine whether or not there is an absolute increase in the concentration of immunoreactive fragments. To the best of our knowledge such studies have not been performed, undoubtedly due to the limited availability of hPTH.

Similar studies with bPTH might not be conclusive if negative because metabolism of bPTH and hPTH in man might not be identical. However, the only study with which we are familiar that even considered the direct measurement of return of fragments following in vivo administration of glandular hormone was from our own laboratory (1). In that study we concluded that a distinction could not be made between a minor heterogeneous component in the injected material which had a slow disappearance rate and the possible return of a metabolic product in vivo. The recent study by Fischer et al. (22) of a peptidase present in several porcine tissues that rapidly converts PTH to a smaller molecular weight component that has biologic activity is probably not relevant since the hormonal fragments in plasma in the present report appear to be without biologic activity.

The limitations of the assay for hPTH in plasma lie partly in the problem of immunochemical heterogeneity, just discussed, and partly in the low sensitivity of most antisera, which enhances the error of measurement at low hormone concentrations. In non-uremic primary adenomatous or carcinomatous hyperparathyroidism, antiserum C-329 measures primarily immunoreactive component A, which appears to be the intact, biologically active hormone. The immunoreactive hormone concentration measured by antiserum 273, however, includes not only component A, which, like glandular fraction II, is probably equipotent in both antisera, but also component B, which is not detected by C-329. Thus, while the use of antiserum 273 provides enhanced sensitivity, it must be appreciated that the immunoreactivity measured includes biologically inactive as well as active hormone and might therefore result in false positive results. Nevertheless, with this antiserum we have been able to distinguish rather well between hyperparathyroid patients and non-hyperparathyroid controls. Previously we reported that 53 of 56 values in patients with proven hyperparathyroidism exceeded the highest level observed in 98% of the controls and that in every case in whom elevated hPTH values were found and the patient was explored, surgical confirmation of parathyroid adenoma or hyperplasia was obtained (19). In that report antiserum 273 was employed for diagnostic evaluation. The present data confirm that a high degree of diagnostic accuracy is obtained wtih antiserum 273 but not with antiserum C-329. It would therefore appear that the use of an antiserum with enhanced sensitivity due to its detection of both biologically inactive and active hormone offers an advantage for preoperative diagnosis of the hyperparathyroid state. However, the use of an antiserum such as 273 to measure the suppressibility of biologically active endogenous hormone by calcium infusion or the disappearance of active hormone following parathyroidectomy cannot produce quantitatively valid data and the use of an antiserum such as C-329 would be preferable.

The complexity of immunoreactive hormonal forms of hPTH in plasma and glandular extracts and the failure of most investigators to evaluate fully the significance of their differing rates of removal has resulted in some confusion concerning the source of the immunoreactive PTH fragments found in the circulation. In the present report more complete analysis of the experimental findings has permitted elucidation of some of these problems.

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