

# Metabolism of Bovine Parathyroid Hormone

## IMMUNOLOGICAL AND BIOLOGICAL CHARACTERISTICS OF FRAGMENTS GENERATED BY LIVER PERFUSION

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**ABSTRACT** The metabolism of bovine parathyroid hormone (PTH) by the perfused rat liver was studied. Labeled hormone, with or without cold hormone, was infused into the circulating perfusion medium containing various calcium concentrations. Perfusate samples at various time periods after the introduction of PTH into the system were chromatographed on Bio-gel P-10; radioactivity and/or immunoreactivity were measured in eluted fractions.

Before the perfusion, all immuno- and radioactivity eluted in a single peak, with an apparent mol wt of 9,500 (peak I). After perfusion for 15 min, two other peaks with approximate mol wt of 7,000 (peak II) and 3,500 (peak III) were discernible. Peak I contained both NH<sub>2</sub>-terminal and COOH-terminal immunoreactivity and was biologically active at all time periods tested. The relative contribution of NH<sub>2</sub>-terminal and COOH-terminal immunoreactivity to the total immunoreactivity remained constant in this peak throughout the perfusion. In every respect, peak I had the characteristics of intact hormone. At all times, peak II consisted of only COOH-terminal immunoreactivity and was biologically inactive. At early time periods, peak III contained predominantly NH<sub>2</sub>-terminal immunoreactivity and was biologically active. With time, the relative contribution of NH<sub>2</sub>-terminal immunoreactivity decreased strikingly while that of COOH-terminal immunoreac-

tivity increased. The three peaks identified in these experiments were analogous in size, biological activity, and immunological characteristics to those we have previously described for fractionated human hyperparathyroid serum.

The rate of metabolism of PTH appeared to be regulated by the calcium concentration in the medium. At a high concentration of calcium (> 11 mg/100 ml), PTH metabolism was greatly retarded. At a low concentration of calcium (< 5 mg/100 ml), the rate of metabolism was greatly increased.

The physiological significance of our observations on the metabolism of PTH by isolated perfused rat liver is not known. However, since such metabolism results in a biologically active fragment, it is suggested that metabolism of intact hormone may be required before full biological expression is possible.

### INTRODUCTION

Despite rapid progress in elucidating the structure of parathyroid hormone (PTH)<sup>1</sup> in several species (1-5), the nature of circulating PTH remains incompletely defined. The possibility that circulating PTH may consist of several distinct immunochemical forms was first suggested by the work of Berson and Yalow in 1968 (6). Since that time, work carried out in several laboratories has provided supporting evidence for this suggestion. However, there remains much doubt concerning the number, biological characteristics, origin, and physiological significance of the various circulating forms of PTH.

Circulating PTH consists of the native hormone (9,500 mol wt) and one or more smaller mol wt im-

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This work was presented in part at the 56th Annual Meeting of the Endocrine Society, Atlanta, June 1974, and at the Fifth International Parathyroid Conference, Oxford, England, July 1974.

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Received for publication 22 October 1974 and in revised form 17 February 1975.

<sup>1</sup> Abbreviations used in this paper: cAMP, cyclic 3',5'-AMP; PTH, parathyroid hormone.

munoreactive fragments. In all experiments reported, a PTH fragment with a mol wt of approximately 7,000 is the predominant circulating immunoreactive species of the hormone in patients with hyperparathyroidism. In the experience of Segre, Habener, Powell, Tregear, and Potts, this 7,000-mol wt fragment is the only one that can be detected in the circulation with a variety of antisera (7). Silverman and Yalow have demonstrated the presence of two fragments in plasma from hyperparathyroid patients: the characteristic 7,000-mol wt moiety and an additional fragment with an approximate mol wt of 4,000 (8). Similar results have recently been reported by Arnaud, Goldsmith, Bordier, and Sizemore (9). Using concentrated sera from hyperparathyroid patients, we have consistently identified two fragments analogous to those observed by Silverman and Yalow (10).

There is less agreement concerning the biological characteristics of fragments. It is known from the work of Keutmann, Dawson, Aurbach, and Potts (11) that the biological activity of PTH resides in the NH<sub>2</sub>-terminal portion of the molecule while the COOH-terminal portion is devoid of biological activity. The smallest fragment identified by Silverman and Yalow (4,000 mol wt) was interpreted by them as representing an NH<sub>2</sub>-terminal fragment (8). However, because it persisted in the circulation for a very long time, they considered this fragment to be biologically inactive. The smallest fragment identified by Arnaud et al. has immunological characteristics of an NH<sub>2</sub>-terminal fragment, but these investigators make no statement concerning probable biological activity (9). The smaller fragment identified in our experiments has an NH<sub>2</sub>-terminal characteristic in that it possesses biological activity, as tested in the renal adenylate cyclase assay (12). All investigators agree that the 7,000-mol wt fragment is immunologically COOH-terminal and hence devoid of biological activity.

The mechanism by which these various immunoreactive forms of PTH arise from native hormone remains obscure. Habener, Powell, Murray, Mayer, and Potts fractionated PTH derived from the thyroid venous effluent of patients with parathyroid adenomas and demonstrated that such samples contained predominantly intact PTH (13). These experiments strongly suggest that PTH is secreted from the glands in intact form and that the fragments are generated by peripheral metabolism. Silverman and Yalow, on the other hand, pointed out that fragments characteristically have a long half-life in the circulation (hours to days) (8), while the intact hormone has a half-life of less than 30 min (14). Hence, under steady-state conditions, fragments may represent the predominant circulating form, even though they were secreted from the glands in much smaller

quantities than was intact hormone. The most persuasive evidence that fragments of PTH can arise from metabolism of intact hormone is based on the work of Segre, Niall, Habener, and Potts (15). They administered <sup>131</sup>I-labeled bovine PTH to cows and dogs and performed careful radio- and immunochemical analysis of gel filtration products at timed intervals. Edman degradation and sequence-specific immunoassay data were closely correlated and demonstrated that the primary site of cleavage was between amino acids 33 and 34. While the secretion of some fragments by the parathyroid glands cannot be excluded on the basis of the available data, peripheral metabolism of exogenous PTH clearly occurs.

In broken cell systems, there is abundant evidence that a variety of tissues contain the enzymatic machinery necessary for the degradation of PTH. In early studies, Fujita, Ohata, Orimo, Yoshikawa, and Maruyama (16, 17) and Martin, Melick, and DeLuise (18, 19) described specific enzymes that degraded PTH in the kidney. More recently, Fang and Tashjian (20) and Fischer, Oldham, Sizemore, and Arnaud (21) have reported that the liver also has the capacity of metabolize PTH.

The objective of this study was to generate PTH fragments in sufficient quantity for further characterization. Only small amounts can be derived from the circulation. We chose to investigate the liver because this organ readily lends itself to *in situ* perfusion. Such perfusions possess advantages over the broken cell system in that they more nearly mimic the physiological state, permit the generation of large amounts of fragments, and make possible examination of factors controlling the generation of fragments.

## METHODS

*Parathyroid hormone.* Purified bovine PTH with biological activity of approximately 3,000 USP u/mg (Munson bioassay) was obtained from Wilson Laboratories, Chicago, Ill. This material was labeled with <sup>131</sup>I (Union Carbide Corp., New York) to specific activities of 200-300  $\mu$ Ci/ $\mu$ g by a modification of the Hunter and Greenwood technic (22) and purified on QUSO G-32 (Philadelphia Quartz Co.) (23). Purity of <sup>131</sup>I-labeled PTH was checked immediately before use by chromatoelectrophoresis to make certain that the preparation did not contain contaminants. For some immunoassays, PTH was labeled with <sup>125</sup>I (New England Nuclear, Boston, Mass.) by the same method. None of the iodinated preparations were tested for biological activity. Tritiated PTH (<sup>3</sup>H]PTH) with a specific activity of approximately 1.6  $\mu$ Ci/ $\mu$ g was prepared according to the method of Zull and Repke (24). This process involves reaction of PTH with high specific activity tritiated methyl ester of acetamidate. [<sup>3</sup>H]PTH was stored under an N<sub>2</sub> atmosphere at -80°C. At the time of use, [<sup>3</sup>H]PTH was separated from "damaged" components by gel chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Although [<sup>3</sup>H]PTH possessing high biological activity can be prepared, the preparation used in these studies had only minimal biological activity. In some

studies, of the synthetic NH<sub>2</sub>-terminal peptide, 1-34 amino acids of bovine PTH (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), was employed.

**Liver perfusions.** Male Sprague-Dawley rats (200-250 g) were anesthetized with sodium pentobarbital intraperitoneally, 5 mg/100 g body wt, and surgically prepared for liver perfusion. The technic utilizes a variable pressure, constant flow apparatus (Vanderbilt University Medical Instrumentation), as has been previously described (25). The perfusion medium (100 ml) consisted of human outdated, washed red blood cells, 4 g of fraction V bovine serum albumin (Pentex Biochemical, Kankakee, Ill.), and 60 mg glucose in Krebs bicarbonate buffer, pH 7.4. The perfusion rate was 7 ml/min. The perfusate was oxygenated with humidified oxygen (95% with 5% CO<sub>2</sub>), and the system was maintained at 38°C. The duration of perfusion varied from 30 min to 2 h. 2-ml samples of perfusate were drawn from the sampling portal at time "0" (see below) and at various time periods after the introduction of labeled and/or unlabeled PTH into the rotating drum of the circuit. Calcium concentrations were altered by varying the amount of calcium chloride in the perfusion medium. Total calcium concentration of medium was measured by atomic absorption spectrophotometry. Viability of the liver preparations was assessed by the arterial-to-venous color difference, the production of bile, and the conversion of [2-<sup>14</sup>C]sodium acetate to cholesterol (26).

During liver perfusion, 80% of radioactivity introduced into the system remained in the perfusing medium. Only 1.5% of radioactivity was recovered in the liver at the termination of perfusion. 10-12% adhered to tubing, the trap, and the drum. 2% appeared in bile. 4-6% of radioactivity could not be accounted for.

**Gel filtration.** Timed samples were centrifuged to remove blood cells, and the supernates were applied to Bio-gel P-10 (Bio-Rad Laboratories, Richmond, Calif) columns (1.5 × 50 cm) equilibrated immediately before use with 10 ml of 2% bovine serum albumin in the elutant buffer, 0.15 M ammonium acetate, pH 5.0. Columns were calibrated with dextran blue (Pharmacia Fine Chemicals, Inc.), [<sup>131</sup>I]PTH, [<sup>3</sup>H]PTH, [<sup>125</sup>I]insulin, [<sup>125</sup>I]glucagon, and Na<sup>131</sup>I. At the end of each experiment, the void volume and the elution profile of at least one of the radioactive hormone markers were again determined to make sure that the column characteristics had not been altered. Eluted fractions (0.5 ml) were counted directly for iodine radioactivity in a Nuclear-Chicago automatic gamma scintillation spectrometer (Searle Analytic Inc., Des Plaines, Ill.) or for tritium in a Packard model 3390 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) after the addition of 15 ml Aquasol liquid scintillation cocktail (New England Nuclear). Loss of radioactivity and immunoreactivity during the fractionation procedure approximated 25-30%. This loss was independent of sampling time.

**Radioimmunoassay.** Immunoreactive PTH was measured in the eluted fractions by using antiserum CH-824 (27) at a final dilution of 1:75,000. Separation of antibody-bound and free [<sup>125</sup>I]bovine PTH was performed by using dextran-coated charcoal (28). In some experiments, the antigenic recognition sites of antiserum CH-824 were modified by preincubation for 3 days with an excess of the synthetic NH<sub>2</sub>-terminal peptide (1-34 amino acids) of bovine PTH or the COOH-terminal fragment 53-84<sup>2</sup> prepared by tryptic digestion of succinylated native hormone as described by

<sup>2</sup> Hormone fragment was kindly supplied by Drs. Gino V. Segre and John T. Potts, Jr.

Niall et al. (2). The 1-34 and 53-84 peptides were used in a concentration calculated to give a final concentration of 20 and 10 ng/assay tube, respectively. The antiserum for blocking studies was preincubated with these peptides at a dilution of 1:15,000, and the blocked antisera were used in a final concentration of 1:75,000. Antiserum blocked with 1-34 peptide did not bind the 1-34 peptide in amounts up to 10 ng and possessed superimposable binding functions for the 1-84 and 53-84 peptides. Similarly, antiserum blocked with the 53-84 peptide did not bind the 53-84 peptide in amounts up to 10 ng and possessed superimposable binding functions for the 1-84 and 1-34 peptides.

**Biological activity.** Rats were anesthetized with pentobarbital and the kidneys removed. The cortex was dissected from medullary tissue with a razor blade and minced. Approximately 200-300 mg of cortical tissue was homogenized in 4.5 ml of cold 0.25 M sucrose. The homogenate was centrifuged at 12,000 *g* for 10 min at 4°C, and the supernate discarded; the particles were washed with cold 0.25 M sucrose, resuspended, and rehomogenized in the cold 0.25 M sucrose. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (29) and the adenylate cyclase activity was assessed by the method of Krishna, Weiss, and Brodie (30). The particulate fraction containing 0.09-0.12 mg protein was incubated at 37°C for 15 min with 2.5 mM ATP, 3.0-3.5 × 10<sup>8</sup> cpm; theophylline, 8 mM; MgCl<sub>2</sub>, 3.0 mM; Tris-HCl, 21 mM, pH 7.7; phosphoenolpyruvate, 0.9 mM; pyruvate kinase, 0.02 mg/ml; potassium chloride, 26 mM; bovine albumin, 0.8 mg/ml; and varying amounts of bovine PTH, immunoreactive fragments, or the equivalent amount of fractionated perfusion medium. The diluent for PTH, 0.01 N acetic acid, was added to all control tubes. The total volume of the incubation mixture was 0.065 ml. The cyclic 3',5'-AMP (cAMP) produced was determined as previously described (31).

## RESULTS

**Control incubations.** To assess the effects of the perfusion medium per se on PTH and to exclude the possibility that fragments were present in the preparation used, labeled hormone was incubated in the medium under various conditions and then subjected to gel filtration. Neither 60 min at 37°C nor 24 h at 4°C had any effect on the elution pattern of [<sup>131</sup>I]PTH. As can be seen in Fig. 1, each yielded a single, identical peak with an elution volume indicative of a molecule of approximately 9,500 mol wt. These results show that the medium itself contained no mechanism for the degradation of PTH and that the [<sup>131</sup>I]PTH preparations used were not contaminated by fragments.

**Patterns of PTH fragments.** Representative elution patterns of immunoreactivity and radioactivity of [<sup>3</sup>H]-PTH at various time periods after the initiation of the liver perfusions are shown in Fig. 2. The "0"-time pattern shown does not represent a true zero sample since 3-5 min were required for introducing the sample into the system and obtaining a sample. In this interval, some metabolism of PTH has already occurred. The "0"-time elution profile shown represents the fractionation of [<sup>3</sup>H]PTH preincubated for 30 min at 38°C in the complete perfusion medium. For this sample, all im-

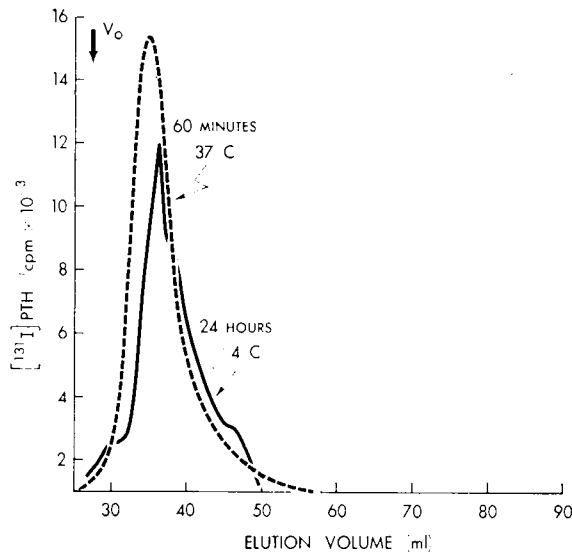


FIGURE 1 Elution profiles on P-10 columns of  $[^{131}\text{I}]\text{PTH}$  incubated in the perfusate medium for 60 min at  $37^\circ\text{C}$  (---) and for 24 h at  $4^\circ\text{C}$  (—). The single, superimposable peaks indicate that the  $[^{131}\text{I}]\text{PTH}$  remained intact and was not affected by the perfusion medium.

munoreactivity and radioactivity eluted in a single peak with an apparent mol wt of 9,500. Radioactivity and immunoreactivity of  $[^3\text{H}]\text{PTH}$  and its metabolic fragments coeluted as discrete, single peaks during the 1st h of perfusion, indicating the utility of a uniformly tritiated hormone for this type of metabolic study. By 15 min, three distinct peaks with apparent mol wts of 9,500, 7,000, and 3,500 were discernible, and by 30 min, the smaller molecular fractions represented the predominant immunoreactive species. Up to 60 min (not shown in Fig. 2) after the introduction of PTH into the liver-perfusing medium, there was a continuous reduction of immunoreactive PTH and radioactivity in the 9,500-mol wt peak I and an increase in peaks II and III. By 90 min, peaks II and III were no longer discrete; immunoreactivity and radioactivity were nonspecifically dispersed throughout the column eluate.

When these same experiments were performed with trace  $[^{131}\text{I}]\text{PTH}$  and cold PTH in the liver perfusion medium (not shown in a figure), the elution profile of radioactivity differed from that observed with  $[^3\text{H}]\text{PTH}$ . Samples taken up to and including 15 min after the introduction of  $[^{131}\text{I}]\text{PTH}$  into the system revealed an elution profile of only two radioactive peaks, 9,500 mol wt and 7,000 mol wt. However, when this column effluent was assayed for immunoreactivity with  $[^{125}\text{I}]\text{PTH}$  as labeled antigen, the three characteristic peaks of approximately 9,500, 7,000, and 3,500 mol wt were again detected. In samples at any time after the 15-min sample,

a radioactive peak with an elution volume characteristic of the 3,500-mol wt peak was detectable.

**Biological activity.** To obtain sufficient material for testing the biological activity of the various fragments generated by the perfused liver,  $10\ \mu\text{g}$  of cold PTH was used with a trace quantity of  $[^3\text{H}]\text{PTH}$ . The radioactive and immunoreactive elution patterns at various times were identical to those obtained with lower doses of PTH. Narrow cuts were made of the tops of each radioactive peak (5 ml). The sample was lyophilized and reconstituted in  $200\ \mu\text{l}$  of 0.01 N acetic acid.  $5\ \text{ml}$  of void volume effluent was lyophilized and reconstituted in  $200\ \mu\text{l}$  of 0.01 N acetic acid.  $25\ \mu\text{l}$  of this material was added to all adenylate cyclase control tubes. The results of testing the concentrated peaks are shown in Table I.

Peak I and peak III were biologically active at all time periods tested as evidenced by the generation of cAMP. Peak II, the 7,000-mol wt fragment, was biologically inactive. There appeared to be little difference in the absolute amount of cAMP generated by the individual peaks at various time periods tested. This could

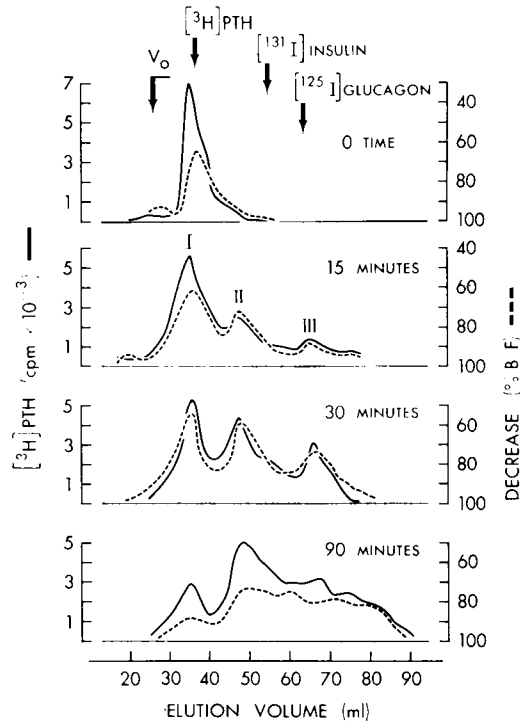


FIGURE 2 Elution profiles on P-10 columns of radioactivity (—) and immunoreactivity (---) of  $[^3\text{H}]\text{PTH}$  at "0"- and various time periods after the introduction of  $2\ \mu\text{g}$  of  $[^3\text{H}]\text{PTH}$  into the liver perfusion system. The concentration of calcium in the medium was  $8.8\ \text{mg}/100\ \text{ml}$ . The arrows at the top of the graphs indicate the elution positions of markers used to calibrate the columns. See text for comment on "0"-time sample. "% B/F" refers to the bound-to-free hormone ratio of samples as a fraction of controls containing only tracer hormone.

TABLE I  
Activation of Rat Renal Cortical Adenylate Cyclase by PTH, Synthetic 1-34 Amino Acids of Bovine PTH, and Liver Perfusion Fragments

	Biological activity*
Control	215 ± 12
1-34 Peptide	540 ± 14
Peak I: 0 time	600 ± 13
15 min	565 ± 30
Peak II: 10 min	200 ± 15
30 min	220 ± 20
Peak III: 10 min	585 ± 32
15 min	566 ± 15
20 min	546 ± 35
30 min	522 ± 26

\* Units of activity are pmol cAMP accumulated/15 min per mg protein. Each value represents the mean ± SE of six samples. The concentration of the 1-34 peptide was  $1 \times 10^{-6}$  M. In testing the peaks 25  $\mu$ l of the concentrated peaks were used.

indicate that the concentrations used were maximal for stimulating the enzyme. Therefore, the dose-response curves of peaks I and III at two separate times were examined (Fig. 3). The dose of 25  $\mu$ l used in the experiment shown in Table I in fact gave a maximal response. The response curves obtained are parallel to each other as well as to the response curve obtained with bovine PTH (12).

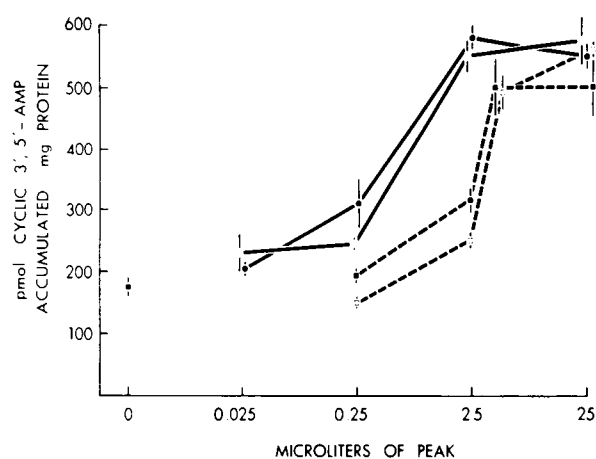


FIGURE 3 Dose-response curves for peak I at "0" (■---■) and at 15 min (□---□), and for peak III at 15 min (●---●) and at 30 min (○---○) after the introduction of PTH into the liver perfusion system. Each value represents the mean ± SE of four samples. ■ at the far left represents control values.

*Immunological characteristics.* The immunological composition of the various peaks at different times was determined by blocking experiments as described in the methods section. In Table II are given the results of one of two experiments of this type performed. The "COOH-terminal specific" antiserum refers to the antiserum blocked with 1-34 peptide so that it recognized carboxy terminal (35-84) antigenic determinants of the PTH molecule. The "NH<sub>2</sub>-terminal specific" assays were performed with antiserum blocked with the 53-84 fragment so that it recognized only the amino terminal 1-52 se-

TABLE II  
Immunological Properties of the Liver Perfusion Fragments

Time after initiation of liver perfusion	Sample tested	COOH-terminal specific	NH <sub>2</sub> -terminal specific	NH <sub>2</sub> -terminal specific / COOH-terminal specific
<i>min</i>				
0	Peak I	14.5	13.5	0.9
15	Peak I	7.8	7.5	0.9
	Peak II	4.7	0.4	0.1
	Peak III	0.3	2.0	6.7
30	Peak I	5.0	3.8	0.8
	Peak II	4.0	0.2	0.1
	Peak III	2.8	3.4	1.2
45	Peak I	3.8	3.0	0.8
	Peak II	3.2	0.2	0.1
	Peak III	4.0	2.5	0.6

The peaks are defined in the text and figures. "COOH-terminal" and "NH<sub>2</sub>-terminal" are operational definitions explained in the text. The concentration of calcium in the medium was 8.8 mg/100 ml in this experiment.

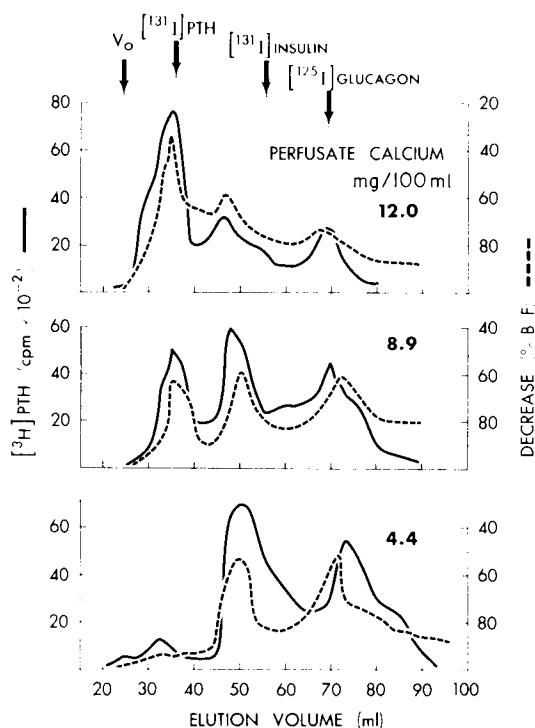


FIGURE 4 Elution profiles on P-10 columns of  $[^3\text{H}]$ PTH radioactivity (—) and immunoreactivity (---) in samples taken 30 min after the introduction of  $[^3\text{H}]$ PTH into the liver perfusion system. The three experiments were performed at widely varying concentrations of calcium. "% B/F" refers to the bound-to-free hormone ratio of samples as a fraction of controls containing only tracer hormones.

quence. At "0" time, the relative contribution of COOH-terminal and  $\text{NH}_2$ -terminal immunoreactivity was approximately equal, as reflected in the ratio shown in the last column. With time, this ratio remained constant while the total immunoreactivity decreased.

Peak II consisted predominantly, if not exclusively, of COOH-terminal immunoreactive material. With time,

this remained relatively constant. The finding of predominant COOH-terminal immunoreactivity in peak II fits well with the absence of biological activity in this peak.

Peak III at the 15-min sample time was composed predominantly of  $\text{NH}_2$ -terminal immunoreactivity ( $\text{NH}_2/\text{COOH} = 6.7$ ). However, with time, the contribution of COOH-terminal immunoreactivity in peak III increased so that by 45 min the  $\text{NH}_2/\text{COOH}$  ratio had decreased to 0.6.

*Effect of calcium concentration of medium.* The influence of the calcium concentration of the perfusing medium on the rate of fragment generation is shown in Fig. 4. Results are given for three widely varying concentrations of calcium after 30 min of liver perfusion. At a concentration of 8.9 mg/100 ml, the elution profile was comparable to that previously depicted in Fig. 1. Raising the calcium concentration to 12.0 mg/100 ml greatly inhibited the metabolism of the hormone; most of the radioactivity and immunoreactivity continued to elute in the position of peak I. By contrast, when the perfusate concentration of calcium was reduced in 4.4 mg/100 ml, virtually all of peak I was converted to peaks II and III.

In other experiments not shown in a figure, the time relationship of the generation of fragments was examined. At a high concentration of calcium in the perfusate (11.2 mg/100 ml), all of the PTH present remained in the form of peak I by 15 min, and even the 30-min sample showed little conversion of the native hormone to the smaller mol wt fragments. By contrast, at a low concentration of calcium in perfusate (4.2 mg/100 ml), virtually all of peak I was converted to fragments in 15 min. The 30-min sample appeared similar to the 15-min sample.

When peaks II and III derived from a low-medium calcium perfusion were analyzed immunologically and for biological activity, it became apparent that the com-

TABLE III  
*Immunological and Biological Properties of the Various Fragments Generated by the Liver at a 4.2 mg/100 ml Perfusate Calcium Concentration*

Time after initiation of liver perfusion	Sample tested	COOH-terminal specific	$\text{NH}_2$ -terminal specific	$\frac{\text{NH}_2\text{-terminal specific}}{\text{COOH-terminal specific}}$	Biological activity*
<i>min</i>					
15	Peak II	4.8	0.20	0.04	45 ± 10
	Peak III	0.42	3.6	8.6	390 ± 13
30	Peak II	3.6	0.11	0.03	Not tested
	Peak III	2.87	0.84	0.29	45 ± 8

\* Units of activity are pm cAMP accumulated/15 min per mg protein. Each value represents the mean ± SE of four samples. For this bioassay the control was 100 ± 12; bovine PTH ( $1 \times 10^{-6}$  M) gave 420 ± 10. The data on biological activity were derived from a separate experiment.

position of the peak III changed drastically with time (Table III). Peak II at both times studied was composed almost entirely of COOH-terminal immunoreactivity and had no biological activity when tested at 15 min. However, peak III at 15 min consisted largely of NH<sub>2</sub>-terminal immunoreactivity and had biological activity, but by 30 min the composition of this peak had shifted to predominantly COOH-terminal immunoreactivity and had become biologically inactive.

## DISCUSSION

The logical tissue for the study of the metabolism of PTH is the kidney, for there is abundant evidence that this organ metabolizes PTH (16-19). In addition, it is one of the recognized target tissues of the hormone. Since perfusion of the intact kidney represents a technically unsolved problem, we selected the liver for study, an organ for which an excellent perfusion system has been perfected. As has been shown recently, PTH activates adenylate cyclase in the liver (32, 33). That liver is one of the tissues that metabolizes PTH has been known since the studies of Davis and Talmage (34) and has been confirmed by more recent investigations (20, 21), but the physiologic significance of this metabolism remains obscure. The present studies confirm unequivocally the previously reported results.

For the purposes of the present study, the failure of the perfusion medium to metabolize PTH represents an important control observation (Fig. 1). In the absence of metabolizing systems, PTH is remarkably stable. In other studies, it has been shown that incubation of PTH with plasma does not result in the generation of PTH metabolites (15), and unpublished observations in our laboratory have confirmed this observation.

The elution pattern of fragments generated by the perfused liver and their immunological and biological characteristics were remarkably consistent. This suggests that the process studied was in fact specific for the metabolism of PTH. Metabolism of PTH in the liver proceeded rapidly. In the few minutes required for the introduction of the hormone into the perfusion system, enough metabolism had taken place so that no true base-line or "0" sample could be obtained. The course of metabolism could be followed for approximately 60 min. Thereafter, the elution patterns were indistinct.

There is a clear analogy between the fragments generated by liver perfusion and those isolated by us in the fractionation of human hyperparathyroid serum (10, 12). This analogy, including approximate molecular size and biological activity, has important implications. First, it suggests that the liver perfusion system mimics the physiologic condition. Secondly, it tends to lend additional credence to our earlier results with concentrated hyperparathyroid sera. Using concentrated sera, we

were concerned that the concentrating procedure might introduce artifactual generation of fragments (12). The present results combined with previously cited work concerning the absence of metabolizing enzymes in plasma or serum render this unlikely. Despite this suggestive evidence, one cannot be certain that PTH fragments generated *in vitro* are identical to those generated *in vivo*.

In these experiments, comparison of the elution patterns obtained with tritiated and iodinated PTH provided the first clue to the initial site of cleavage of the hormone. With tritiated PTH, three radioactive peaks became evident within 15 min, but with iodinated PTH, only two peaks, 9,500 and 7,000 mol wt, respectively, were discernible. The tritium label is located in many loci throughout the linear chain of the hormone while the iodine label is predominantly on the tyrosine residue in position 43. Detection of radioactive peak III at the early time periods with tritium but not with iodine suggests that this peak does not include the midportion of the molecule. By itself, this observation does not distinguish between the NH<sub>2</sub>- and COOH-terminal portions of the molecule. However, in view of Segre's data concerning the subsequent immunologic characteristics of the fragments (15), there seems little doubt that the initial peak III detected with tritiated but not with iodinated hormone represents an NH<sub>2</sub>-terminal portion of the hormone. The appearance of an iodinated peak III at later time periods probably reflects secondary cleavages of the hormone.

In all probability, peak I represented intact bovine PTH (amino acids 1-84). At all time periods, it co-eluted with labeled bovine PTH markers and had an apparent mol wt of 9,500. It was biologically active at all time periods tested. Although its half-time of disappearance from the system approximated 20 min in most experiments, no clear statement can be made about biologic half-life of this peak since the system used was too complex and contained too many variables.

Peak II remained remarkably constant in all experiments regardless of the times at which it was tested. It was predominantly COOH-terminal in composition and never contained detectable biological activity. Again, the data do not permit a concrete statement concerning biologic half-life though it did appear that the half-time of disappearance of this peak was on the order of hours rather than minutes.

Peak III is the most intriguing and difficult to analyze. At early time periods, it represented predominantly NH<sub>2</sub>-terminal immunoreactivity and possessed biological activity. By 30 min, it continued to have biological activity, but its composition had changed so that it contained increasing amounts of COOH-terminal activity. This indicates that secondary cleavages were

taking place in the COOH-terminal portion of the molecule and that the resultant fragments were appearing in peak III. These data also show that no statement concerning biological activity can be made on the basis of the elution profile of a particular peak. They confirm our earliest suggestion that peak III is in part generated by further metabolism of peak II (10).

The evidence that the concentration of calcium in the perfusion medium is an important determinant of the rate of metabolism of PTH is firm. Our data confirm and extend the original observations by Fischer et al. (21). Less certain is whether this represents a physiologically important determinant. Metabolism of PTH was largely blocked with a high concentration of calcium in the perfusion medium and was markedly accelerated when the concentration was low. At low concentrations of calcium, the metabolism was in fact so rapid that within 15 min little if any of peak I remained. Fortunately, this experimental design permitted more concrete conclusions about the half-life of NH<sub>2</sub>-terminal, biologically active material within peak III. With the depletion of peak I, the kinetics of the disappearance of biologically active material are simpler. As shown in Table III, between the 15- and 30-min samples, the NH<sub>2</sub>-terminal immunoreactivity of peak III decreased drastically, suggesting an extraordinarily rapid half-life. This rapid half-life is reminiscent of the half-life of disappearance of injected 1-34 terminal peptide (8).

Although the present studies unequivocally demonstrate the occurrence of peripheral metabolism of PTH in the liver, they do not exclude the possibility of secretion of fragments by the parathyroid glands. It is possible that glandular secretion and peripheral cleavage both contribute to the population of circulating immunoreactive fragments; one mechanism or the other may become dominant depending upon physiological circumstances. It is tempting to speculate that in the intact animal, under conditions of hypocalcemia, the parathyroid glands as well as peripheral tissues generate fragments and that under this circumstance secretion of fragments from the glands becomes important. That parathyroid tissue contains the enzymatic machinery for the metabolism of PTH and is calcium controlled is evident from the studies of Fischer et al. (21). In addition, Silverman and Yalow have shown that fragments analogous to those found in hyperparathyroid plasma can be extracted from parathyroid tissue (8).

The present data leave unanswered the critical issue: what is the biological significance of the metabolism of PTH? It may merely represent a catabolic disposal of the active hormone. This seems highly unlikely since metabolism generates a biologically active fragment. The most intriguing possibility is that intact PTH is a "prohormone," which first must be cleaved before

full expression of biological activity is possible. This is not an original suggestion (35). Strangely, there is no concrete information whether intact PTH possesses biological activity since all methods for testing of biological activity require exposure of the hormone to systems capable of metabolizing it. The early work of Parsons and Robinson (36) showing that intact PTH passed through the circulation of an intact animal (cat) had a more rapid action on bone in vitro than intact PTH suggests that the hormone must be activated before biological expression is evident.

The notion that metabolism of PTH may be a required prerequisite for the expression of biological activity is reinforced by our data and those of others concerning the control of metabolism by calcium (21). Teleologically, it would make good sense if the entire biological machinery were geared toward maintenance of a normal serum calcium concentration. Under conditions of hypercalcemia of nonparathyroid origin, metabolism of PTH is blocked and, hence, expression of biological activity of the hormone is also blunted. Under conditions of hypocalcemia, metabolism of the hormone resulting in the generation of a biologically active fragment is accelerated.

#### ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants AM-16768 and HL-13715-04 and by the Damon Runyon Cancer Fund, DRG-1120.

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