Biochem. J. (1969) **111**, 509
Printed in Great Britain

Metabolism of Parathyroid Hormone

DEGRADATION OF ^{125}I-LABELLED HORMONE BY A KIDNEY ENZYME

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(*Received* 11 *October* 1968)

A study was made of the enzymic degradation of ^{125}I-labelled parathyroid hormone by rat kidney microsomes. Incubation with microsomes resulted in rapid destruction of the labelled hormone. The microsomal factor was not separable by dialysis, and the reaction was favoured by pH values in the physiological range. Velocity of the reaction varied directly as the substrate concentration, and additional crude parathyroid hormone (trichloroacetic acid-precipitated, 3·68mg./ml.) inhibited destruction of labelled hormone. There was much less inhibition with added trichloroacetic acid-precipitated calcitonin (3·92mg./ml.) and virtually none with added pig insulin (3·80mg./ml.). Gel filtration of control medium on P6 (Bio-Gel) yielded one radioactive peak at the void volume. After incubation with microsomes three further peaks were obtained on gel filtration. Only the void-volume peak contained intact ^{125}I-labelled parathyroid hormone, indicating that the microsomal enzyme degraded labelled hormone to a number of smaller fragments.

Incubation of parathyroid extract with rat kidney slices results in complete loss of detectable biological activity of the hormone, whereas other tissues have relatively little effect on the extract (Orimo, Fujita, Morii & Nakao, 1965). Vajda, Martin & Melick (1969) showed that homogenates of rat kidney rapidly destroyed bovine ^{131}I-labelled parathyroid hormone, and that activity was maximal in the microsomal fraction of the kidney and was abolished by prior heating at 60° for 30min.

The present paper describes further experiments designed to study the nature of the kidney factor and its mode of action on labelled parathyroid hormone.

EXPERIMENTAL

Chemicals. AnalaR-grade chemicals were used in all procedures. Crystalline bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Parkville, Vic., Australia. [^{125}I]Iodide was obtained from The Radiochemical Centre, Amersham, Bucks.

Hormones. Purified bovine PTH* was kindly supplied by Dr J. T. Potts, jun., and Dr G. D. Aurbach, National Institutes of Health, Bethesda, Maryland, U.S.A. It was prepared by the method of Aurbach & Potts (1964). The pig insulin used was Lilly batch no. 81894, and crude calcitonin, potency 550 M.R.C. milli-units/mg., was prepared by phenolic extraction and precipitation with trichloroacetic acid (Martin & Melick, 1967). Trichloroacetic acid-precipitated PTH (potency 200units/mg.) was prepared in this Laboratory by the method of Aurbach (1959).

* Abbreviation: PTH, parathyroid hormone.

Animals. Wistar albino rats of either sex were used, and were starved overnight before experiments.

Tissue homogenization and fractionation. Rats were stunned and decapitated, the kidneys quickly removed and homogenized manually in an all-glass homogenizer with 0·25M-sucrose at 4°. After spinning at 700*g* for 10min. in the small rotor of a Sorvall model RC2 centrifuge, the supernatant fraction was centrifuged at 5000*g* for 15min. The supernatant from the mitochondrial fraction was then centrifuged for 1hr. in a Beckman model L ultracentrifuge at 54000*g* in the 30 rotor. After decantation the sedimented microsomes were suspended in 10ml. of 0·25M-sucrose and centrifuged for 30min. in the 50 rotor at 148000*g*. The remaining button constituted the microsomal fraction. Electron microscopy (by courtesy of Dr K. D. Muirden) of the mitochondrial and microsomal fractions showed that the tissue-fractionation procedure was effective. The microsomal preparation was suspended in 0·25M-sucrose and frozen in divided samples. Samples were stored at −20° and were found to retain activity for several weeks despite frequent thawing and refreezing. The protein concentration of preparations was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

^{125}I-labelling of hormones. Labelling of parathyroid hormone and insulin with ^{125}I was carried out by the procedure of Hunter & Greenwood (1962), and the ^{125}I-labelled peptides were purified by adsorption to Quso G32 (Philadelphia Quartz Co.) and by elution with 20% (v/v) acetone in 1% (v/v) acetic acid (Yalow & Berson, 1966).

Chromatoelectrophoresis. The technique of chromatoelectrophoresis (Yalow & Berson, 1960) was used to check the labelled hormone preparations. With this method ^{125}I-labelled PTH remains at the origin, whereas peptide breakdown products migrate towards the anode with the plasma proteins, and free iodide migrates still further ahead

of this fraction. Strips were scanned in a Packard radiochromatogram scanner. The same chromatoelectrophoretic technique was used to measure intact ^{125}I-labelled PTH after incubation. Samples (100 μl.) from flasks were applied to paper (Whatman no. 3 MC) at 4°. After chromatoelectrophoresis (0·06 M-veronal buffer, pH 8·6; 700 v) for 90 min. the strips were dried and scanned. The origin and first ½ in. of each strip was cut and counted in a Nuclear–Chicago well scintillation counter.

Trichloroacetic acid preparation. In many experiments ^{125}I-labelled PTH was measured as the trichloroacetic acid-precipitable radioactivity. Samples (200 μl.) were added to 1·8 ml. of 10% trichloroacetic acid, centrifuged at 3000 rev./min. for 10 min. in an MSE centrifuge and washed once with 2 ml. of 10% trichloroacetic acid. The precipitates were counted in a Nuclear–Chicago automatic well scintillation counter. A comparison of the chromatoelectrophoretic method with the trichloroacetic acid-precipitation method of measuring intact ^{125}I-labelled PTH revealed that there was very close agreement between the results achieved with the two procedures (Vajda *et al.* 1969). For this reason trichloroacetic acid precipitation alone was used in the later experiments in this series. Where both methods were used, this is indicated in the legends to Figures and Tables.

Incubation. Experiments were carried out in 25 ml. conical flasks in a shaker bath at 37°. Incubation mixtures consisted of 4 ml. of 0·1 M-phosphate buffer (KH_2PO_4, 13·6 g./l., K_2HPO_4, 17·4 g./l.) pH 7·3, 1 ml. of microsomal preparation, 1 ml. of normal human serum or 1 ml. of 10% bovine serum albumin, and 100 μl. of ^{125}I-labelled PTH, about $1·5 \times 10^6$ counts/min., of specific radioactivity as indicated in each experiment. Control flasks contained 1 ml. of 0·25 M-sucrose in place of the microsomal protein. The serum or bovine serum albumin was added to prevent the ^{125}I-labelled PTH adhering to glass, which it does avidly at this pH. Zero time was taken as that at which labelled hormone was added to the incubation medium. Small equal samples were taken from each flask at zero time and at other times as specified in different experiments. When ^{125}I-labelled PTH was to be measured as radioactivity remaining at the origin after chromatoelectrophoresis, samples were taken from the flasks in Pasteur pipettes, and frozen immediately in small glass tubes placed in liquid N_2. These were thawed in the cold-room immediately before chromatoelectrophoresis. When trichloroacetic acid-precipitable radioactivity was to be measured, 200 μl. samples were pipetted from the flasks directly into 1·8 ml. of 10% trichloroacetic acid.

Gel filtration. Gel filtration was carried out on polyacrylamide, P6 (Bio-Gel), in 0·1 N-acetic acid. Column size was 1 cm. × 30 cm., flow rate 2 ml./hr.; fractions were collected in tubes in a refrigerated fraction-collector (Paton Industries, Beaumont, S. Austral., Australia). Effluent protein was measured as E at 280 mμ in a Unicam SP.500 spectrophotometer. Radioactivity of column fractions was measured by counting 100 μl. samples in a Nuclear–Chicago automatic well scintillation counter.

RESULTS

The rate of destruction of ^{125}I-labelled PTH was rapid (Fig. 1), almost 50% of the labelled hormone being destroyed in 10 min. Increasing the substrate concentration by adding increasing amounts of labelled hormone led to a progressive increase in reaction velocity (Fig. 2). The maximum velocity of the reaction was not reached in these experiments.

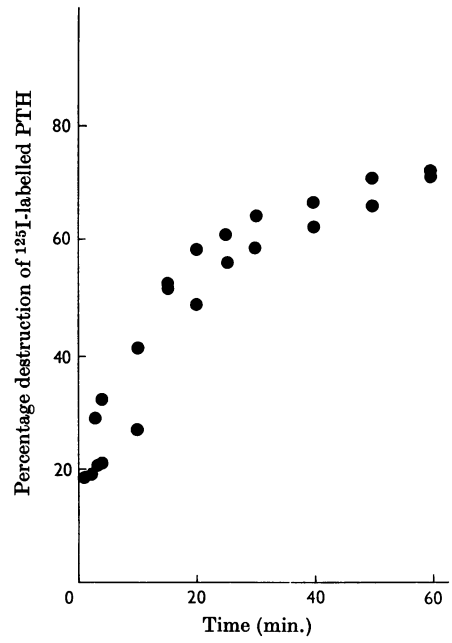

Fig. 1. Percentage destruction of ^{125}I-labelled PTH. In each of two flasks ^{125}I-labelled PTH ($1·5 \times 10^6$ counts/min., specific radioactivity 180 mc/mg.) was incubated with 1 ml. of human serum, 4 ml. of 0·1 M-phosphate buffer and 1 ml. of microsomes (700 μg. of protein) in 0·25 M-sucrose. Small equal samples were taken from each flask at short time-intervals, and frozen in liquid N_2. After thawing at 4°, 100 μl. samples were subjected to chromatoelectrophoresis. Intact ^{125}I-labelled PTH was measured as the radioactivity remaining at the origin.

The result of an examination of the specificity of the microsomal degradation of ^{125}I-labelled PTH is given in Fig. 3. A large excess of trichloroacetic acid-precipitated PTH almost completely inhibited the destruction of labelled PTH. A similar concentration of trichloroacetic acid-precipitated calcitonin caused considerably less inhibition and crystalline pig insulin had very little effect on the rate of the reaction.

A comparison was made of the effects of microsomal and mitochondrial fractions from liver and kidney on pig ^{125}I-labelled insulin and PTH. The results in Table 1 show that after incubation for 15 min. there was virtually no effect of either kidney fraction on the ^{125}I-labelled insulin, whereas considerable destruction of ^{125}I-labelled PTH was caused by the kidney microsomal fraction and somewhat less by the mitochondrial fraction. The liver fractions had no effect on ^{125}I-labelled PTH.

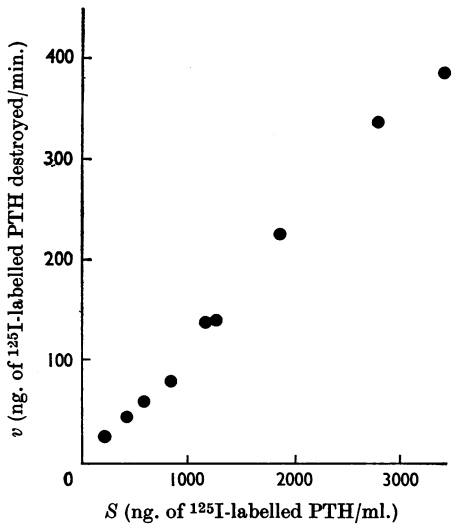

Fig. 2. Effect of increasing concentration (S) of ^{125}I-labelled PTH on the rate of destruction (v) of ^{125}I-labelled PTH. Incubation flasks contained 0·5ml. of 10% bovine serum albumin, 2ml. of 0·1M-phosphate buffer and 0·25ml. of microsomes (250μg. of protein) in 0·25M-sucrose in addition to the labelled hormone, of specific radioactivity 36mc/mg. Samples (200μl.) were taken from each flask at 0 and 15min., and intact ^{125}I-labelled PTH was measured by precipitation with 10% trichloroacetic acid. As the total ^{125}I-labelled PTH content of each flask was known, the rate of destruction/min. was calculated from the observed percentage destruction at 15min.

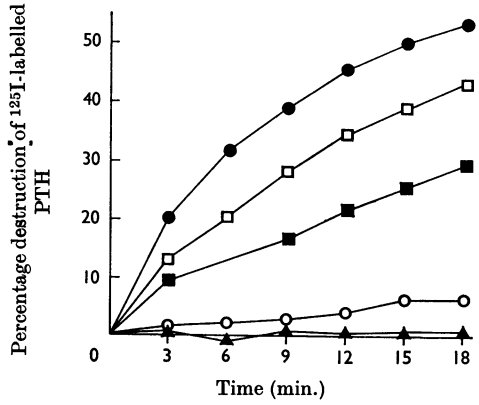

Fig. 3. Effects of added pig insulin, trichloroacetic acid-precipitated calcitonin and trichloroacetic acid-precipitated PTH on the destruction of ^{125}I-labelled PTH by rat kidney microsomes. All flasks contained 0·5ml. of 10% bovine serum albumin, 1·7ml. of 0·1M-phosphate buffer and $1{\cdot}5 \times 10^6$counts/min. of ^{125}I-labelled PTH. Other contents are represented as follows: ●, 250μg. of microsomal protein in 0·25ml. of 0·25M-sucrose, and 0·3ml. of 0·1N-acetic acid; □, 250μg. of microsomal protein in 0·25ml. of 0·25M-sucrose, and 11·1mg. of pig insulin in 0·3ml. of 0·1N-acetic acid; ■, 250μg. of microsomal protein in 0·25ml. of 0·25M-sucrose, and 10·9mg. of trichloroacetic acid-precipitated calcitonin in 0·3ml. of 0·1N-acetic acid; ○, 250 μg. of microsomal protein in 0·25ml. of 0·25M-sucrose, and 11·0mg. of trichloroacetic acid-precipitated PTH in 0·3ml. of 0·1N-acetic acid; ▲, 0·25ml. of 0·25M-sucrose and 0·3ml. of 0·1N-acetic acid. Intact ^{125}I-labelled PTH was measured by precipitation with 10% trichloroacetic acid.

Earlier experiments (Vajda *et al.* 1969) have shown the lack of effect of the rat kidney microsomal fraction on ^{125}I-labelled human growth hormone and the very slight effect on ^{131}I-labelled insulin.

The process was slowed considerably by acid conditions, and an optimum pH in the physiological range was apparent (Table 2). After dialysis at 4° for 48hr. against four changes of 0·25M-sucrose the microsomal fraction retained its capacity to destroy ^{125}I-labelled PTH (Table 3).

Gel filtration on P6 (Bio-Gel) in 0·1N-acetic acid was used to study the products of kidney microsomal degradation of ^{125}I-labelled PTH. Fig. 4 shows the elution profile after chromatography of 0·4ml. of control medium consisting of ^{125}I-labelled PTH in buffer, bovine serum albumin and sucrose, incubated for 45min. There was one radioactive peak indicating the emergence of labelled PTH (mol.wt. 8600) and albumin at the void volume of the column. The same preparation after incubation for 45min. in the microsomal preparation yielded four peaks (Fig. 5). With longer incubation there was a decline in peak I (^{125}I-labelled PTH) and an increase in the lower-molecular-weight fractions. In particular, peak IV increased with prolonged incubation. The results of trichloroacetic acid precipitation of equal samples from the different peaks are shown in Table 4. When the pooled freeze-dried column

Table 1. *Effects of kidney and liver mitochondria and microsomes on ^{125}I-labelled parathyroid hormone and ^{125}I-labelled insulin*

Incubation flasks contained 1ml. of human serum. Intact ^{125}I-labelled PTH and ^{125}I-labelled insulin were measured by precipitation with trichloroacetic acid. Specific radioactivities: ^{125}I-labelled PTH, 65mc/mg.; ^{125}I-labelled insulin, 180mc/mg.

Incubation system	Destruction of hormone (% in 15min.) ^{125}I-labelled PTH	^{125}I-labelled insulin
Control	0·6	−1·2
Kidney microsomes	42·4	4·4
Kidney mitochondria	27·0	4·0
Liver microsomes	0·1	0·8
Liver mitochondria	6·6	20·7

Table 2. *Effect of* pH *of incubation on the destruction of* ^{125}I*-labelled parathyroid hormone by rat kidney microsomal preparation*

Medium contained 10% bovine serum albumin. Intact ^{125}I-labelled PTH was measured by precipitation with trichloroacetic acid. Specific radioactivity of ^{125}I-labelled PTH was 220 mc/mg.

Buffer	pH	Destruction of ^{125}I-labelled PTH (% in 15 min.) Control	Microsomes
Sørensen's citrate	2·4	1·7	1·5
Sørensen's citrate	3·2	−2·5	5·6
Sørensen's citrate	4·2	−0·3	12·1
Sørensen's phosphate	5·3	1·8	47·3
Sørensen's phosphate	6·3	2·6	60·1
Sørensen's phosphate	7·3	2·0	59·7
Sørensen's phosphate	8·2	−0·9	50·8
Sørensen's glycine	9·3	0·2	37·1

Table 3. *Effect of dialysis on microsomal activity*

Microsomal suspension was dialysed in Visking tubing (25/32) against four changes of 0·25 M-sucrose at 4° for 48 hr. Flasks 2 and 3 contained equal quantities of microsomal protein. Medium contained 10% bovine serum albumin; intact ^{125}I-labelled PTH was measured by precipitation with trichloroacetic acid. Specific radioactivity of ^{125}I-labelled PTH was 240 mc/mg.

Flask	Destruction of ^{125}I-labelled PTH (% in 40 min.)
1. Control	−1·5
2. Non-dialysed microsomes	77·4
3. Dialysed microsomes	77·1

fractions were examined by chromatoelectrophoresis, only peak I was found to contain material that remained at the origin. With the control flask, all radioactivity remained at the origin. Incubation with the microsomal preparation gave a front peak of radioactivity, most of which remained at the origin. However, there was a small mobile fraction in this peak on chromatoelectrophoresis, and not all the radioactivity in this peak was precipitated by trichloroacetic acid (Table 2). The remaining three peaks contained no material remaining at the origin, and it should be noted that even in the fraction of lowest molecular weight, peak IV, there was no evidence for the presence of free iodide on chromatoelectrophoresis.

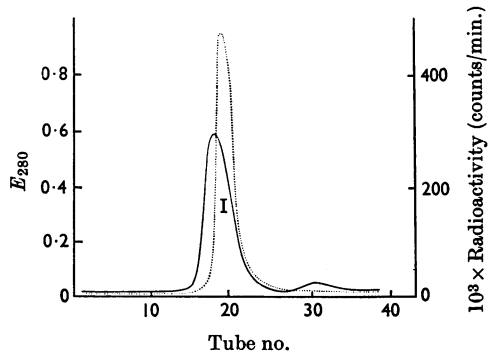

Fig. 4. Gel-filtration chromatography of 1 ml. of control medium on a column (30 cm. × 1 cm.) of polyacrylamide P6 (Bio-Gel) in 0·1 N-acetic acid. Fractions (30 drops) were collected at a flow rate of 8 ml./hr., examined spectrophotometrically at 280 mμ, and 100 μl. samples were counted in a Nuclear–Chicago automatic well scintillation counter. ^{125}I-labelled PTH (10^7 counts/min., specific radioactivity 210 mc/mg.) had been incubated for 45 min. with 0·5 ml. of 10% bovine serum albumin, 2 ml. of 0·1 M-phosphate buffer and 0·25 ml. of 0·25 M-sucrose. —, E_{280}; ····, counts/min.

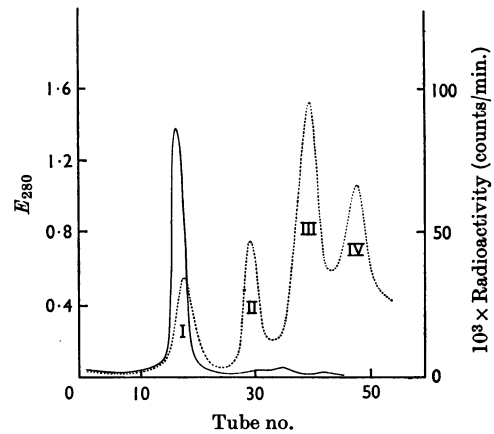

Fig. 5. Gel-filtration chromatography of 1 ml. of medium after incubation of ^{125}I-labelled PTH with microsomes. Gel-filtration conditions were as described in Fig. 4. ^{125}I-labelled PTH (10^7 counts/min., specific radioactivity 180 mc/mg.) had been incubated for 45 min. with 0·5 ml. of 10% bovine serum albumin, 2 ml. of 0·1 M-phosphate buffer and 0·25 ml. of microsomes (280 μg. of protein) in 0·25 M-sucrose. —, E_{280}; ····, counts/min.

DISCUSSION

The results of these experiments show that the microsomal fraction of rat kidney contains a non-diffusible factor that rapidly degrades ^{125}I-labelled PTH to a number of peptide fragments. The reaction is favoured by pH in the physiological range, and the reaction velocity is increased by increasing the substrate concentration. These findings provide further evidence that the micro-

Table 4. *Trichloroacetic acid solubility of P6 (Bio-Gel) column fractions*

The trichloroacetic acid solubility of the ^{125}I-labelled peptides was studied by examining a number of tubes from individual peaks after gel filtration (see Figs. 4 and 5). Samples (200μl.) from fraction-collector tubes were added to 200μl. of human serum, precipitated with 3·6ml. of 10% trichloroacetic acid and washed once. Radioactivity of the precipitates was expressed as a percentage of the total counts in the 200μl. samples. Means ± S.E.M. of four tubes from each column peak are given.

Column	Peak	Radioactivity precipitated by trichloroacetic acid (% of total)
Control flask (Fig. 4)	I	95·2±0·34
Microsome flask (Fig. 5)	I	76·1±0·66
	II	0·8±0·20
	III	1·0±0·38
	IV	0·5±0·16

somal factor is a proteolytic enzyme. This was suggested after the demonstration that the process was dependent on temperature and on the quantity of microsomal protein, and that the activity was destroyed by prior heating at 60° for 30min. (Vajda *et al.* 1969).

There was competitive inhibition of ^{125}I-labelled PTH degradation when an excess of trichloroacetic acid-precipitated PTH was included in the incubation medium, although we have no evidence of the relative susceptibility of the native and labelled hormones to the enzyme. Narahara & Williams (1957) found that the addition of labelled glucagon was slightly more effective than unlabelled glucagon in suppressing degradation of the ^{125}I-labelled hormone, suggesting that the manipulations involved in iodination caused slight changes in the glucagon that rendered it a better substrate for proteolysis. In our experiments some competitive inhibition occurred with an excess of trichloroacetic acid-precipitated calcitonin also, and very little was seen with insulin. Competition between insulin and glucagon for a liver enzyme has been claimed (Tomizawa & Williams, 1955), and between insulin and oxytocin for a renal cortical enzyme (Camu & Conard, 1966). The possibility of competition between PTH and calcitonin for the kidney microsomal enzyme awaits study with purer hormone preparations. Although there is evidence that the kidney causes insulin degradation (Mirsky & Broh-Kahn, 1949), assessed at 75% of the liver activity (Williams, Hay & Tjaden, 1959), nevertheless kidney microsomes had little effect on ^{125}I-labelled insulin. Therefore insulin must be degraded by a kidney mechanism separate from the one we have studied for PTH.

The formation of a number of ^{125}I-labelled fractions after incubation of ^{125}I-labelled PTH with the microsomal preparation suggests that the enzyme acts at more than one location in the parathyroid hormone molecule, since the latter contains only one tyrosine residue available for iodination (Potts, Aurbach & Sherwood, 1966). The results of gel filtration on P6 (Bio-Gel) indicated that at least three smaller fragments of the parathyroid hormone molecule were formed. Although ^{125}I-labelled PTH emerged at the void volume, remained at the origin on chromatoelectrophoresis and was fully precipitated by trichloroacetic acid, the radioactivity in peak I after incubation of ^{125}I-labelled PTH with microsomes was partly mobile on chromatoelectrophoresis and not fully precipitated by trichloroacetic acid. The explanation could be that a fragment of the molecule had been formed of molecular weight greater than 4600, the exclusion limit of P6 (Bio-Gel). If this were so, however, it is likely that the fragment would be precipitable by trichloroacetic acid. A more probable explanation is that proteolytic digestion continued albeit slowly even in 0·1N-acetic acid, since the enzyme would also emerge at the void volume of the column. When pooled fractions of peak I were freeze-dried and reconstituted in plasma for chromatoelectrophoresis the mobile component became more prominent. Freeze-drying of the column peak from a control incubation, on the other hand, did not give rise to any change in the chromatoelectrophoretic pattern.

Studies of the metabolism of peptide hormones have revealed tissue enzymes concerned with the proteolytic breakdown of the hormone molecules (Mirsky & Broh-Kahn, 1949). The liver insulinase (Mirsky, Perisutti & Dixon, 1954) was found to be considerably more active than the kidney enzyme in degrading insulin (Williams *et al.* 1959). Vajda *et al.* (1969) found kidney most active in destroying ^{125}I-labelled PTH, with much less activity in skeletal muscle, still less in liver, lung and spleen. The earliest experiments of Mirsky's group (Mirsky & Broh-Kahn, 1949; Mirsky & Perisutti, 1953) were done by using a biological assay for insulin. Their findings were confirmed fully when they used ^{131}I-labelled insulin. Most of the work on tissue destruction of peptide hormones since then has been achieved with labelled hormones. The present experiments confirm the results obtained by Orimo *et al.* (1965), with biological assay, and indicate the presence in rat kidney of a proteolytic enzyme that degrades parathyroid hormone *in vitro* in amounts far in excess of the estimated secretion rate of the hormone *in vivo* (Melick, Aurbach & Potts, 1965). This provides further evidence of the importance of the kidney in parathyroid hormone metabolism.

We are grateful to Professor V. M. Trikojus for helpful advice in the preparation of the manuscript, to Dr J. T. Potts, jun., and Dr G. D. Aurbach for supplying pure bovine parathyroid hormone, and to Dr K. D. Muirden for carrying out electron microscopy of the tissue fractions. This investigation was supported by a grant from the National Health and Medical Research Council of Australia.

REFERENCES

Aurbach, G. D. (1959). *J. biol. Chem.* **234**, 3179.

Aurbach, G. D. & Potts, J. T., jun. (1964). *Endocrinology*, **75**, 290.

Camu, F. & Conard, V. (1966). *Arch. int. Pharmacodyn.* **162**, 247.

Hunter, W. M. & Greenwood, F. C. (1962). *Nature, Lond.*, **194**, 495.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Martin, T. J. & Melick, R. A. (1967). *Med. J. Aust.* **2**, 519 (abstr.).

Melick, R. A., Aurbach, G. D. & Potts, J. T., jun. (1965). *Endocrinology*, **77**, 198.

Mirsky, I. A. & Broh-Kahn, R. H. (1949). *Arch. Biochem.* **20**, 1.

Mirsky, I. A. & Perisutti, G. (1953). *Endocrinology*, **52**, 698.

Mirsky, I. A., Perisutti, G. & Dixon, F. J. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 228.

Narahara, H. T. & Williams, R. H. (1957). *Endocrinology*, **60**, 285.

Orimo, H., Fujita, T., Morii, H. & Nakao, K. (1965). *Endocrinology*, **76**, 255.

Potts, J. T., jun., Aurbach, G. D. & Sherwood, L. M. (1966). *Recent Progr. Hormone Res.* **22**, 101.

Tomizawa, H. H. & Williams, R. H. (1955). *J. biol. Chem.* **217**, 685.

Vajda, F. J. E., Martin, T. J. & Melick, R. A. (1969). *Endocrinology*, **84** (in the Press).

Williams, R. H., Hay, J. S. & Tjaden, M. B. (1959). *Ann. N.Y. Acad. Sci.* **74**, 513.

Yalow, R. S. & Berson, S. A. (1960). *J. clin. Invest.* **39**, 1157.

Yalow, R. S. & Berson, S. A. (1966). *Nature, Lond.*, **212**, 357.