Metabolism of Parathyroid Hormone

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

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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, 3.68 mg./ml.) inhibited destruction of labelled hormone. There was much less inhibition with added trichloroacetic-acid-precipitated calcitonin (3.92 mg./ml.) and virtually none with added pig insulin (3.80 mg./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 $^{125}$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 30 min.

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 200 units/mg.) was prepared in this Laboratory by the method of Aurbach (1969).

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 0.25 M sucrose at 4°. After spinning at 700 g for 10 min. in the small rotor of a Sorvall model RC2 centrifuge, the supernatant fraction was centrifuged at 5000 g for 15 min. The supernatant from the mitochondrial fraction was then centrifuged for 1 hr. in a Beckman model L ultracentrifuge at 54000 g in the 30 rotor. After decantation the sedimented microsomes were suspended in 10 ml. of 0.25 M sucrose and centrifuged for 30 min. in the 50 rotor at 145000 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 0.25 M 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 Quo G 32 (Philadelphia Quartz Co.) and by elution with 20% (v/v) acetic acid (Yalow & Berson, 1966).

Chromato-electrophoresis. The technique of chromatooelectrophoresis (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 radio-
chromatogram scanner. The same chromatoelectrophoretic
technique was used to measure intact $^{125}$I-labelled PTH
after incubation. Samples (100 $\mu$L) from flasks were applied
to paper (Whatman no. 3 MC) at 4°. After chromato-
electrophoresis (0-06 M-veronal buffer, pH 8-6; 700 V) for
90 min. the strips were dried and scanned. The origin and
first $\frac{1}{4}$ 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 $\mu$L) 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 (KH₂PO₄, 13.6 g./
l., K₂HPO₄, 17.4 g./l.) pH 7-3, 1 ml of microsomal prepara-
tion, 1 ml of normal human serum or 1 ml of 10% bovine
serum albumin, and 100 $\mu$L 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
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 N₂. These were thawed in the
cold-room immediately before chromatoelectrophoresis.
When trichloroacetic acid-precipitable radioactivity was to
be measured, 200 $\mu$L samples were pipetted from the flasks
directly into 1-8 ml of 10% trichloroacetic acid.

Gel filtration. Gel filtration was carried out on poly-
crylamide, P6 (Bio-Gel), in 0-1% acetic acid. Column size
was 1 cm. x 30 cm.; flow rate 2 ml/hr.; fractions were
collected in tubes in a refrigerated fraction-collector (Paton
protein was measured as E at 280 m$\mu$L in a Unicam SP.500
spectrophotometer. Radioactivity of column fractions was
measured by counting 100 $\mu$L 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.

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 calci-
tonin 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 micro-
somal 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.
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 $^{125}$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 $4^\circ$ for 48 hr. against four changes of 0.25 M sucrose the microsomal fraction retained its capacity to destroy $^{125}$I-labelled PTH (Table 3).

Gel filtration on P6 (Bio-Gel) in 0.1 N 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 0.4 ml. of control medium consisting of $^{125}$I-labelled PTH in buffer, bovine serum albumin and sucrose, incubated for 45 min. 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 45 min. 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

![Graph](image-url)
Table 2. Effect of pH of incubation on the destruction of 125I-labelled parathyroid hormone by rat kidney microsomal preparation

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

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Control</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorensen's citrate</td>
<td>2.4</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Sorensen's citrate</td>
<td>3.2</td>
<td>-2.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Sorensen's phosphate</td>
<td>4.2</td>
<td>-0.3</td>
<td>12.1</td>
</tr>
<tr>
<td>Sorensen's phosphate</td>
<td>5.3</td>
<td>1.8</td>
<td>47.3</td>
</tr>
<tr>
<td>Sorensen's phosphate</td>
<td>6.3</td>
<td>2.6</td>
<td>60.1</td>
</tr>
<tr>
<td>Sorensen's phosphate</td>
<td>7.3</td>
<td>2.0</td>
<td>59.7</td>
</tr>
<tr>
<td>Sorensen's phosphate</td>
<td>8.2</td>
<td>-0.9</td>
<td>50.8</td>
</tr>
<tr>
<td>Sorensen's glycine</td>
<td>9.3</td>
<td>0.2</td>
<td>37.1</td>
</tr>
</tbody>
</table>

Destruction of 125I-labelled PTH (% in 15 min.)

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°C for 48 hr. Flasks 2 and 3 contained equal quantities of microsomal protein. Medium contained 10% bovine serum albumin; intact 125I-labelled PTH was measured by precipitation with trichloroacetic acid. Specific radioactivity of 125I-labelled PTH was 240 mc/mg.

Destruction of 125I-labelled PTH (% in 40 min.)

<table>
<thead>
<tr>
<th>Flask</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>-1.5</td>
</tr>
<tr>
<td>2. Non-dialysed</td>
<td>77.4</td>
</tr>
<tr>
<td>3. Dialysed microsomes</td>
<td>77.1</td>
</tr>
</tbody>
</table>

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.

DISCUSSION

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

<table>
<thead>
<tr>
<th>Column</th>
<th>Peak</th>
<th>(% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control flask</td>
<td>I</td>
<td>95.2 ± 0.34</td>
</tr>
<tr>
<td>(Fig. 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsome flask</td>
<td>I</td>
<td>76.1 ± 0.66</td>
</tr>
<tr>
<td>(Fig. 5)</td>
<td>II</td>
<td>0.6 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1.0 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.5 ± 0.16</td>
</tr>
</tbody>
</table>

The formation of a number of 125I-labelled fractions after incubation of 125I-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 125I-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 125I-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.

Serum and plasma, which contain 125I-labelled PTH, can be subjected to proteolysis with trypsin, chymotrypsin, and other proteolytic enzymes. There is evidence that trypsin and chymotrypsin can split the hormone into smaller fragments (Williams & Periutti, 1954). 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.

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REFERENCES
