A DETAILED STUDY OF THE URINARY PEPTIDES IN A PATIENT WITH OSTEOMALACIA AND HYPERPARATHYROIDISM

ROSE CAHILL, R. SMITH* AND R. G. WESTALL

Medical Unit, University College Hospital Medical School, London

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SUMMARY

1. The urinary peptides and amino acids have been examined in a 28-year-old female with osteomalacia and severe parathyroid bone disease. Forty ninhydrin positive substances were identified.

2. These substances included nine hydroxyproline containing peptides of relatively simple composition. Of these eight contained 4-hydroxyproline, two contained cis-3-hydroxyproline and one had trans-3-hydroxyproline. Two larger peptides which contained a high proportion of 4-hydroxyproline residues were also found. Free 4-hydroxyproline, free hydroxylysine and a hydroxylysine complex were found.

3. Of the remaining twenty-six substances, which may not be derived from collagen, alaninol is reported as an urinary constituent for the first time, six are less common but have been reported previously whilst the remaining nineteen are well-known urinary constituents.

4. The significance of these findings in relation to collagen breakdown is discussed.

The urinary excretion of total hydroxyproline (THP), i.e. the free hydroxyproline plus that released by acid hydrolysis, is regarded as a reliable indicator of changes in collagen metabolism and particularly the metabolism of bone collagen (Sjoerdsma et al., 1965; Prockop & Kivirikko, 1967). The THP excretion has been measured in many disorders, and is particularly increased where bone resorption is increased, as in Paget’s disease (Krane, Munoz & Harris, 1967) and in hyperparathyroidism with bone disease (Smith, 1967). It is logical to try to establish the composition of the various hydroxyproline containing peptides, but few attempts have been made. Previous work, much of which was preliminary, and which includes the detailed study of Westall (1955), on 100 litres of normal urine, is reviewed by Smiley & Ziff (1964). In 1967 Anderson et al. presented data which suggested that the nature of the hydroxyproline containing peptides excreted in osteomalacia did not differ from normal. Nevertheless

*Present address: Nuffield Orthopaedic Centre, Headington, Oxford.
Correspondence: Dr R. G. Westall, St. Lawrence’s Hospital, Caterham, Surrey.
a detailed investigation of the chemical nature of the urinary peptides derived from collagen in a patient with increased THP excretion has not been previously reported. The present communication describes such an investigation in a patient who had osteomalacia and extensive bone disease predominantly due to parathyroid overactivity. The THP excretion is increased in some patients with osteomalacia, an increase which is roughly proportional to the degree of parathyroid overactivity (Smith & Dick, 1968b, c); a temporary rise in THP excretion may occur when vitamin D is given (Smith & Dick, 1968a). In the patient to be described the large increase in THP excretion at the time of study may be considered to be due predominantly to an increase in bone collagen resorption (Avioli & Prockop, 1967; Prockop & Kivirikko, 1967; Smith, 1969), due to extensive parathyroid bone disease. We have characterized at least twelve collagen derived substances in the urine and consider that our results contribute to a better understanding of collagen breakdown in vivo.

PATIENT

The clinical and biochemical findings in this patient have been reported by Davies, Dent & Watson (1968, case 158, and Fig. 3), and by Smith & Dick (1968b, case 10.D.S.). She was born in 1937 and was admitted to the metabolic ward at University College Hospital under the care of Professor C. E. Dent in 1965, with osteomalacia due to gluten sensitive enteropathy. She had a severe proximal muscle weakness. Radiological examination and a bone biopsy showed gross parathyroid bone disease and osteomalacia. At this time her daily THP excretion varied considerably, with a mean value of 800 mg, the highest value being found shortly after starting treatment with vitamin D$_2$ which was given for 6 weeks only. About 25 litres of urine were collected at this stage, but after many months work the results and isolated specimens were destroyed in a fire. The investigation described here was done on 38 litres of urine collected nearly 2 years later. At this time (May–June 1966) the patient had improved clinically and was on a gluten-free diet which was also low in gelatin (Sjoerdsma et al., 1965). However, her parathyroid bone disease had progressed and her ionized serum calcium concentration was 6.4/100 ml. She was considered to have tertiary hyperparathyroidism (Davies et al., 1968). A parathyroid adenoma was subsequently removed (Mr D. R. Davies) and her THP excretion fell to normal.

EXPERIMENT PROCEDURE AND RESULTS

These are described together. The plan of separation of the amino acids and peptides (Fig. 1) is described briefly.

Initial fractionation

Thirty-eight litres of urine were passed through two consecutive columns containing Zeo Karb cation exchange resin 225, 8% crosslinked, 100–200 mesh, in the H$^+$ form. The first column was 75 cm long and 7.5 cm wide and the second column, connected in series, was 45 cm long and 3.8 cm wide. The amino acids and peptides which were retained by the cation exchange resin were displaced by passing 0.2 M ammonia through the columns until the ammonia solution emerged at full strength at the outlet of the second column. No attempt was made to fractionate the eluate at this stage. The eluate which was collected as soon as a positive
FIG. 1. Scheme for the separation of urinary amino acids and peptides. For explanation see text.
ninhydrin colour reaction was obtained, was reduced in volume to about 1 litre by distillation under reduced pressure at 40° to remove the free ammonia. The concentrate was then acidified to pH3 with HCl and the distillation repeated to remove CO2. The concentrate was then diluted to 5 litres with distilled water and passed through two consecutive columns (61 cm long by 5.0 cm wide and the second 45 cm long by 3.8 cm wide) containing Dowex 2 anion exchange resin in the hydroxyl form. This was an 8% cross-linked resin which had a particle size between 100 and 200 mesh. The amino acids and peptides which were retained by the resin were displaced by applying 0.1 M HCl to the columns. As soon as the eluate gave a positive ninhydrin colour reaction, fractions (15 ml) were collected and the collection continued until unchanged 0.1 M HCl emerged at the column outlet. 240 fractions were obtained (A fractions, Fig. 1).

The 240 fractions were stored frozen. Each sample was examined, before and after hydrolysis (5.5 M HCl, 16 hr at 105° in a sealed capillary tube) by one-way paper chromatography using (i) phenol-NH3 and (ii) lutidine-water as solvent systems, the substance being revealed with ninhydrin reagent. In addition, two-way chromatography using the same solvents and automatic amino acid analysis (Locarte Co. machine, 50 cm column; buffers, 33 ml/hr pH 3.25–150 min, pH 4.25–210 min, pH 6.65–240 min, 0.2 N NaOH, 150 min) were carried out on certain selected fractions. By this means an overall picture of the fractionation was obtained. The positions of the well-known constituents and areas which were rich in peptide material could be selected for further study. Fig. 2 summarizes the positions of the various substances found and includes the peptides which were characterized after procedures to be described later. On the basis of this information the primary fractions (A fractions, Fig. 1) were further investigated in groups, according to the following general scheme. The positions of unknown substances were plotted by their position on the chart of the automatic amino acid analyser. If the peak position was unchanged after acid hydrolysis then the substance was assumed to be a free amino acid; however, if the peak disappeared and fresh peaks appeared then the substance was considered to be a peptide or a conjugated amino acid. At the same time an attempt was made to correlate the substance giving a peak on the analyser chart (Table 1) with a position on the two-way paper chromatogram (Fig. 3). Further fractionations, using ion-exchange columns of smaller size, were also carried out in an endeavour to secure enriched fractions of the unknown substances.

**Analysis of fractions**

**Group I** (Fractions A 10–26). These pooled fractions were re-refractionated on a small column of Amberlite cation exchange resin C.G. 120 in the H+ form. The retained amino acids were displaced with 0.1 M NH3 and ninety-five fractions (B fractions) were collected. These fractions contained a number of well-known constituents including lysine, 5-hydroxylysine, ornithine, proline, 4-hydroxyproline, glycine, alanine, β-alanine, β-aminoisobutyric acid and carnosine. In addition there were several unknown substances. Thus, fraction B 17 produced a well-defined peak on the amino acid analyser (A.A.A.) chart 115 min after the start, in a position between serine and glutamic acid. This substance was unchanged by acid hydrolysis and was subsequently found to be sarcosine. Fractions B 60–69 contained another unknown compound which gave a peak on the A.A.A. chart at 380 min between tyrosine and histidine. This substance was obtained pure, in small quantity, by elution with water from samples run on high voltage paper electrophoretograms using pyridine acetate buffer at pH 5.4 (5 kv, 60 mA, 50 cm long, 30 min run, Locarte Co. machine). After acid hydrolysis it yielded 5-hydroxylysine only.
This substance was termed 'hydroxylysine complex'. A third unknown substance was detected in fractions B 70–85. This substance was stable to acid hydrolysis and differed only slightly from ethanolamine in its position on the A.A.A. chart and in its behaviour on paper chromatograms. We suspected that it might be a propanolamine. Test runs with the three propanolamine isomers (2-amino-propan-1-ol; 3-amino-propan-1-ol; 3-amino-propan-2-ol) showed that the unknown substance behaved identically with 2-amino-propan-1-ol (alaninol). It gave a peak on the A.A.A. chart just after the ammonia peak.

![Diagram showing the order of displacement of urinary ampholytes from a column of Dowex 2×8% resin.](image)

**Group II** (Fractions A 27–107). These fractions contained mainly glycine and alanine. There were traces of unknown substances but these could not be further concentrated by fractionations.

**Group III** (Fractions A 108–116). These pooled fractions were refractionated on a small column of the cation exchange resin Zeo Karb 225. Thirty-one (5 ml) fractions were obtained (B fractions, Fig. 1). These contained glycine, alanine, valine, leucine, isoleucine and 1-N-methylhistidine. Fractions 18–22 contained an unknown substance which gave a brown coloured spot with ninhydrin on paper chromatograms and gave a peak on the A.A.A. chart at 243 min just after valine. Acid hydrolysis and end group analysis by dansylation (Gray, 1967) proved this substance to be prolyl-4-hydroxyproline.
Group IV (Fractions A 150-163). These fractions contained a relatively large amount of a substance which gave a peak in the leucine position on the A.A.A. chart. On paper chromatograms it reacted with ninhydrin to give a bright yellow colour which slowly changed to purple within 30 min at room temperature. The pooled fractions were refractionated on an Amberlite C.G. 120 column and fifty-three (5 ml) fractions were collected. The unknown substance (Y1) was mainly concentrated in fractions B 6-27 but there was still contamination with asparagine, histidine, valine and leucine. A further refractionation on a column of the anion exchanger Dowex 2 gave a few fractions (C fractions) with the unknown in chromatographically pure form. Acid hydrolysis and end group analysis by dansylation showed it to be glycyl-proline. This was confirmed by running it as a mixture with a synthetic sample of glycyl-proline on the A.A.A. where it emerged as a single peak at 289 min (almost exactly in the leucine position). Two other unknown substances (Y2 and P) were present in the B fractions 30-40. They could be readily observed on paper chromatograms where Y2 gave a yellow spot with ninhydrin which slowly changed to purple, but at a much slower rate than glycyl-proline. P gave an immediate purple colour. P was separated by cutting out and elution from a paper chromatographic strip after running in lutidine. Acid hydrolysis and end group analysis by dansylation showed the substance to be alanyl-4-hydroxyproline. It gave a peak on the A.A.A. chart at 216 min between alanine and valine. The substance Y2, subsequently shown to be glycyl-4-hydroxyproline, was not as easily separable from these fractions as from those of Group V.

### Table 1. Time of emergence of the common amino acids and of the isolated peptides on the automatic amino acid analyser

<table>
<thead>
<tr>
<th>Substance</th>
<th>Minutes</th>
<th>Substance</th>
<th>Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>29</td>
<td>α-Aminoacidic acid</td>
<td>191</td>
</tr>
<tr>
<td>trans-3-Hydroxyproline</td>
<td>63</td>
<td>(Glu2-cis-3-Hyp) (b)</td>
<td>193</td>
</tr>
<tr>
<td>β-Aspartylglycine</td>
<td>70</td>
<td>Ala-4-Hyp</td>
<td>216</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>75</td>
<td>Cystine</td>
<td>231</td>
</tr>
<tr>
<td>β-Hydroxyasparagine</td>
<td>80</td>
<td>Valine</td>
<td>238</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>83</td>
<td>Pro-4-Hyp</td>
<td>243</td>
</tr>
<tr>
<td>Threonine</td>
<td>93</td>
<td>Methionine</td>
<td>261</td>
</tr>
<tr>
<td>cis-3-Hydroxyproline</td>
<td>93</td>
<td>Leu-4-Hyp</td>
<td>269</td>
</tr>
<tr>
<td>Serine</td>
<td>100</td>
<td>Isoleucine</td>
<td>276</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>115</td>
<td>Leucine</td>
<td>288</td>
</tr>
<tr>
<td>(Glu2-cis-3-Hyp) (a)</td>
<td>129</td>
<td>Gly-Pro</td>
<td>289</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>141</td>
<td>Tyrosine</td>
<td>335</td>
</tr>
<tr>
<td>Proline</td>
<td>148</td>
<td>Phenylalanine</td>
<td>352</td>
</tr>
<tr>
<td>Gly-4-Hyp-trans-3-Hyp</td>
<td>151</td>
<td>Hydroxylysine complex</td>
<td>380</td>
</tr>
<tr>
<td>Glycine</td>
<td>173</td>
<td>Hydroxylysine</td>
<td>469</td>
</tr>
<tr>
<td>α-Aspartylglycine</td>
<td>178</td>
<td>Histidine</td>
<td>486</td>
</tr>
<tr>
<td>Gly-4-Hyp</td>
<td>178</td>
<td>Lysine</td>
<td>505</td>
</tr>
<tr>
<td>(Gly2-Glu2-Asp-Ala-Pro-Hyp4)</td>
<td>172-178</td>
<td>Ammonia</td>
<td>522</td>
</tr>
<tr>
<td>Ser-4-Hyp</td>
<td>181</td>
<td>Alaninol</td>
<td>528</td>
</tr>
<tr>
<td>(Glu2-4-Hyp)</td>
<td>186</td>
<td>Arginine</td>
<td>676</td>
</tr>
<tr>
<td>Alanine</td>
<td>191</td>
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Group V (Fractions A 164–180). These fractions were pooled and refractionated on a column of Zeo Karb 225 resin in the H⁺ form. After displacement with 0·1 M NH₃ 125 B fractions (Fig. 1) were collected. Fractions 90 onwards contained mainly histidine. Tyrosine and phenylalanine spread over a wide range from fractions 20–90 with smaller amounts of glycyl-proline and alanyl-4-hydroxyproline. Subfractions 15–20 had a relatively high amount of β-hydroxyasparagine. This substance has been found in normal urine (Tomanga et al., 1963). It gives an orange colour with ninhydrin and lies just to the right of serine on our two-way paper chromatograms. The identity of our substance was confirmed by comparison with an authentic specimen (kindly sent to us by Professor Tomanga). On the A.A.A. chart it produced a peak at 80 min, that is just 3 min before aspartic acid. In the first 50 B fractions we detected four more unknown substances. By further fractionation on small ion exchange columns to produce higher concentrations and by paper chromatographic elution techniques we were able to isolate enough to establish their identity. The first two were seryl-4-hydroxyproline and leucyl-4-hydroxyproline. Their structures were proved by acid hydrolysis and end group analysis. The other two substances were glycine peptides. One was glycine-4-hydroxyproline (Y₂ mentioned in Group IV above) and the other was glycyl-4-hydroxyprolyl-trans-3-hydroxyproline. The structures of these are tentative as we did not have enough material to carry out end group analyses, but since they give yellow spots with ninhydrin on paper chromatograms, as do the other proven glycyl peptides, we strongly suspect that glycine is in the primary position having the free amino group. The positions of these peptides on the A.A.A. chart are given in Table 1.
Group VI (Fractions A 181–200). The major amino acid components of this group were tyrosine, phenylalanine, tryptophan, histidine, \( \alpha \)-aminoadipic acid and glutamic acid. There were further traces of the peptides found in the previous group. Glutamyl-4-hydroxyproline and \( \beta \)-aspartyl-glycine were also found (see Group VII).

Group VII (Fractions A 201–240). This group contained the most acidic components of the primary fractionation with aspartic and glutamic acids present in large amounts. It also contained a number of unknown substances which could be demonstrated by high voltage paper electrophoresis (Fig. 4). Some ninhydrin positive material streaked from the start line towards the anode—'streak spot'. There were also two well-defined spots (2 and 3), spots due to glutamic (4) and aspartic (6) acids and a brown coloured spot as well as trace amounts of other ninhydrin reacting substances. The behaviour of 'streak spot' suggested that it might be a large peptide. Hence the pooled fractions were concentrated by distillation under reduced pressure to 250 ml and placed in a sac made of cellophane dialysis tubing (25 mm diameter). Dialysis against repeated changes of distilled water and concentration of the diffusate showed that almost all the ninhydrin positive material was dialysable (Fig. 4). The small amount of material which remained inside the sac did not give a colour with ninhydrin on paper but yielded a wide selection of amino acids after acid hydrolysis (Table 2). The recovered dialysable material was refractionated on a column of Amberlite C.G. 120 resin and 105 fractions (B fractions) were obtained.

Subfractions B 0–10. These were acidic and emerged before aspartic and glutamic acids; they gave only a weak colour with ninhydrin. Fractions 1–6 were mixed and passed through a small column of Sephadex G 10 followed by elution with water. Fifty (1 ml) C fractions were collected. Fractions C 30–40 were mixed and a 1 ml sample was run on the amino acid analyser. Two well-defined peaks were obtained, at 129 min and 193 min respectively. By repeating this procedure but collecting 1 ml samples of the eluate at the time of the emergence of these peaks, hydrolysing the samples and then re-running the hydrolysates on the analyser, we established that each of the two original peaks represented a peptide with a composition glutamic acid-\( \text{cis}-3 \)-hydroxyproline in the ratio of 2:1. Comments on these two peptides will be made later in the Discussion.

Subfractions B 10–38. In addition to aspartic and glutamic acids and a small quantity of
Urinary peptides in osteomalacia

substance 3 (Fig. 4) which we subsequently identified as \(\alpha\)-amino adipic acid, there was also the substance which gave a brown colour with ninhydrin on paper. It was present in highest concentration in fractions 22 and 23. After further fractionation of these two fractions on a small column of Dowex 2 resin in the acetate form we obtained a crystalline product. It produced a peak on the A.A.A. chart at 70 min and gave equimolar yields of aspartic acid and glycine after acid hydrolysis. Dansylation tests established that it was aspartyl-glycine.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues</th>
<th>Amino acid</th>
<th>Residues</th>
</tr>
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<tbody>
<tr>
<td>Aspartic</td>
<td>154</td>
<td>Methionine</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>143</td>
<td>Isoleucine</td>
<td>4</td>
</tr>
<tr>
<td>4-Hydroxyproline</td>
<td>7</td>
<td>Leucine</td>
<td>15</td>
</tr>
<tr>
<td>Threonine</td>
<td>22</td>
<td>Tyrosine</td>
<td>4</td>
</tr>
<tr>
<td>Serine</td>
<td>50</td>
<td>Phenylalanine</td>
<td>4</td>
</tr>
<tr>
<td>Proline</td>
<td>7</td>
<td>Hydroxylysine</td>
<td>7</td>
</tr>
<tr>
<td>Glycine</td>
<td>365</td>
<td>Lysine</td>
<td>55</td>
</tr>
<tr>
<td>Alanine</td>
<td>128</td>
<td>Histidine</td>
<td>9</td>
</tr>
<tr>
<td>Valine</td>
<td>18</td>
<td>Arginine</td>
<td>18</td>
</tr>
</tbody>
</table>

We later identified another aspartyl-glycine which was less acidic and which gave a purple colour with ninhydrin on paper. We therefore concluded that the brown spot was \(\beta\)-aspartyl-glycine.

**Subfractions B 30–70.** Four further unknown substances could be detected in this sub-group. Fractions 49 and 50 had a high concentration of three of these unknowns. Subfractionation of these two combined samples on a column of Dowex 2 in the acetate form produced 200 (1 ml) C fractions. Analysis of these fractions showed that fractions 175–190 contained aspartic acid only, 142–157 contained \(\alpha\)-aspartyl-glycine, 80–100 contained glutamic acid only and 40–60 contained two unknowns admixed at this stage. Fractions 40–60 (after removal of free acetic acid by vacuum distillation) were fractionated on a Sephadex G 10 column by elution with water. 20 \(\times\) 1 ml fractions were collected. Fractions 11–18 contained a substance which gave a peak on the A.A.A. chart at 186 min and which, after acid hydrolysis, produced glutamic acid and 4-hydroxyproline in the proportion of 2:1. The earlier fractions 1–10 contained the substance we have called 'streak spot'. This substance gave rather a broad peak on the A.A.A. chart between glutamic acid and glycine and on acid hydrolysis gave (Gly₂, Glu₂, Asp, Ala, Pro, Hyp₂)n. The remaining unknown peptide could not be purified under the conditions of column refractionation. However, we isolated a chromatographically pure sample by cutting out and elution from paper after running a high voltage paper electrophoretogram (similar to that shown in Fig. 4). This sample gave a peak on the A.A.A. chart between 172 and 178 min and on acid hydrolysis yielded (Gly₂, Glu₂, Asp, Ala, Pro, Hyp₂)n.

**DISCUSSION**

This investigation was undertaken primarily to characterize collagen-derived peptides in the urine. Additionally a number of substances were found which may not be derived from collagen or have any relevance to the patient's disease. These are dealt with first.
Non collagen derived substances

Carnosine (β-alanyl-histidine) and anserine (β-alanyl-1-N-methylhistidine) occur in muscle tissue and in normal urine. Our patient excreted much more carnosine than anserine but this may not be abnormal. Myopathy in osteomalacia is rarely associated with evidence of muscle breakdown (Smith & Stern, 1967, 1969) and in our patient the muscle weakness had been cured before the urine was collected. The excretion of sarcosine (Stein, 1953) was probably normal. β-hydroxyasparagine is of interest since it gives a characteristic colour on paper chromatograms which had led us to call it 'peach spot' for a number of years until it was identified by Tomanga et al. (1963). It must be regarded as a normal urinary constituent but our patient was a heavy excretor of this substance. The presence of α-amino adipic acid, first reported in urine by Stein (1953), was confirmed. It is difficult to say how often this substance occurs in urine since it occupies a position on our paper chromatogram identical to that of the more abundant glutamic acid and produces a peak coincident with alanine on the A.A.A. chart. We found appreciable amounts of the α- and β-aspartyl-glycines. These may be derived partly from diet and partly from endogenous sources in the same way as the β-aspartyl and γ-glutamyl dipeptides described by Buchanan, Haley & Markiw (1962) and Dorer, Haley & Buchanan (1966).

A considerable amount of 'ultra violet light absorbing' and fluorescent material was found in many of the fractions and was recognized when paper chromatograms were examined before spraying with ninhydrin. These occurred although the more common purines such as uric acid, xanthine and hypoxanthine were eliminated in the course of the many fractionations. Several of the substances have been isolated and crystallized and will be investigated later. Finally, amongst those substances which are not clearly derived from collagen there is alaninol. We are not aware that this has been previously recognized in human urine. It may be of little significance; however, alaninol is closely related to 2-aminopropionaldehyde which, together with aspartyl α-semialdehyde, has been isolated from tropocollagen (Blumenfeld & Gallop, 1962).

Collagen derived substances

Amino acids and simple peptides. An abnormally large amount of 5-hydroxylysine, which must arise from collagen, was found in the basic fractions of the original fractionation. There was also a large amount of the 'hydroxylysine complex' which produced one of the large unknown peaks when the untreated urine was run on the A.A.A. This complex did not crystallize from almost pure fractions and on acid hydrolysis produced 5-hydroxylysine as the only ninhydrin positive product. No associated carbohydrate was detected (Cunningham, Ford & Sigrist, 1967). Its identity remains obscure but it is possible that it could be derived from the cross-linking area of the collagen molecule and be related to hydroxylysino norellucine recently isolated from this part of collagen (Bailey & Peach, 1969). There was only a small amount of free 4-hydroxyproline in the urine of this patient. This is unusual since the proportion of THP which is in the free form may be considerably increased in hyperparathyroidism (Kalima et al., 1968).

Large amounts of glycyproline (equivalent to an excretion rate of about 1 g/day) were found and we assume that most of this was derived from collagen. This substance was found in the urine of a child with severe bone disease and hyperphosphatasia (Seakins, 1963). Its structure was proved by Scriver (1964).
Of the simple peptides containing 4-hydroxyproline, prolylhydroxyproline was first found in urine by Westall (1955). It has been found since by Jagenburg (1959), Meilman, Urivetsky & Rapoport (1963) and Kibrick et al. (1964). As far as we are aware the other hydroxyproline peptides that we found are reported as urinary constituents for the first time.

3-Hydroxyproline, a minor constituent of collagen (Eastoe, 1955; Ogle, Arlinghaus & Logan, 1962) was not found in the free state but was found in peptide form. Dr Irreverre kindly sent us authentic samples of cis and trans 3-hydroxyproline which separate well from each other, and from 4-hydroxyproline, on the A.A.A. (Table 1). One of our peptides (Gly-4-Hyp-trans-3-Hyp)n could be derived from collagen which is said to contain the trans isomer. The two peptides with the formula (Glu₂-cis-3-Hyp)n give the same products after hydrolysis but differ appreciably in their isoelectric points. We can only tentatively suggest that perhaps in one case the glutamyl linkage involves the α-carboxyl and in the other the γ-carboxyl group. Previously cis-3-hydroxyproline has only been found in hydrolysates of the antibiotic, telomycin (Meister, 1965).

Larger peptides. We did not expect to find peptides of very large molecular weight since proteins and large peptides are not recovered from the ion exchange resins under the conditions that we used. However, a small amount of high molecular weight material was recovered from the non-dialysable fraction of Group VII. This may be a mixture of similar types and may not be derived from collagen at all, but since it had a high content of glycine and glutamic and aspartic acids we considered that it would be of interest to record its amino acid composition (Table 2). The peptide which gave a compact spot at position 2 on the H.V.E. strip (Fig. 4), and a single peak on the A.A.A. chart, contained thirteen amino acid residues (Gly₂, Glu₄, Asp, Ala, Pro, Hyp₄)n. Streak spot, the other large peptide, appeared to have eleven amino acid residues. However, from its behaviour both on the H.V.E. strip and on the A.A.A., we think it likely that it is not a single substance. Its composition (Gly₄, Glu₂, Asp, Ala, Pro, Hyp₂)n is similar to the peptide at position 2; however, it is not so acidic and has two less glutamic acid residues.

The significance of the collagen derived peptides

In this patient it can be assumed that the peptides containing hydroxyproline and the hydroxylsine are derived predominantly from the collagen of bone matrix. The bone is the main affected tissue (although the skin may not be entirely normal (Dent & Garretts, 1960)). Relative to the amount excreted, the dietary contribution to the urinary hydroxyproline is insignificant but it cannot be ruled out as a factor when considering some of the other peptides that we found. Since the osteomalacia had been cured, the main bone abnormality was due to parathyroid overactivity, which increases bone resorption (Owen & Bingham, 1967; Avioli & Prockop, 1967). In our patient we consider, as in the rhesus monkey (Avioli & Prockop, 1967), that the collagen peptides are derived largely from breakdown of old ‘mature’ insoluble bone collagen, whilst the contribution from the newly formed collagen is not known. How collagen breaks down in vivo is not clear (Evanson, Jeffrey & Krane, 1967) but it is presumably due to the action of specific collagenases, and the structure of the peptides that we have described may give some clue about the method of breakdown. The relative amounts of the peptides will be less useful since they need not bear any relation to the amounts at the bone resorbing surface, mainly because of renal factors (Anderson et al., 1967). Thus the action of collagenase with the specificity of a bacterial collagenase (Bornstein, 1967) would produce particularly glycylproline,
and one with the specificity of tadpole collagenase (Kang et al., 1966; Seifter & Gallop, 1966) could additionally produce leucylhydroxyproline. Bacterial collagenase attacks the non-polar regions of the collagen molecule, which contains the Gly-Pro-X sequence, and appears to leave the polar regions virtually intact (Seifter et al., 1964). Our acidic larger peptides could be derived from such polar regions, and their approximate amino acid composition corresponds better with such parts of the collagen molecule than with the large collagen fragments found in the urine in Paget’s disease (Krane et al., 1967). The significance of the structure of these peptides will only become apparent when the full amino acid sequence of human collagen and the specificity of its collagenases are known.

Since both the laboratory conditions and the clinical disorder differ from one worker to another it is difficult to directly compare results. We have found new peptides but we have been unable to identify others previously reported. Thus, in comparison with the work of Meilman et al. (1963) on normal subjects and patients with connective tissue diseases, we have not encountered the diketopiperazine of prolylhydroxyproline; we have not found the two peptides (Mechanic et al., 1962) reported in rheumatoid arthritis (Hyp$_4$, Pro$_4$, Glu) and Hyp-(Hyp-Pro$_3$-Glu$_3$). We found no associated carbohydrate or glycoprotein, in contrast to others (Bourrillon & Vernay, 1966) and we did not confirm the cystine containing peptides reported by Anderson et al. (1967). Furthermore the pattern of peptide excretion is very different from that described by Goodman et al. (1968) in a patient with a disorder resembling lathyrism.

Finally, although we have every confidence in the identification of the substances that we have described, we realize that these identifications must be regarded as tentative until such time as amounts become available to carry out elemental analyses in the classical way.

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A more detailed description of the experimental work forms part of a thesis to be submitted to the University of London by Rose Cahill, in partial fulfilment of the requirements for the Ph.D. degree.

REFERENCES


Urinary peptides in osteomalacia


