PEPTIDURIA IN AN UNUSUAL BONE DISORDER

ISOLATION OF TWO PEPTIDES

BY

J. W. T. SEAKINS

*From the Department of Chemical Pathology, The Hospital for Sick Children,
Great Ormond Street, London*

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In his investigations on the amino acids and ampholytes in normal urine, Westall (1955) found some 30 ninhydrin positive peptides which were excreted in amounts of the order of 5 mg. or less per day. He suggested that these peptides might either arise from dietary or endogenous protein degradation, or represent specific fragments required for protein synthesis, or physiologically active substances, and that they might occur in larger amounts in pathological urines and in tissue fluids. Jagenburg (1959) also found small amounts of peptides in the urine of normal adults and children.

Peptidurias have subsequently been reported in cerebromacular degeneration (Bessman and Baldwin, 1962), in a patient with rheumatoid arthritis (Mechanic, Skupp, Safier and Kibrick, 1960) and in familial ataxia with skin lesions (Pelc and Vis, 1960). In the last, the patients excreted a peptide containing proline and hydroxyproline.

This paper describes the isolation and characterization of two peptides from the urine of a child in whom the principal findings were a generalized osteopathy, massive new bone formation, and a grossly raised serum alkaline phosphatase. The clinical features of this patient have already been described (Marshall, 1962), and a preliminary report on the isolation of the peptides has been given (Seakins, 1962). Clinically similar cases have been described by Swoboda (1958) and Choremis, Yannakos, Papadatos and Baroutsou (1958). In the former there was a mild aminoaciduria and in the latter the amino acid pattern was stated to be normal. Stransky, Mabilangen and Lara (1962) also describe a case which shows many similarities with this patient. Unfortunately, their patient died before biochemical studies were complete.

Experimental

Paper Chromatography. Routine paper chromatography of urinary amino acids was performed on 25.5 cm.

square sheets of Whatman no. 54 paper using as solvents *n*-butanol-acetic acid-water (120 : 30 : 50 v/v) followed by *p*-cresol-0.3% aqueous ammonia (100 : 14 v/v) in an atmosphere of ammonia. The chromatograms were dried at 45° C. and subsequently dipped in 0.1% ninhydrin in acetone containing 0.01% ascorbic acid, and either steamed immediately after dipping, or the colours allowed to develop overnight at room temperature, or dipped in isatin reagent (Atfield and Morris, 1961). Under the first conditions proline and hydroxyproline give orange colours with red fluorescence, instead of the usual yellow colours. Confirmatory chromatograms were run in *n*-butanol (70 ml.)—pyridine (60 ml.)—water (50 ml.)—diethylamine (2 ml.) followed by phenol—water (4 : 1) in an atmosphere of ammonia on Whatman no. 1. A volume of urine containing 0.1 mg. creatinine was chromatographed.

Chromatography of phenylthiohydantoin derivatives was carried out on Whatman no. 1 paper (25.5 × 25.5 cm.) using *n*-heptane-pyridine (7 : 3 v/v) as solvent. After steaming to remove residual pyridine, chromatograms were viewed under ultraviolet light, and then sprayed with a mixture of equal volumes of 1% aqueous starch glycollate containing 3% sodium azide and 0.5% iodine in 8% potassium iodide solution. Phenylthiohydantoin derivatives are revealed as white spots on a brown background.

Peptides, etc., were hydrolysed in 6*N*-HCl in sealed tubes overnight at 100° C.

Isolation of the Peptides. The procedure of Westall (1952) has been modified as follows: The urine (1,260 ml. representing four days' output, collected while the child was receiving norethandrolone) was made 1% with respect to acetic acid (pH 4.6) and shaken with acetic acid deactivated charcoal (5 g. per litre). The clear pale yellow filtrate and washings were combined, and slowly passed down a column of Zeo-Karb 225 (SRC 10, H⁺). The amino acids and other ampholytes were displaced from the washed resin with 5*N*-NH₃. The filtrate was concentrated to one-tenth of the original volume (pH 8.4) and three volumes of ethanol added, and kept at 0° C. overnight. The precipitate was separated by centrifugation and washed with 75%

aqueous ethanol. Partial evaporation of the ethanolic supernatant solutions gave a pale yellow viscous liquid which after dilution (2 litres) was subjected to displacement chromatography using 0.2N-NH₃ on a series of coupled columns of Zeo-Karb 225 (SRC 10, H⁺) (23 × 2.5 cm., 14 × 2.0 cm., 11 × 1.0 cm., 8 × 0.75 cm.). Fractions (100 drops, nominally 8 ml.) were collected and samples (10 µl.) analysed by one- and two-dimensional paper chromatography. Appropriate fractions were combined to give three main fractions, namely a fore-run (16 ml.) containing glycine, serine, alanine and glutamine, together with traces of other amino acids and peptides; a 'peptide fraction' (104 ml.) containing 'Y', yellow pigment, creatinine and creatine, and traces of other amino acids; a basic fraction (30 ml.) containing lysine, arginine, histidines and basic peptide(s).

The peptide fraction was passed down a column of De-Acidite FF (SRA 67, acetate) which removed some of the yellow pigment and acidic peptides. The effluent and washings were taken to dryness (4.74 g.) and dissolved in 0.2M-ammonium formate buffer pH 3.5 (100 ml.) to which formic acid (10 ml.) was added. The solution was subjected to elution chromatography (Hirs, Moore and Stein, 1952) on a column (90 × 5 cm.) of Zeo-Karb 225 (SRC 8) at pH 3.5 using 0.2M-ammonium formate (pH 3.5) as eluting buffer. The flow rate was 300 ml. per hour. Fractions (nominally 10 ml.) were collected and appropriate ones (after desalting on Zeo-Karb 225 (H⁺)) were examined by one- and two-dimensional paper chromatography. The major components of the fractions 3.03-3.75 litres and 4.01-4.66 litres were respectively the peptides (Pro, Hypro), Y₁ and (Gly, Pro), Y₂, and each was free from the other peptide. (The intermediate fraction 3.75-4.01 litres contained both peptides.) These two fractions were desalted on Zeo-Karb 225 (H⁺) (0.95 and 1.16 g. respectively). From the first fraction a quantity (250 mg.) of crystalline material was isolated which was ninhydrin negative and appeared to be resistant to acid and alkaline hydrolysis. Further purification of both fractions was effected by one-dimensional chromatography on Whatman 3 MM paper (25.5 cm. square, 40 mg. per sheet) using *n*-butanol-acetic acid-water as solvent. The peptides were isolated pure as shown by paper chromatography in 70% yield. These figures indicate an output of 200 mg. of Y₂ and 100 mg. Y₁ per day, which is in reasonable agreement with the values previously obtained by visual comparison (Seakins, 1962).

Characterization of the Peptides. Hydrolysis of Y₁ gave proline and hydroxyproline, and Y₂ glycine and proline. The peptides Y₁ and Y₂ were then subjected to Edman degradation as follows, which is modified from Smith (1960): The peptide (0.05 mmole) was dissolved in a mixture of water (2 ml.), pyridine (2 ml.) and dioxan (2 ml.) and adjusted at 40° C. to pH 9 with 0.05N-NaOH. Phenylisothiocyanate (0.2 ml.) was added, and the pH maintained by the addition of further quantities of sodium hydroxide. After one hour the reaction mixture was cooled, and pyridine and excess reagent were extracted with benzene (7 × 6 ml.). The aqueous layer was taken

to dryness and dissolved in 2N-HCl in acetic acid (5 ml.). The solution was kept at 30° C. for 1 hour by which time cyclization of the phenylthiocarbamyl derivative to the amino acid phenylthiohydantoin (PTH) was complete. The solution was freeze-dried and the residue dissolved in water (4 ml.). The phenylthiohydantoin derivative was extracted into ethyl acetate (3 × 4 ml.), and subsequently examined by paper chromatography. The aqueous layer was also examined for amino compounds before repeating the Edman degradation.

Peptide Y₁ gave proline as the *N*-terminal amino acid; hydroxyproline only was recovered from the aqueous layer. Repetition of the Edman degradation gave the PTH of hydroxyproline only. Peptide Y₁ is therefore prolylhydroxyproline. Similarly, peptide Y₂ gave glycine as the *N*-terminal amino acid, and proline as the *C*-terminal amino acid. Peptide Y₂ is therefore glycyproline.

Chromatographic Properties. Both authentic glycyproline and Y₂ gave yellow changing to mauve reactions with ninhydrin, and weak yellow water-soluble colorations with isatin. Y₁ gave a weak mauve reaction with ninhydrin after steaming which did not fluoresce in ultraviolet light, and a strong blue reaction with isatin.

In the following solvent systems proline, authentic glycyproline, Y₁ and Y₂ had the same R_F values: *n*-butanol-acetic acid-water (R_F, 0.38); *p*-cresol-NH₃ (R_F 0.80); *n*-butanol-pyridine-water-diethylamine (R_F, 0.45); phenol-NH₃ (R_F 0.85) and in *n*-butanol-ethyl acetate-acetic acid-water (distance travelled by spots 33 cm.). In the solvent system *tert*-pentanol-water-diethylamine (50 : 50 : 2 v/v, upper layer) after a 90-hour run Y₁ and Y₂ had R_{proline} 0.67, and the distance travelled by proline was 9 cm. But in the solvent system phenol-*n*-butanol-acetic acid water (100 : 50 : 20 : 45 v/v) some separation was achieved, proline (R_F, 0.86); Y₁ (R_F, 0.77); Y₁ and glycyproline (R_F, 0.72); hydroxyproline (R_F, 0.59). High voltage electrophoresis at pH 2 separated proline from glycyproline which ran with Y₂.

The amino acids in a specimen of the child's urine (10 ml.) were subjected to simple elution chromatography (Hirs *et al.*, 1952) on a column (50 × 1 cm.) of Zeo-Karb 225 (SRC 11) at pH 3.6. Fractions (5 ml.) were collected and examined by paper chromatography. The chromatographic behaviour of a synthetic mixture of amino acids (hydroxyproline, proline, glycine, valine, leucine, glycyproline and phenylalanine) was similarly studied. Y₂ emerged at the same position as glycyproline, i.e. after leucine and before phenylalanine; Y₁ with valine.

Examination of Urines. Amino acid chromatography of the urine of the patient showed the presence of a spot (Y) which had the same chromatographic parameters as proline, gave a yellow changing to mauve reaction with ninhydrin and a blue isatin reaction (Fig. 1). A number of ninhydrin positive spots which disappeared on hydrolysis were also present (Fig. 2). The excretion of glycine was also raised. The urine was examined weekly while the child

Fig. 1.—Amino acid chromatogram of 0.1 ml. urine de-salted by ion-exchange resin. Solvent (1) *n*-butanol-acetic acid-water. Solvent (2) cresol-ammonia. The area encircled by a broken line is a slow reacting peptide.

was receiving cortisone (average 50 mg./day for 90 days), oxymetholone (5 mg./day for 30 days), norethandrolone (average 15 mg./day for 80 days), vitamin D (average 200,000 units/day for 60 days). During the last treatment the alkaline phosphatase fell from 600 to 110 King-Armstrong units/100 ml. of serum. This amino acid pattern remained constant from the age of 2 years when the child was first examined until his death at the age of 4 years. The serum amino acid pattern was normal.

Following parturition, the uterus undergoes considerable involution, and rapid katabolism of uterine collagen occurs (Woessner, 1962). The urinary amino acid patterns given by a pregnant woman (8 months) and 48 hours after delivery were normal and no isatin positive spots were detected.

The following also were studied: two cases of Marfan's syndrome, in which sometimes an increased excretion of bound hydroxyproline is reported (Prockop and Sjoerdsma, 1961); two mild cases of Paget's disease which is also reported to give an increased excretion of bound hydroxyproline (Dull, Causing and Henneman, 1962); one case of pseudoxanthoma elasticum; two cases

of atypical rickets with high alkaline phosphatase (c. 100 King-Armstrong units per 100 ml. serum). All were normal. One severe case of Paget's disease, however, showed the presence of small amounts of both peptides (Y_1 and Y_2).

Discussion

As the patient's serum amino acid pattern was normal, it is probable that the two peptides, glycylproline and prolylhydroxyproline partially inhibited the tubular reabsorption of glycine. It has been reported (Scriver, Schafer and Efron, 1961) that infusion of glycine or proline or hydroxyproline increases the clearances of the other two amino acids, and furthermore the two peptides (Y_1 and Y_2) and proline have similar chromatographic properties.

Westall (1955) examined 100 litres of normal urine, and reported the presence of a peptide containing proline and hydroxyproline. No peptide with the properties of glycylproline was described.

FIG. 2.—The same as Fig. 1, but after hydrolysis.

Jagenburg (1959) also reported the presence in normal urine of a peptide which contained proline and hydroxyproline, and another which was very similar to, but not identical with, glycylproline. An examination of their data makes it likely that the peptide prolylhydroxyproline (Y_1) found in the patient's urine is identical with the above two peptides and probably with that described by Pelc and Vis (1960).

The collagens of skin and bone are chemically very similar, but differ considerably in amino acid composition from the other mammalian proteins. Glycine accounts for one-third of the amino acid residues, while alanine, proline and hydroxyproline each account for 10% of the remaining residues in collagen (Bowes, Elliot and Moss, 1955; Eastoe, 1956). Grassmann, Nordwig and Hörmann (1961) have shown that the three sequences: -Gly-Pro-Gly-, -Gly-Pro-Ala- and -Gly-Pro-Hypro-, between them, account for 94% of the proline and 74% of the

glycine of collagen. Ogle, Arlinghaus and Logan (1961) have further demonstrated that 23% of the hydroxyproline of collagen is present in the last peptide. Each peptide contains the sequence -Gly-Pro-.

Gerber and Altman (1961) have suggested that collagen synthesis proceeds by the addition of preformed peptides to existing collagen chains. Although this hypothesis has recently been criticized (Prockop, 1962), it is suggested that on the basis of the above facts, the peptide, glycylproline, is an important intermediate in the synthesis of (bone) collagen. Failure or partial failure to incorporate this peptide into the developing collagen chain would result in the rapid excretion of the unused peptide and the production of an abnormal collagen. Of the collagens, which as a class of proteins are almost metabolically inert (Neuberger, Perrone and Slack, 1951), bone collagen undergoes the most rapid turnover (Neuberger and Slack, 1953; Gerber,

Gerber and Altman, 1960). Collagen is remarkably resistant to attack by the usual proteolytic enzymes, with the exception of collagenase, but is readily converted into gelatin which is not resistant to enzymic attack by processes involving the minimal cleavage of peptide bonds (Steven and Tristram, 1962). Small changes in structure would greatly increase the susceptibility of collagen to enzymic degradation. It is suggested that in this patient there was a considerable increase in the turnover of his bone collagen, some fragments of which escaped complete degradation to amino acids and appeared in the urine. This rapid turnover of collagen is reflected in the raised excretion of bound hydroxyproline (Prockop and Sjoerdsma, 1961) in particular, the excretion of prolylhydroxyproline, a minor constituent of normal urine, and the grossly raised level of serum alkaline phosphatase. It is also possible that the process of calcification might be disturbed (White, Handler, Smith and Stetten, 1959). Such a mechanism would explain the failure of the various treatments to alter the course of the disease.

Several problems remain unanswered. If synthesis of bone collagen is abnormal, why is the skin, for example, apparently not so affected? Further investigations to identify other abnormal urinary constituents and to study the fate of intravenously administered ^{14}C -L-proline, and the comparative chemistry of bone specimens are in hand.

Summary

The isolation and characterization of two peptides in the urine of a child suffering from a generalized osteopathy are described. They are glycylproline and prolylhydroxyproline. The relation between these peptides and the disease process has been examined. It is tentatively suggested that this process represents an error of collagen synthesis.

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