Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 109 MARCH 1962 No. 3

SECTION MEETINGS

CLEVELAND, OHIO	
University Hospitals	December 18, 1961 January 15, 1962
DISTRICT OF COLUMBIA	
Georgetown University Med. School	February 1, 1962
ILLINOIS	
Furniture Club of America	November 28, 1961
University of Illinois Medical School	January 23, 1962
IOWA	
State University of Iowa	January 30, 1962
MINNESOTA	
University of Minnesota	January 5, 1962

Hydroxyproline Peptides of Urine in Arthritic Patients and Controls on a Collagen-Free Diet.* (27240)

ANDRE C. KIBRICK,† CAROLE Q. HASHIRO AND LENORE B. SAFIER
Biochemistry Section, Laboratory Service, New York Veterans Administration Hospital, and Department of Pathology, New York University Medical Center, New York

The excretion of the amino acid, hydroxyproline, in the urine during a collagen-free diet has been utilized to determine differences in the degradation of endogenous collagen by patients with so-called collagen diseases(1). Although the amount of total hydroxyproline excreted in many of these diseases did not differ significantly from that excreted in normal subjects, Udenfriend found increased total urinary hydroxyproline in the Marfan syndrome(2). Since this amino acid is excreted almost entirely in the bound form, a clue to differences in the endogenous metabolism of collagen in other clinical conditions may be found in the types of peptide of hydroxyproline appearing in the urine. As previously described(3), 2 such peptides were isolated from a patient with rheumatoid arthritis, one consisting of 2 hydroxyproline, 3 proline and 9 glutamic acid residues, and the other of 4 hydroxyproline, 4 proline and one glutamic acid residue. The absence of either glycine or alanine from these peptides argues against their direct derivation from collagen.

We report here results of further studies of urinary hydroxyproline peptides obtained on

* This investigation was supported in part by a research grant from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S.

† Present address: Institute for Muscle Disease, New York.

a collagen-free diet in 3 other patients with rheumatoid arthritis and in 2 control subjects. Part of this study consists of attempts to isolate the peptides for characterization as in previous work. Since this proved difficult, other methods were substituted by which several peptides could be separated more readily in small amounts so that at least the amino acid composition could be determined. A peptide containing only proline and hydroxyproline has been found in urine of all 3 patients with arthritis, was absent in one of the controls, and was found in smaller amount in the other control. A peptide with this composition was also found by Westall in 100 liters of urine from a normal subject(4). This may point to a difference in collagen breakdown between arthritic patients and controls.

Experimental. The 3 patients studied were males, 35, 60 and 36 years old, who had had rheumatoid arthritis in well-defined form for 2, 18 and 7 years, respectively. The 2 controls were men of 48 and 69 years, one with a brain syndrome and alcoholism, and the other with papillary adenocarcinoma in the lymph nodes. They were placed on the collagen-free diet(1), and the urine was discarded for 4 days. Then 3 separate 24-hour samples were collected and preserved with merthiolate in the cold. As in the previous work(3), total urine for 3 days was desalted on columns of one kilogram of Dowex 50 resin (×8, 200-400 mesh) in the H^+ form. The dried salt-free ampholytes weighed 22.5 to 64.8 g and contained a total of 72.7 to 545 mg of α amino nitrogen (unhydrolyzed) and 11.9 to 64.7 mg of hydroxyproline.

Exploratory ion exchange chromatography similar to that of the earlier work was performed on the desalted control urines in 0.9 × 115 cm columns containing 20 and 25 g of NH_4^+ Dowex 50 resin (×2, 200-400 mesh). The columns were developed with pH 2.0 buffer, pH was increased in 0.5 increments with buffers of ammonium formate and ammonium acetate up to pH 5.0, and elution was continued finally with 1 N ammonium hydroxide. Essentially all of the hydroxyproline was eluted with the initial buffer as in the earlier work. The α amino nitrogen continued to be eluted at higher pH values, but all was removed at pH 4.5. None was detected with the pH 5 buffer and none was detached from the resin with 1 N ammonia. Preparatory ion exchange chromatography was performed only at pH 2.0. As in the earlier work, these columns were 3.5 × 85 cm and contained 125 g of NH_4^+ Dowex 50 (×2, 200-400 mesh). The desalted urine solids (10 to 21 g according to α amino nitrogen content) from patients and controls were dissolved and charged on the columns. The columns were then developed with pH 2.0 formate buffer at a rate of 10 ml per hour, and 5 ml samples collected. Every third or fourth tube was sampled and analyzed for hydroxyproline by the Stegemann method (5) after removal of buffer and hydrolysis with hydrochloric acid. The curves obtained from all urines in the preparatory columns showed a large part of the total hydroxyproline in peak I. This was either a sharp single peak, or a peak consisting of 2 rather sharp components. The tubes of peak I were combined and recharged, after concentration, on smaller columns of 0.9 × 115 cm containing 15 to 20 g of the same NH_4^+ resin. The chromatograms were developed with 0.2 M ammonium formate buffer at pH 2.0 at a rate of 1-2 ml/hour and 1 ml fractions collected. The curves for hydroxyproline obtained from the smaller columns were also composed of one rather sharp peak, either of one or 2 components.

As in the earlier work, fractions from the small columns were pooled, the solvent removed *in vacuo,* and streaked across 7 × 22 inch sheets of Whatman No. 1 filter paper. These were chromatographed in a single direction by descending technic in the first of one of 3 solvent systems. The systems used were: n-butanol-0.2 N T.C.A., isoamyl alcohol-pyridine-water (35:35:30), and n-butanol-acetic acid-water (4:1:5). The peptide bands indicated by the chlorine gas method (6), or in certain instances the Clorox method (7), on ¼ inch strips cut from the sides, were eluted by the wick method and analyzed for hydroxyproline. The hydroxyproline bands were then obtained from several papers and combined for further purification in the other solvent systems. The purified peptides

were sufficient for only one complete analysis for amino acid composition by paper chromatography.

A less time-consuming procedure similar to that of Westall(4) was substituted to determine the content of hydroxyproline peptides in urine. It was found that sufficient salt-free urine solids could be chromatographed in 2 dimensions on washed† 16-inch-square Whatman No. 3 MM filter paper by the ascending technic in the systems, n-butanol-acetic acid-water (4:1:5) and isoamyl alcohol-pyridine-water (35:35:30), to permit detection of the hydroxyproline peptides by a light spraying with ninhydrin followed by the chlorine gas method. After elution of the cut-out spots by the wick method, it was found that a modified Troll-Cannan method for hydroxyproline(8) was possible.

This method was also used after fractionating the urinary solids in small columns containing 7.2 g of NH_4^+ Dowex 50 resin, as in the preparatory experiments. The solids (0.8 to 2.5 g) were charged on the columns, which were then developed overnight with pH 2.0 buffer at a rate of 40 drops per hour. Samples of 20 drops per tube were collected, and 2 tubes, close to the maximum of peak I, were individually processed for removal of buffer. They were then spotted on washed 16-inch-square Whatman No. 3 MM paper and chromatographed, as with the whole urinary solids. In addition to analyzing the eluted peptide spots, after hydrolysis, for hydroxyproline by the modified Troll-Cannan method, their amino acid composition was determined by chromatography on paper in the systems: n-butanol-acetic acid-water and phenol-water. During this work, similar peptides were separated in several urines. It was evident that frequently the peptide #15, which later proved to yield equal amounts of proline and hydroxyproline, and to have the same R_f values of 0.55 in both the n-butanol-acetic acid-water system, and the isoamyl alcohol-pyridine-water system, did not always stain. The presence of peptide #15 was evidenced by the finding of proline and hydroxyproline in this region in spite of absence of staining at times. It appears that this peptide is an unreactive form since neither the amino end group nor the free amino acids can be detected without hydrolysis. This may exist as the diketopiperazine, which forms readily from the dipeptide prolyl-hydroxyproline(9), or either the acetyl or a conjugate with another substituent. We believe that the material that stains is a non-peptide impurity that appears to be present in the latter portion of peak I from the Dowex resin.

It was readily apparent that the proline-hydroxyproline peptide #15 of the 2-dimensional chromatograms of peak I from Dowex was of special interest, since it was found in considerable amount in all of the patients' urine, but was not detected in one of the control urines, and was present in smaller amount in the other. Further study of this peptide was undertaken. It was found that a urea complex, which also stains in the chlorine gas method could be largely eliminated by gel filtration(10). A solution of 9 g of one of the arthritic urine solids (P 2) at pH 6.0 (ammonium acetate) was passed through a column containing 155 g of Sephadex G-25‡, and 1-2 ml fractions were collected. Aliquots of the fractions were analyzed for hydroxyproline and α amino N as usual, and in addition tested by one dimensional paper chromatography for presence of the urea complex. The latter portions of peak II from Sephadex containing the urea complex, were reprocessed twice through the gel until the complex was largely removed. Purified peak II from Sephadex was then chromatographed on NH_4^+ Dowex 50 ($\times$2, 200-400 mesh) with pH 2.0 buffer, as in the preparatory experiments. This yielded a curve for hydroxyproline consisting of a sharp peak I containing 32% of the total hydroxyproline from the Dowex resin, followed by 3 other peaks. Total hydroxyproline content of peak I could be accounted for almost entirely as the proline-hydroxyproline peptide, essentially free of other hydroxyproline contaminants.

Results and discussion. The amino acid

† The sheets were soaked for 15 minutes in a tray of 0.2% Versene at pH 8.5. After drying in air, they were washed for 6 days by continuous elution from troughs of water in a chromatocab.

‡ Sephadex in gel filtration, Pharmacia, Uppsala, Sweden.

composition of the purified peptides isolated in the initial section from the controls, is as follows: 1, HyPro; 2, HyPro, Pro, Asp, Gly, Ser, Ala or Tyr; 3, HyPro, Pro, Asp, Gly, Ala; 4, HyPro, Pro, Asp, Gly, Lys; 5, HyPro, Pro, Gly, Ser, Thr, Ala or Tyr. Little can be concluded from these results. On the other hand, both patient and control urines chromatograph on the Dowex resin quite similarly. The proportion of hydroxyproline in

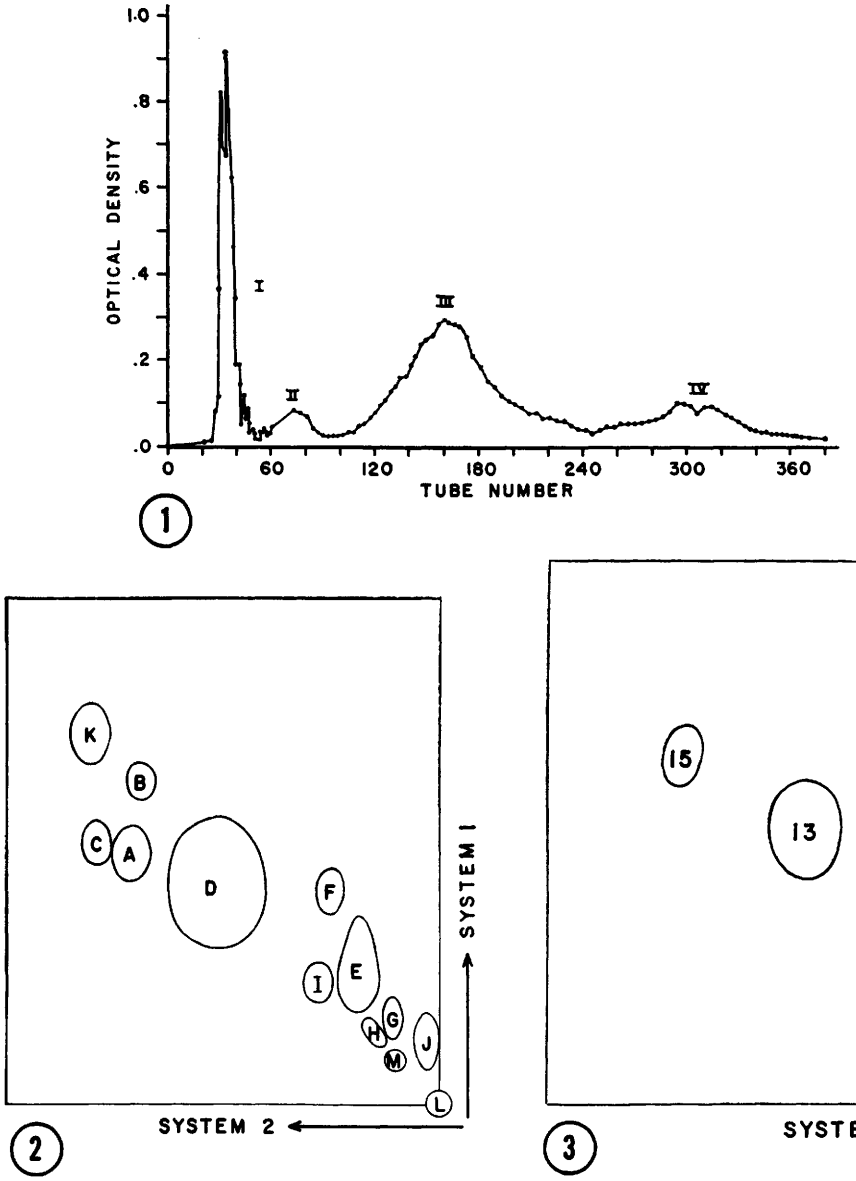

FIG. 1. Preparatory chromatography of desalted urine solids from patient P1 on Dowex 50. Hydroxyproline peaks are shown. Column: 3.5 × 85 cm. Resin: 125 g. Eluant: 0.2M. Ammonium formate, pH 2.0.

FIG. 2. Composite chromatogram of hydroxyproline peptides for whole urine desalted solids in the systems, (1) n-butanol-acetic acid-water (4:1:5), and (2) isoamyl alcohol-pyridine-water (35:35:30).

FIG. 3. Composite chromatogram of hydroxyproline peptides for peak I fractions from Dowex 50 resin in the systems, (1) n-butanol-acetic acid-water (4:1:5), and (2) isoamyl alcohol-pyridine-water (35:35:30).

the various peaks from the preparatory columns is quite similar. There is also general uniformity in the liquid volumes at which the various peaks appear. A representative curve of the chromatograms appears in Fig. 1. Average values of hydroxyproline in the 4 peaks are as follows: Patients: 1) 45.2% 2) 10.7% 3) 27.6% 4) 13.7%; Controls: 1) 34.7% 2) 6.7% 3) 33.8% 4) 20.1%.

Fig. 2 is a composite chromatogram of the hydroxyproline peptides in whole urine solids of all patients, including that of the previous study(3), and control subjects. The peptides appear in the urines examined as follows: P1, A,D,E,F,I,J,K,L,M; P2, A,D, E,I,J,L; P3, A,C,D,E; C1, D,E,F,G,H; C2, A,C,D,E,F,M. The urine of the previous study contained only B. Fig. 3 is a similar composite chromatogram of Peak I from the Dowex columns. Peptide 15 is indicated as a spot although, as mentioned earlier, it is questionable whether the form in which it exists actually stains. Only in the case of peptide 15 is there common agreement in amino acid composition. It may also be significant that this peptide appears in all the patients, while it is present in smaller amount in one of the controls, and entirely absent in the other control urine. Since the former is from a patient with adenocarcinoma, it may be that collagen is broken down in this case because of the well-known tissue destruction of malignant tumors. The peptides of Fig. 3 appear as follows: P1, 15,13; P2, 15,13; P3, 15,13,26,36; C1, 13,3,23,4; C2, 15,26,4. The peptides previously isolated(3) containing proline, hydroxyproline and glutamic acid have not been found in these chromatograms.§

During gel filtration, peak 1 from the Sephadex column starts in the outside volume, Vo., continuing until approximately the maximum in Vo, then extends into the inside volume, Vi. This peak 1 on chromatography on paper does not migrate from the origin in either n-butanol-acetic acid-water nor isoamyl alcohol-pyridine-water. This may indicate that the material contained in peak 1 consists of rather large molecules. On paper chromatography of this peak in the n-butanol-T.C.A. system, 2 spots were obtained, the one remaining at the origin containing all of the hydroxyproline. When the same peak was chromatographed on Dowex 50 resin, hydroxyproline appeared in 2 distinct peaks, indicating that it contains more than one component. Hanson and Fittkau(11) also found relatively large peptides containing hydroxyproline in pooled urine from normal subjects on a regular diet. Peak 2 is all contained in the inside volume, Vi, and includes all of peptide 15. Chromatography of this material of peak 2 on the NH_4^+ Dowex 50 columns yielded an initial peak (peak I from Dowex) which contains roughly 18% of the total hydroxyproline of the original urine solids, of which 90% is peptide 15. Analysis by chemical methods of a small amount of this peptide, obtained by 2 dimensional chromatography on paper from one of the arthritic patients (P3), was found to yield a one-to-one ratio of proline to hydroxyproline. The presence of the proline-hydroxyproline linkage has been demonstrated in collagen (12,13). That this peptide is probably a dipeptide, if it is directly derived from collagen, would be indicated by the conclusions of Grassmann, Nordwig and Hörmann(14). That it comprises such a large portion of the total hydroxyproline of arthritic urine on a collagen-free diet, and that it is absent in one of the control urines and present in smaller amount in the other is of interest for further study.

Summary. The amino acid composition of 5 hydroxyproline peptides isolated from urine of control subjects on a collagen-free diet is reported. There is no evidence of the 2 peptides previously isolated from a patient with rheumatoid arthritis. The chromatographic pattern of hydroxyproline from Dowex 50 ion exchange columns is shown to be quite similar for the 3 arthritic urines and 2 controls on the collagen-free diet. Hydroxyproline peptides separated by 2-dimensional chromatography on paper from such columns have been analyzed for amino acid composition. One peptide containing only proline and hydroxyproline, present in all of the patients,

§ Peptide B of Fig. 2 does not seem to have this composition.

was not detected in one of the 2 control urines. The significance of this finding is discussed, and a procedure is described by which this peptide can be separated essentially free of other hydroxyproline contaminants.

We are indebted to the physicians of the Medical Service for permission to study these patients.

1. Ziff, M., Kibrick, A. C., Dresner, E., Griebetz, H. J., *J. Clin. Invest.*, 1956, v35, 579.

2. Mitoma, C., Smith, T. E., Davidson, J. D., Udenfriend, S., DaCosta, F. M., Sjoerdsma, A., *J. Lab. Clin. Med.*, 1959, v53, 970.

3. Mechanic, G., Skupp, S. J., Safier, L. B., Kibrick, A. C., *Arch. Biochem. Biophys.*, 1960, v86, 71.

4. Westall, R. G., *Biochem. J.*, 1955, v60, 247.

5. Stegemann, H., *Z. physiol. Chem.*, 1958, v311, 41.

6. Rydon, H. N., Smith, P. W. G., *Nature*, 1952, v169, 922.

7. Dutcher, J. D., Pan, S. C., *Anal. Chem.*, 1956, v28, 836.

8. Troll, W., Cannan, R. K., *J. Biol. Chem.*, 1953, v200, 803.

9. Smith, E., *The Chemical Structure of Proteins*, London, 1953, Churchill Co., p194.

10. Porath, J., Flodin, P., *Nature*, 1959, v183, 1657.

11. Hanson, H., Fittkau, S., *Z. physiol. Chem.*, 1958, v313, 152.

12. Kroner, T. D., Tabroff, W., McGarr, J. J., *J. Am. Chem. Soc.*, 1955, v77, 3356.

13. Schrohenloher, R. E., Ogle, J. D., Logan, M .A., *J. Biol. Chem.*, 1959, v234, 58.

14. Grassmann, W., Nordwig, A., Hörmann, H., *Z. physiol. Chem.*, 1961, v323, 48.

Received November 16, 1961. P.S.E.B.M., 1962, v109.

Diffusion of Penicillinase from *Staphylococcus aureus*.* (27241)

LEON D. SABATH AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

There is conflicting evidence as to whether or not penicillinase produced by *Staphylococcus aureus* leaves the cell. Favoring the production of some extracellular enzyme are reports of penicillinase activity in filtrates (1,2) and supernatant fluids(3,4) of broth cultures of staphylococci and its diffusion from colonies of such organisms grown on agar(5). On the other hand, other workers have failed to demonstrate penicillinase in filtrates of staphylococcal cultures passed through Seitz, Mandler, Berkefeld or sintered glass filters(3,6,7) and the presence of bacterial cells in the supernatant fluids of centrifuged cultures was not definitely excluded(2, 7). The evidence from agar diffusion data is likewise inadequate because the findings could also be interpreted as indicating that penicillin diffuses to the staphylococcal cells where it is destroyed.

This report presents more convincing evidence from agar diffusion and filtration experiments that staphylococcal penicillinase does indeed leave the cell.

* Aided by a grant from Nat. Inst. Health.

Agar diffusion. Petri plates, 100 mm in diameter, containing 15 ml of 2% nutrient agar that had been allowed to dry, were marked off into quadrants. A point near the center of each quadrant was touched lightly with the tip of platinum wire that had been dipped into a broth culture of *Staph. aureus* and the plates were incubated at 37°C. The age of the broth cultures varied from 6 hours to 7 days and time of incubation from 12 to 24 hours in different experiments. The resulting colonies with their surrounding and underlying agar were each cut out with a piece of 6-7 mm glass tubing used like a cooky-cutter and aspirated with the aid of a rubber bulb attached to the upper, cotton plugged end. The agar plates with the punched out holes were then incubated at 37°C for another 24 hours to detect any contaminants or any staphylococci that were not removed by this procedure. Plates showing