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Research Article

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3-Hydroxyproline Content of Normal Urine

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ABSTRACT Values for total 3-hydroxyproline and 4-hydroxyproline were obtained from 24-h urine specimens of 18 healthy human subjects of both sexes, whose ages ranged from the first to the sixth decade in age. Urinary 3-hydroxyproline levels, not earlier described to our knowledge, were determined by an isotope-dilution method requiring considerable purification and utilizing the amino acid analyzer for final measurement. 3-Hydroxyproline averaged 3% of the corresponding 4-hydroxyproline in individual urine samples. Like 4-hydroxyproline, 3-hydroxyproline excretion is increased in the second decade, and there is generally good correlation between the two values in individual urines. A hydroxyprolinemic subject excreting greatly elevated 4-hydroxyproline levels did not excrete excessive 3-hydroxyproline, consistent with independent catabolic pathways for the two compounds. 3-Hydroxyproline appears to be selectively excreted relative to 4-hydroxyproline when compared with the probable total body content of each amino acid. Possible explanations are: a more rapid turnover of basement membrane collagen than interstitial collagen or, alternatively, relatively greater resistance to the proteolytic cleavage of peptides containing 3-hydroxyproline.

INTRODUCTION

3-Hydroxyproline was first isolated in 1961 (1) bovine tendon collagen and identified in 1962, from the original source (2) and, in independent studies, from sponge protein (3) and the antibiotic, Telomycin (4). It is now known to occur in several collagen types, notably in basement membrane collagens (5), in which its frequency may be as high as 20–25 residues/1,000 (sources cited in reference 6). Interstitial collagens (types I–III [7]), in contrast, contain no more than 1–2 residues of 3-hydroxyproline/1,000. In the completely sequenced α-1 chain of type I collagen (8), the single residue of 3-hydroxyproline is present in the sequence Gly-3-Hyp-4-Hyp; the same tripeptide sequence appears also to account for all or most of the 3-hydroxyproline in basement membrane collagen of kidney cortex (9).

The literature on urinary 4-hydroxyproline, recently reviewed in reference 10, includes many studies of altered 4-hydroxyproline levels under conditions affecting collagen metabolism. Estimation of 3-hydroxyproline in urine, however, has not yet been reported, although such values might prove to be indicators of altered basement-membrane collagen metabolism. The present paper reports measurements of total urinary 3-hydroxyproline for healthy human subjects of both sexes over a considerable age range. Although the number of determinations has been limited by the demanding nature of the analytical procedures, they provide the first information available on the excretion of 3-hydroxyproline.

METHODS

Specialized and radioactive compounds. Unlabeled *trans*-3-hydroxy-L-proline, *trans*-[3-*hydroxy*-U-^{14}C]L-proline, and *trans*-[3-*hydroxy*-G-^{3}H]L-proline were obtained as previously noted (6). The ^{14}C- and ^{3}H-tracers had sp act = 5.6×10^5 and 1.3×10^7 dpm/μmol, respectively. [4-*hydroxy*-2-^{14}C]D,L-Proline (4×10^7 dpm/μmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and purified by ion-exchange chromatography (amino acid analyzer).

In 16 trials, our amino acid analyzer integration factor (area units of the 440 nm peak/μmol) for standard 3-hydroxyproline averaged 5.1, with an SD = 0.37. The integration factor for 4-hydroxyproline, in contrast, was 3.5±0.71 (16 trials). The 3-hydroxyproline color yield is relatively sensitive to changes in the ninhydrin solution; 3-hydroxyproline standards were run at frequent intervals and always when a new ninhydrin solution was used.

Urine collection. Urine samples were collected from healthy volunteers. The 24-h collection began after the first morning urine and included the first morning urine of the following day. No attempt was made to control the diet or activity of these subjects. Urine was collected without preservatives and was stored at −15°C until use.

Hydrolysis of urine and nitrous acid treatment. 3-Hydroxyproline was measured by an isotope-dilution procedure in which the final step was automatic amino acid analysis. Because of the small quantity of 3-hydroxyproline in urine, extensive purification was necessary to remove chromatographically interfering compounds, in turn requiring the initial

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addition of tracer radioactive 3-hydroxyproline to correct for losses. The urine sample, usually 50 ml, was evaporated to a dry residue (flash evaporator), taken up in 15 ml of 6 N HCl, and hydrolyzed in a sealed tube at 110°C (heating block) for 20–24 h. To the hydrolysis mixture was added tracer 3-hydroxyproline, either the ^{14}C-compound (9×10^4 dpm) or the ^{3}H-compound (9.5×10^5 dpm). Tracers were added after hydrolysis because of the probable loss of ^{3}H during prolonged acid hydrolysis (6) and the earlier indication (6) that endogenous 3- or 4-hydroxyproline is not destroyed during comparable acid hydrolysis of tissues.

The hydrolyzed urine was treated with 4 g of a 2:1 mixture of Dowex-1 × 8 (Cl^- form) and Norit A (Fisher Scientific Co., Pittsburgh, Pa.) as described earlier (11), stirred at room temperature for 15 min and filtered. Half the filtrate, representing 25 ml of the original urine, was further processed, the remainder being reserved as a duplicate if desired. To remove the HCl, the filtrate was taken to dryness several times (flash evaporator) and the dry residue was extracted, first with 5 ml, then 3 ml, of ethanol:water (80:20).

Primary amino acids were removed selectively by nitrous acid deamination (12). The ethanol extracts were combined, dried, and treated with 20 ml of an HNO_2 solution freshly made by addition of 6 ml of 40% $NaNO_2$ to 20 ml of 12 N HCl in ice. After 10 min at room temperature, the urine-HNO_2 mixture was filtered, the filtrate evaporated to a yellow residue, and the residue taken up in 4 ml of 6 N HCl and heated for 90 min at 110°C (heating block). The hydrolysis mixture was evaporated to dryness repeatedly until the pH was 1.5–2.0, the residue was taken up in 5–10 ml of water, and loaded on a Dowex-50 H^+-X8 column (1 × 30 cm). The column was washed with several column volumes of water and eluted with 0.23 N NH_4OH; radioactive 3-hydroxyproline emerged with the first alkaline effluent and the fractions containing significant radioactivity were pooled.

Chromatographic separation and analysis of 3-hydroxyproline. The pooled fractions above were evaporated to dryness, taken up in 1 ml of 0.01 N HCl and applied to a column (0.9 × 58 cm) of amino acid analyzer resin (CD-1, Durrum Instrument Corp, Sunnyvale Calif.). The column was eluted with Durrum's Pico Buffer A' (Buffer A adjusted to pH 2.95 [9]), collecting 1-ml fractions. Radioactive 3-hydroxyproline was generally eluted in several milliliters between fractions 35 and 45. These fractions were pooled, radioactivity determined, adjusted to pH 1.5–2.0, and an aliquot was applied to a small amino acid analyzer column (0.6 × 58 cm) of the same resin. The column was eluted with Buffer A' as noted above, except that the column effluent was not collected but analyzed colorimetrically in the usual way. The 3-hydroxyproline peak usually appeared at about 1 h and was often the only peak seen on the amino acid analyzer record. Under favorable circumstances, this peak permitted a reliable calculation of 3-hydroxyproline content in the aliquot applied, while the radioactivity (usually 10–15% recovery of that initially added) permitted correction for losses on purification. In all urines, the peak seen on the recorder coincided with the radioactive tracer 3-hydroxyproline added. For a number of urines, however, the procedure described above did not eliminate other ninhydrin-reactive components which appeared to coelute with 3-hydroxyproline. Their presence was detected by an altered ratio of absorbance at 440 nm to that at 570 nm. For 3-hydroxyproline standards, this ratio averaged 2.66 with an SD = 0.18 (six determinations), and was independent of sample size, from 50 to 375 nmol. For 3-hydroxyproline derived from some urines, however, the 440/570 nm absorbance ratio was as low as 0.9, indicating the presence of contaminating ninhydrin-reactive compounds with relatively high 570 nm absorbance. When the 440/570 nm absorbance ratio was lower than 2 (see below), one of two alternative procedures was used to further purify the urinary 3-hydroxyproline. In some cases, subjecting the sample to a second treatment with nitrous acid, followed by hydrolysis, sufficed. In other cases, the pooled radioactivity-containing effluent from the first chromatographic separation on Durrum DC-1 resin was desalted by passage through a Dowex 50 H^+-X8 column (2 × 40 cm). After the column was washed with water and the radioactivity was eluted with 0.23 N NH_4OH, pooled radioactive fractions were dried and subjected to high voltage paper electrophoresis (buffer: 90% formic acid/glacial acetic acid/water, 22:82:1,000; pH 1.85; 50 V/cm; 2 h). The radioactivity was located by a strip scanner (Packard Instrument Co. Inc., Downers Grove, Ill.); this portion of the paper was eluted with water and separate aliquots were counted for radioactivity and applied to the amino acid analyzer. This procedure always yielded a 3-hydroxyproline recorder peak with the correct 440/570 nm ratio, permitting calculation of the original urinary content.

Nature of interfering components. No systematic attempts were made to identify the ninhydrin-reactive compounds with occasionally appeared to coelute with 3-hydroxyproline during the final chromatographic steps. They appeared to represent trace components and, from their elution behavior, were not among the usual amino acids of proteins, making possible a large number of structures, derived from the diet or from bacterial action in the intestine, as well as from the pool of normal metabolites. Methionine sulfoxide, a possibility from its elution position, was excluded by treatment of samples with performic acid. Failure of this step to raise the low 440/570 nm ratio of the 3-hydroxyproline peak, or to form the earlier eluted sulfone, argued against the presence of the sulfoxide.

In several electrophoretic runs (both on separate individual urines and on the pooled urine of four adults), the 3-hydroxyproline band was separated from three distinct components, migrating more rapidly toward the anode and seen as purple bands after guide strips were sprayed with ninhydrin. This pattern was similar on all such runs, and in each case the appropriate regions of the paper were eluted with water and chromatographed on the amino acid analyzer. Each area yielded a peak whose 570/440 nm absorbance ratio was typical of a primary amino acid, i.e., a value at least 7. On this basis, an apparent 3-hydroxyproline peak with a 440/570 nm ratio of at least 2 would have its 440 absorbance altered only negligibly by coelution of any or all of these contaminating compounds. For this reason, it appeared safe to evaluate the 440-absorbance peak of 3-hydroxyproline with a 440/570 nm absorbance ratio of at least 2.

Determination of total 4-hydroxyproline in urine. The method was modified from that of Cleary and Saunders (13) which utilizes ion-retardation resin to remove interfering salts. For this measurement, 5 ml of concentrated HCl was added to a 5-ml sample of urine, and the mixture was hydrolyzed under the conditions described for the determination of 3-hydroxyproline. To an aliquot of the filtered hydrolyzed urine, corresponding to 2 ml of the original amount of urine, was added 18,000 dpm of the [^{14}C]4-hydroxyproline tracer and the sample was then treated as described by Cleary and Saunders (13) except for two modifications. One modification involved collecting radioactive fractions from the ion retardation column (rather than collecting an arbitrary elution volume as in the original method) and correcting for incomplete recovery of the tracer. The second modification was the addition to samples of an internal standard to correct for the interference with chromogen formation by other urine components. In our hands, omission

Table I
Total 3-Hydroxyproline and 4-Hydroxyproline in Urine

Subject	Age	Sex	3-Hydroxyproline	4-Hydroxyproline	Ratio 4-Hyp/3-Hyp
			mg/24 h		
J. S.	9	F	2.2	67	30
R. S.	10	M	2.2	48*	22
D. P.	11	F	1.6	36*	23
G. R.	13	F	1.3*	82	63
J. B.	14	M	3.3*	114	35
M. P.	14	M	7.0*	172	25
J. P. S.	15	M	4.5	152	25
M. R.	17	M	2.1	172	82
D. B.	22	M	1.7	51*	30
H. L.	23	F	1.4*	383*	—‡
H. S.	24	M	1.5*	36	24
P. F.	25	F	1.5*	58	39
R. P.	32	F	1.3	28	22
M. L.	37	F	4.8*	40*	8
J. M.	37	M	1.5*	39	26
J. L.	53	F	1.3*	31	24
B. A.	54	F	1.4*	37*	25
E. A.	57	M	1.7*	27	16
Mean±SD			2.4±0.16	70±53§	31±17

* Average of two or more determinations on same urine.
‡ Ratio not calculated: see text.
§ Does not include high value for subject H. L.: see text.

of either modification would have yielded significantly lower values in many samples.

Precision and recovery data for 3-hydroxyproline. A number of 3-hydroxyproline values (Table I) were averaged from two to six determinations. In most cases, agreement was good between two or more samples from the same urine. In one subject (B. A. [Table I]), six determinations gave values of 0.9, 1.2, 1.3, 1.4, 1.5, and 2.2 mg for the 24-h urine, yielding a mean ±SD of 1.4±0.48.

Recovery of added 3-hydroxyproline was also tested with B. A.'s urine (Table I). To 50-ml aliquots of hydrolyzed urine, containing an estimated 0.47 μmol of 3-hydroxyproline, was added 1.2 or 3.0 μmol of 3-hydroxyproline. The quantity of total 3-hydroxyproline determined for the smaller supplement was 88% of that expected, for the larger supplement, 109% of that expected.

Equivalence of ^{3}H and ^{14}C in 3-hydroxyproline tracers. Because of the higher specific activity of [^{3}H]3-hydroxyproline, we preferred this tracer to the ^{14}C-labeled tracer; use of the ^{14}C-tracer required a small correction for the quantity of the tracer itself, usually 10–20% of the total 3-hydroxyproline measured. We had some concern, however, over the possibility of loss of tritium through exchange, particularly during treatment with nitrous acid, since loss of carbon-bound tritium has been observed during the nitrosation of [^{3}H]-dimethylamine.[1]

To examine this possibility, the recovery of radioactivity from [3-*hydroxy*-U-^{14}C]proline and from [3-*hydroxy*-G-^{3}H]proline was measured after adding both tracers in their usual quantities to hydrolyzed samples of urine, which were then carried through the entire procedure up to the final analytical determination of 3-hydroxyproline. In four such trials with 2 different urine samples, recovery of the two isotopes were ^{14}C, 12.2%, ^{3}H, 9.4%; [^{14}C] 13.3%; ^{3}H, 12.4%; ^{14}C, 8.9%, ^{3}H, 8.2%; ^{14}C, 9.1%, ^{3}H, 8.6%. These data appeared to validate the use of the ^{3}H-tracer as equivalent to that of the ^{14}C-tracer in correcting for losses of endogenous 3-hydroxyproline during purification.

RESULTS

Table I summarizes our data on 18 subjects arranged in order of age. The interindividual variation is greatest for 4-hydroxyproline (coefficient of variation = 75), somewhat less for 3-hydroxyproline (coefficient of variation = 67) and least for the ratio of 4-hydroxyproline/3-hydroxyproline for each individual (coefficient of variation = 55), suggesting that the two values are correlated within individuals. This is more clearly seen in Fig. 1 in which, for each individual, the 4-hydroxyproline value is plotted against the 3-hydroxyproline value. These data yield an $r = +0.84$, omitting the aberrant values for M. L. and for H. L., a known high excretor of 4-hydroxyproline (see below). Table I also shows the elevated values for 4-hydroxyproline characteristic of the second-decade age group, in subjects G. R., J. B., M. P., J. P. S. and M. R. This peak in urinary 4-hydroxyproline during

[1] New England Nuclear Corporation, private communication, 1974.

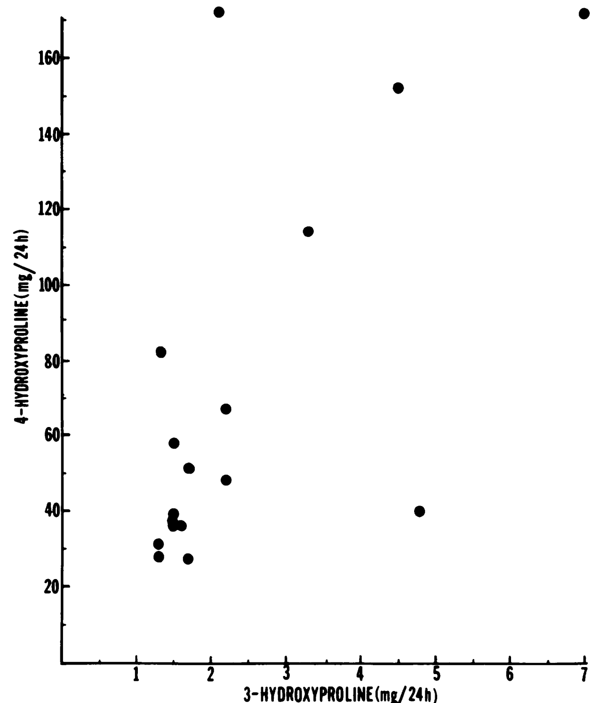

FIGURE 1 The relation between the 24-h excretion of 3-hydroxyproline and 4-hydroxyproline, from the data of Table I. Values for H. L. were not plotted because of the known abnormality in 4-hydroxyproline excretion in this subject.

the mid- to late teens (14) or in the second decade (15) has been reported earlier, although absolute values in the two studies cited are in agreement neither with each other nor with our values for 4-hydroxyproline. It is notable that for three of our five teen-age subjects, the 3-hydroxyproline values are also elevated compared with adults or younger children. To examine the possibility that it is principally data for this age group that determines the high correlation coefficient, the latter value was calculated omitting, in addition to M. L. and H. L. (Table I), the five sets of values for teen-agers, G. R., J. B., M. P., J. P. S., and M. R. (Table I). The correlation still appeared good, with a coefficient of +0.75.

Urinary 3-hydroxyproline in hydroxyprolinemia. Subject H. L. (Table I) has been known since childhood to excrete abnormally large quantities of free 4-hydroxyproline, attributed to a deficiency of 4-hydroxyproline oxidase, the enzyme catalyzing the first step in 4-hydroxyproline catabolism (16). Earlier studies of the excretion and metabolism of 4-hydroxyproline in this subject have been reported in detail (17–19), and our values for free and total urinary 4-hydroxyproline are consistent with earlier measurements. Of particular interest in this subject is the 24-h value for total urinary 3-hydroxyproline (averaged from two independent measurements of 1.3 and 1.5 mg), which indicates no elevation compared with other adults in our series.

DISCUSSION

The data presented on total urinary 3-hydroxyproline in man represent the first report, to our knowledge, of excretion levels of this compound. These values were obtained by an arduous method, each measurement being an isotope-dilution procedure utilizing the amino acid analyzer as the final assay step. The multiple steps of purification required were dictated by the small quantity of 3-hydroxyproline present in urine, the presence of other ninhydrin-reactive compounds in urine that are eluted close to 3-hydroxyproline, and the lack of a relatively specific assay method such as the colorimetric procedure for 4-hydroxyproline (10). With these limitations, any presently available method would appear to require radioactive 3-hydroxyproline as a tracer, since significant purification would be necessary with consequent unpredictable losses of the endogenous compound.

The present method, however, yields reasonably precise values and permits good recovery of added 3-hydroxyproline. The identification of 3-hydroxyproline in urine is based on coincidence of its elution position from the amino acid analyzer column with the authentic tracers, the correct 570/440 nm absorbance of this peak, and the comigration on paper electrophoresis of the urinary compound with the authentic tracer.

One clear conclusion, from the data for H. L. (Table I), is the independence of catabolic pathways for 3-hydroxyproline and 4-hydroxyproline. Subject H. L., who excretes a very large quantity of free 4-hydroxyproline resulting from a blocked first step in 4-hydroxyproline catabolism, does not excrete elevated levels of 3-hydroxyproline. This conclusion is consistent with findings in our laboratory[2] that although free 3-hydroxyproline is rapidly oxidized by the intact rat, it does not appear to be converted to a Δ^1-pyrroline product analogous to the first product of oxidation of 4-hydroxyproline or of proline (16).

Values for total 3-hydroxyproline are not widely variable among individuals, somewhat less so, in fact, than parallel values for 4-hydroxyproline in the same urines. For all urines measured (excluding that of H. L.), 3-hydroxyproline as a fraction of 4-hydroxyproline in the same urine averages 3.2%, the range being 1.2–12.5%. It is of interest to compare this average ratio to the only values available for total body content of 3-hydroxyproline relative to 4-hydroxyproline. The latter data, obtained for mice (6), indicate that total body 3-hydroxyproline is only 1.5% of 4-

[2] Adams, E., and C. Chung. Unpublished data.

hydroxyproline. If this ratio, determined for mice, also holds for man, it would appear that 3-hydroxyproline is selectively excreted relative to 4-hydroxyproline.

Two explanations for selective excretion of 3-hydroxyproline can be considered. One is the possibility that, in aggregate, basement membrane collagen with its much higher ratio of 3-hydroxyproline/4-hydroxyproline, turns over more rapidly than the aggregate of interstitial collagen types. An alternative explanation depends on the postulation, for which evidence exists (9), that 3-hydroxyproline is present in collagen in the unique sequence, Gly-3-Hyp-4-Hyp. If this sequence is relatively resistant to proteolytic breakdown compared with the aggregate of sequences in which 4-hydroxyproline is released, this would also predict selective excretion of 3-hydroxyproline. Thus, small peptides containing 4-hydroxyproline are rapidly excreted in the urine (20, 21), while free 4-hydroxyproline is rapidly metabolized (22). It may be reasonably assumed that 3-hydroxyproline peptides would be handled by the kidney in a manner similar to 4-hydroxyproline peptides, and evidence already cited[2] indicates that free 3-hydroxyproline, like free 4-hydroxyproline, is rapidly metabolized. The relative resistance to proteolytic cleavage of a given peptide containing 3- or 4-hydroxyproline could, therefore, determine the balance between excretion or catabolism. A specific example is the high proportion of total urinary 4-hydroxyproline in the peptide Pro-4-Hyp, attributable by the findings of Weiss and Klein (23) to the resistance of this peptide to peptidase action.

In gel-permeation studies[3] we have found that most of the 3-hydroxyproline in unhydrolyzed human urine is present in a fraction corresponding in size to tripeptides; this fraction coelutes from the amino acid analyzer column with synthetic tritiated Gly-3-Hyp-4-Hyp (9, 24). While the endogenous compound(s) has not yet been unequivocally identified, our earlier studies (9) make the above tripeptide a plausible excretion product accounting for much or most of the urinary 3-hydroxyproline. This would provide a basis for the second explanation (noted above) for the selective excretion of 3-hydroxyproline. Further examination of this possibility will require direct observations on the stability of this tripeptide administered to animals or humans.

A question of physiological and potential clinical interest concerns the source of urinary 3-hydroxyproline. Even if it is present chiefly or exclusively in the specific tripeptide sequence cited, this peptide could arise either from interstitial or basement membrane collagen (9). Whereas basement membrane collagen is relatively rich in 3-hydroxyproline, the small fraction of this collagen compartment (1–2% of total collagen based on mouse data (6)) means that a high relative turnover rate would be required for its major contribution to the urinary 3-hydroxyproline pool. As an example, we can assume that basement membrane collagen in aggregate contains 20 residues/1,000 of 3-hydroxyproline and represents 1% of the total collagen; whereas interstitial collagen, representing 99% of collagen, contains only 1 residue of 3-hydroxyproline/1,000. For assumed equal turnover rates of total basement membrane and total interstitial collagen, only about one-fifth of peptide 3-hydroxyproline would originate from basement-membrane collagen.

Our data cannot answer the questions raised above. However, the general correlation between urinary 3-hydroxyproline and 4-hydroxyproline and, particularly, the increase in both hydroxyprolines during adolescence, when bone remodeling may be a major source of the elevated hydroxyproline in urine, suggests that much of the 3-hydroxyproline in urine may normally be derived from the turnover of interstitial collagen. Whether this is the case, and if so, whether experimental or disease-related alteration of basement membrane turnover can be detected by an altered level of urinary 3-hydroxyproline, are the subjects of futher studies which are now planned or in progress.

[3] Ren, P., and E. Adams. Unpublished data.

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