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URINARY HYDROXYPROLINE PEPTIDES *

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Collagens from widely different animal sources all contain the amino acid hydroxyproline (hypro). Except for a small amount found in elastin, hypro has been found in no other animal protein and therefore may be considered an *in vivo* label of collagen. In the absence of exogenous sources (diet rich in gelatin or collagen), individuals excrete a more or less constant amount of hypro. This occurs predominantly in the peptide form (1–3). Free hypro in the urine, even when large amounts are ingested, never exceeds a few per cent of the total hypro excretion (1). On a diet free of hypro sources, the amounts of urinary hypro are consistent with the estimated small turnover rates of collagen (4).

Ziff, Kibrick, Dresner, and Gribetz (1) reported that the total amount of bound hypro in the urine was essentially unchanged in patients with rheumatoid arthritis or other disorders of connective tissue, as compared to normal controls. High values of bound urinary hypro have been reported present in children (1), Marfan's syndrome (5), burn patients (6), and hyperparathyroidism (7).

Information concerning the nature and number of the hypro peptides in the urine and their variation in disease states remains fragmentary. Westall (8) demonstrated the presence of a urinary peptide containing equal amounts of proline (pro) and hypro. Mechanic, Skupp, Safier, and Kibrick (9) identified two peptides containing (hypro)$_4$(pro)$_4$(glu) and hypro-[(hypro)(pro)$_3$-(glu)$_9$], isolated from the urine of a patient with rheumatoid arthritis on a hypro-free diet. Recently, Kibrick, Hashiro, and Safier (10) found no evidence of these two peptides among the five peaks of hypro peptides obtained by ion-exchange chromatography from the urine of three patients with arthritis and two normal individuals.

The diketopiperazine of pro-hypro was identified in the urine of rachitic children by Plaquet, Biserte, and Boulanger (11), who suggested that it originated during the isolation procedure by cyclization of the dipeptide pro-hypro. Evidence for this compound was also found by Kibrick and associates (10).

A preliminary report from our laboratory demonstrated that urinary hypro peptides were separable into five peaks by ion-exchange chromotography (3). We, therefore, compared the urinary hypro excretion of patients with various disorders of connective tissue and that of normal subjects to determine whether qualitative differences in their chromatographic patterns could be demonstrated. Similar studies were made in a normal individual when a large exogenous collagen (gelatin) source was added to the diet. Comparable data were obtained from studies on normal postpartum women on a hypro-free diet during involution of the uterus and consequent large endogenous collagen breakdown. In particular, demonstration that the hypropeptides isolated from the urine contained sequences known to occur in the collagen molecule would support the use of hypro peptide excretion as an index of collagen breakdown. Qualitative differences in the excretion pattern of these peptides would be suggestive of either differences in collagen structure or collagen metabolism.

MATERIALS AND METHODS

Nine individuals hospitalized under supervised dietary control were the subjects of the study reported in this paper: a normal male, a female with systemic lupus erythematosus, a female with scleroderma, a male with Marfan's syndrome, a male with extensive morphea without systemic involvement, two postpartum women, a female with rheumatoid arthritis, and a girl with Hurler's syndrome. All except the last were adults and each was a clinically characteristic case of the specific disease syndrome. Twenty-four hour urine specimens were collected under toluene and stored in a cold-room at 2 to 4° C. Not more than one week was allowed to

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elapse from the time of collection to the time of the preparation of the concentrate described below. The specimens were collected while the individuals were on a diet from which hydroxyproline sources, such as meat, fish, ice cream, and so forth, were eliminated. A specimen was also collected from the normal individual while he was on a diet supplemented with one pound of meat, one-half pound of fish, and 30 g of gelatin.

Preparation of urine concentrates. The 24-hour urine specimen was adjusted to pH 2 with hydrochloric acid, diluted to 2 L, stored at 5° C overnight, and filtered. The filtrate was passed through a 500 ml resin-bed of Dowex 50 X 8 (20–50 mesh) in the hydrogen form, enclosed in a glass column of 40 to 60 mm diameter. The column was washed with at least 3 L of distilled water, and the adsorbed ampholytes were eluted with 1N KOH, 3 L of the latter being sufficient to elute all of the adsorbed hydroxyproline compounds. The eluate was carefully neutralized with 60 per cent perchloric acid in an ice bath, the potassium perchlorate precipitate filtered off, and the filtrate concentrated by flash evaporation at a temperature of 30° C. The concentrate was adjusted to 50 ml and stored in a refrigerator. The hypro content of these concentrates accounted for 90 per cent of the hypro in the original untreated urine.

Fractionation of hydroxyproline compounds. Columns (1 × 150 cm) of Dowex 50W X 2 (200–400 mesh) were prepared according to the method of Moore and Stein (12). One-tenth M acetic acid was used to equilibrate the columns, after which they were charged with 2 to 5 ml samples of the urine concentrates adjusted to pH 2. A single charge contained not more than 20 μmoles of unhydrolyzed Ninhydrin leucine equivalents. Larger amounts impaired the separation of the hypro peaks. The columns were then mounted over a fraction collector and a gradient elution was started with 150 ml of 0.1 M acetic acid (pH 3.0) in the mixing flask and 0.1 M acetic acid containing .04 M potassium acetate (pH 4.25) in the dropping funnel. After 100 ml had been collected, the mixing flask was filled with the pH 4.25 potassium acetate buffer, and the dropping funnel was filled with the same buffer to which 0.15M KCl had been added. All of the solutions used for equilibration and elution contained Brij 35 detergent (12). Two ml of eluate was collected per tube and samples of alternate tubes were removed for Ninhydrin and hypro analysis. For the first 100 ml the column flow rate was 3 to 4 ml per hour, after which it was adjusted to 6 to 8 ml per hour. The temperature of the column was maintained at 30° C.

The eluates falling under a given hypro peak were combined and concentrated *in vacuo* and stored in the refrigerator for subsequent investigations.

Chemical assays. Hydroxyproline assays on samples of the original urine and of diluted concentrates as well as combined column eluates were carried out by the chloramine T procedure of Stegemann (13). Controls consisted of duplicate samples containing all reagents except chloramine T. Samples containing internal standards yielded recoveries of 104 to 107 per cent. Hypro eluted from the column accounted for 96 to 100 per cent of the original column charge. Free hypro, usually too low to detect in the original urine, was determined on unhydrolyzed concentrated samples and total hypro on samples which were hydrolyzed in 6 N HCl (sealed vials, 110° C, 12 hours). For successive assays on column eluates a modification of the Neuman-Logan procedure (14) was employed after the samples were hydrolyzed in 1.25 N NaOH (100° C, 10 hours). Ninhydrin assays were made using the method of Rosen (15). The pro-

TABLE I

Urinary hydroxyproline excretion

Subject	Sex	Age	Days on hypro-free diet	Total peptide hypro	Total free hypro	% Total urinary peptide hypro in peak				
						A	C	D	E	F
				mg/24 hrs	*mg/24 hrs*					
M.U. Normal	M	29		52	2	6	5	20	65	4
M.U. Normal	M	29	2	30	1	3	4	27	64	2
A.B. Lupus erythematosus	F	35	2	44	1	3		42	41	14
A.G. Scleroderma	F	50	3	52	1	8	10	12	49	21
D.P. Hurler's syndrome	F	13	5	26	1	5	8	11	71	4
M.M. Rheumatoid arthritis	F	43	5	34	1	7	7	14	56	5
A.A. Marfan's syndrome	M	20	5	80	1	2	9	16	60	17
A.D. Morphea	M	29	3	63	1	6		21	60	7
A.D. Morphea	M	29	4	60	1	8		17	70	5
A.D. Morphea	M	29	5	62	1	2		40	50	8
A.D. Morphea*	M	30	5	59	1	6		28	58	7
D.S. Postpartum	F	24	3	57	2	1	3	17	70	10
D.S. Postpartum	F	24	4	41	1	8	10	12	61	9
D.S. Postpartum	F	24	5	37	1	9	3	27	60	1
C.D. Postpartum	F	21	3	66	2	2		22	66	10
C.D. Postpartum	F	21	4	47	1	9	8	18	60	5
C.D. Postpartum	F	21	5	44	1	25	14	15	44	2

* On specimen collected one year after previous ones.

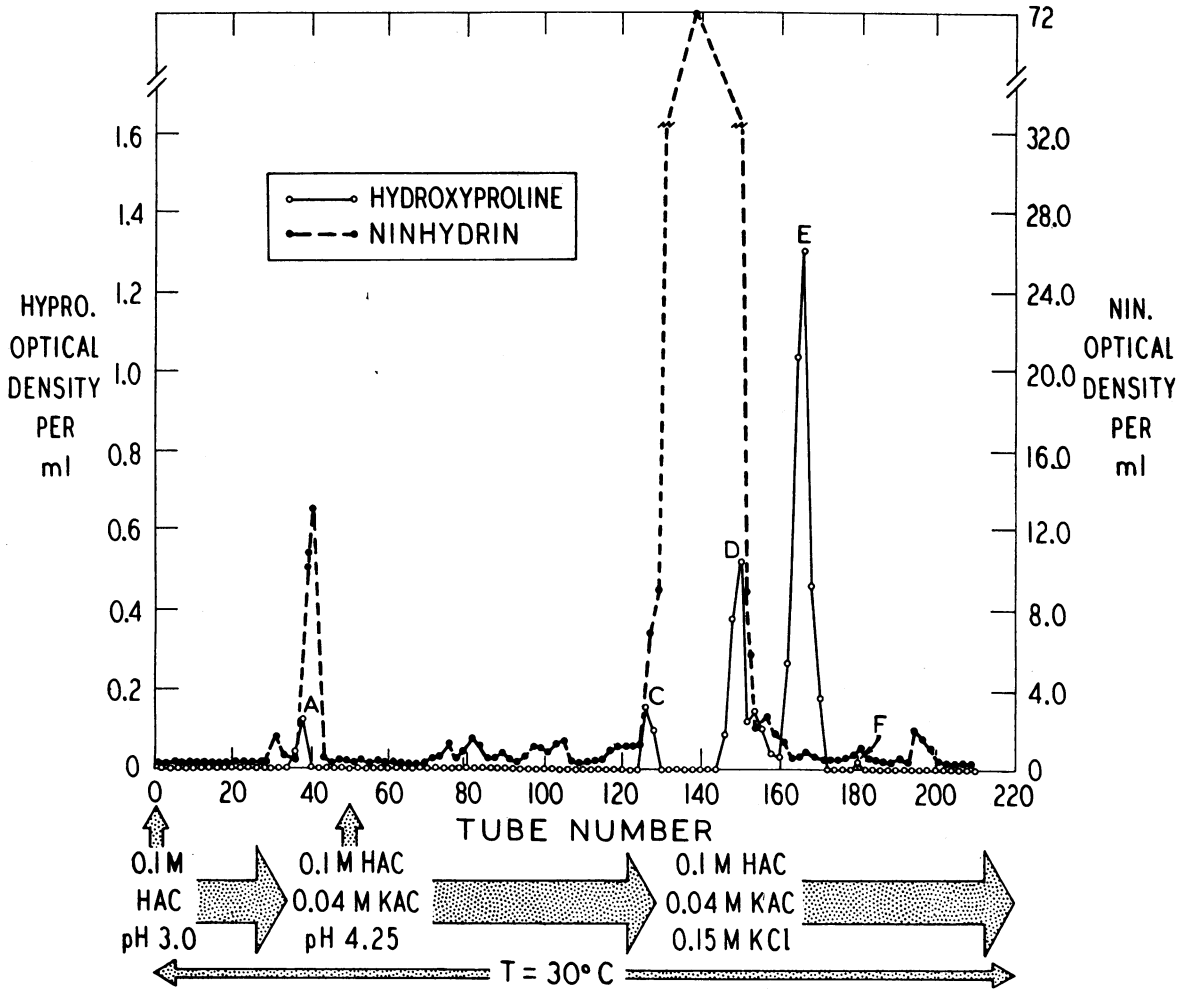

FIG. 1. ELUTION PATTERN OF URINARY HYDROXYPROLINE AND NINHYDRIN OF A NORMAL ADULT MALE ON HYPRO-FREE DIET.

cedure of Piez, Irreverre, and Wolf (16) was used for proline assays.

Paper electrophoresis and chromatography. Paper electrophoresis was carried out on Whatman 3 mm paper and, unless otherwise stated, in Spinco, Durrum-type cells, at 300 volts, for 3 hours, at pH 4.0 (pyridine : acetic acid : water, 8 : 35 : 957). For chromatography the same paper was employed and all chromatograms were developed with an ascending front. The solvents usually employed were: *a*) butanol : acetic acid : water (4 : 1 : 5), *b*) t-amyl alcohol saturated with phthalate buffer pH 6.0, *c*) 1.5 M phosphate buffer, pH 6.0, *d*) ethanol : water : ammonia (80 : 20 : 1), *e*) phenol buffer, 100 : 20 wt/vol; buffer = 6.3 per cent sodium citrate, 3.7 per cent KH_2PO_4 with 0.002 per cent 8-hydroxyquinoline.

RESULTS

Free and bound hydroxyproline values for the specimens collected from the subjects utilized in this investigation are given in Table I. Bound or peptide hydroxyproline was highest in the urine of the man with Marfan's syndrome and lowest in the child with Hurler's syndrome (26 mg). The addition of collagen sources to the diet of the normal subject resulted in an increase in both free and peptide hypro. In the postpartum specimens (days of diet upon which collections were made corresponded to the days postpartum), both free and bound hypro were highest on the first days of collection and decreased on the second and third days. In the patient with widespread morphea, the free and hypro peptide excretion was relatively constant during the three days of collection, as well as one year later when another collection was made.

Figures 1 and 2 show the superimposed Ninhydrin and hydroxyproline elution patterns obtained from the fractionation of the urine concentrates prepared from the normal subject on the hypro-free diet (Figure 1) and on the supplemented diet (Figure 2). The hypro-containing peaks are lettered A to F for comparative purposes. The difference between the two patterns lies essentially in the magnitude of the peaks; it can be seen that dietary supplementation resulted in larger hypro peaks, but no new peptide hypro peaks appeared. Peak B, which contains only free hypro and includes all of the free hypro in the urine, is absent in Figure 1 (hypro-free diet). This is probably due to the lack of sensitivity of the assay method, since a small amount of free hypro was present in the concentrate charged on the column. All of the peptide hypro charged on the column is accounted for by the five peaks shown (A, C, D, E, and F).

The hypro elution pattern of the urine concentrate from the patient with Marfan's syndrome is illustrated in Figure 3 with the Ninhydrin curve omitted for clarity. All of the hypro peptides were present in relatively larger amounts than in normal urine. Figure 4 shows the pattern obtained from the urine of one of the postpartum patients, collected on the third day postpartum; a relatively large amount of free hypro is present, as can be seen from the prominence of peak B, and in this instance peak C was not detected. On the fifth day, Figure 5, peak B was too low to detect and peak C was present in this patient.

The patterns shown are typical of those ob-

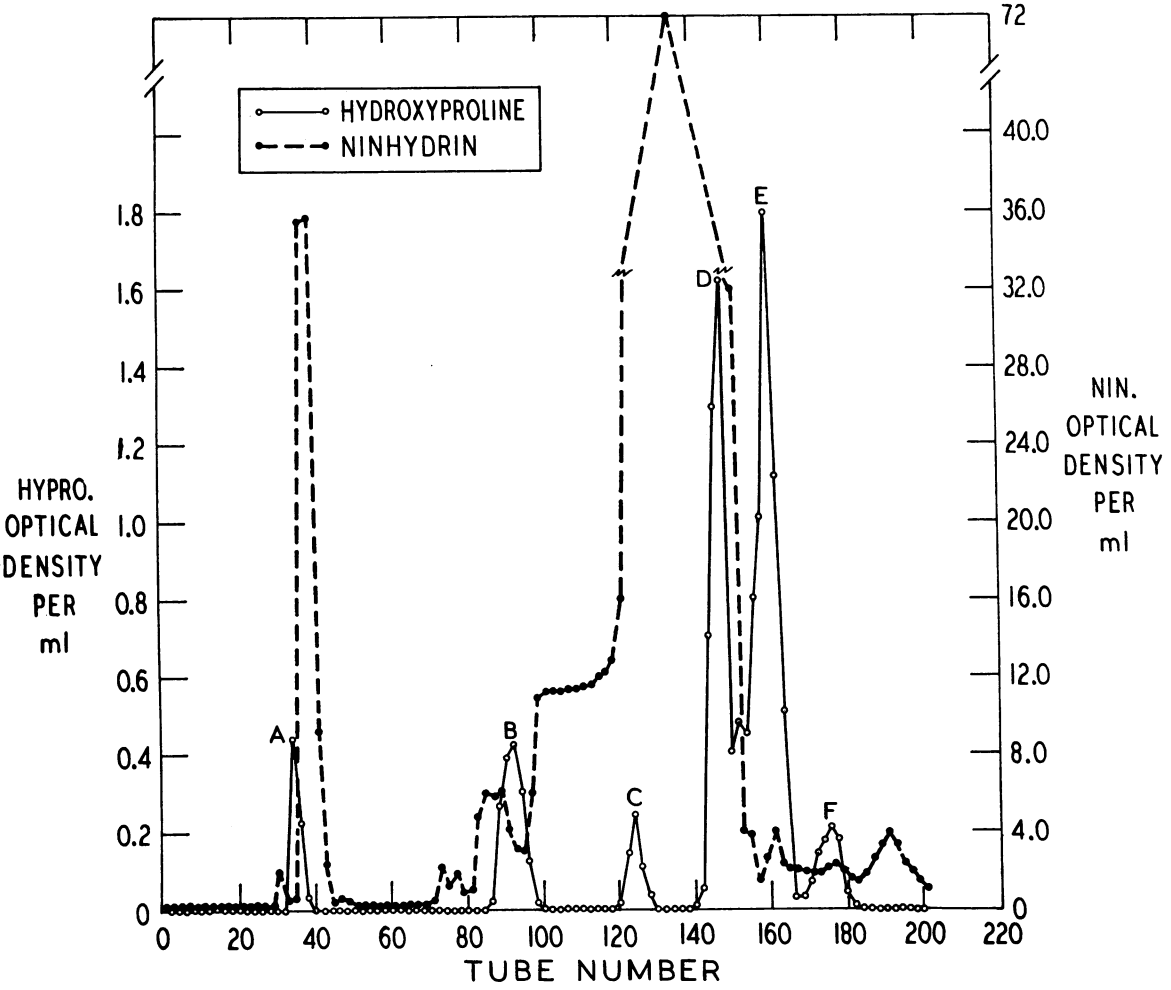

FIG. 2. ELUTION PATTERN OF URINARY HYDROXYPROLINE AND NINHYDRIN OF A NORMAL ADULT MALE ON DIET RICH IN HYPRO.

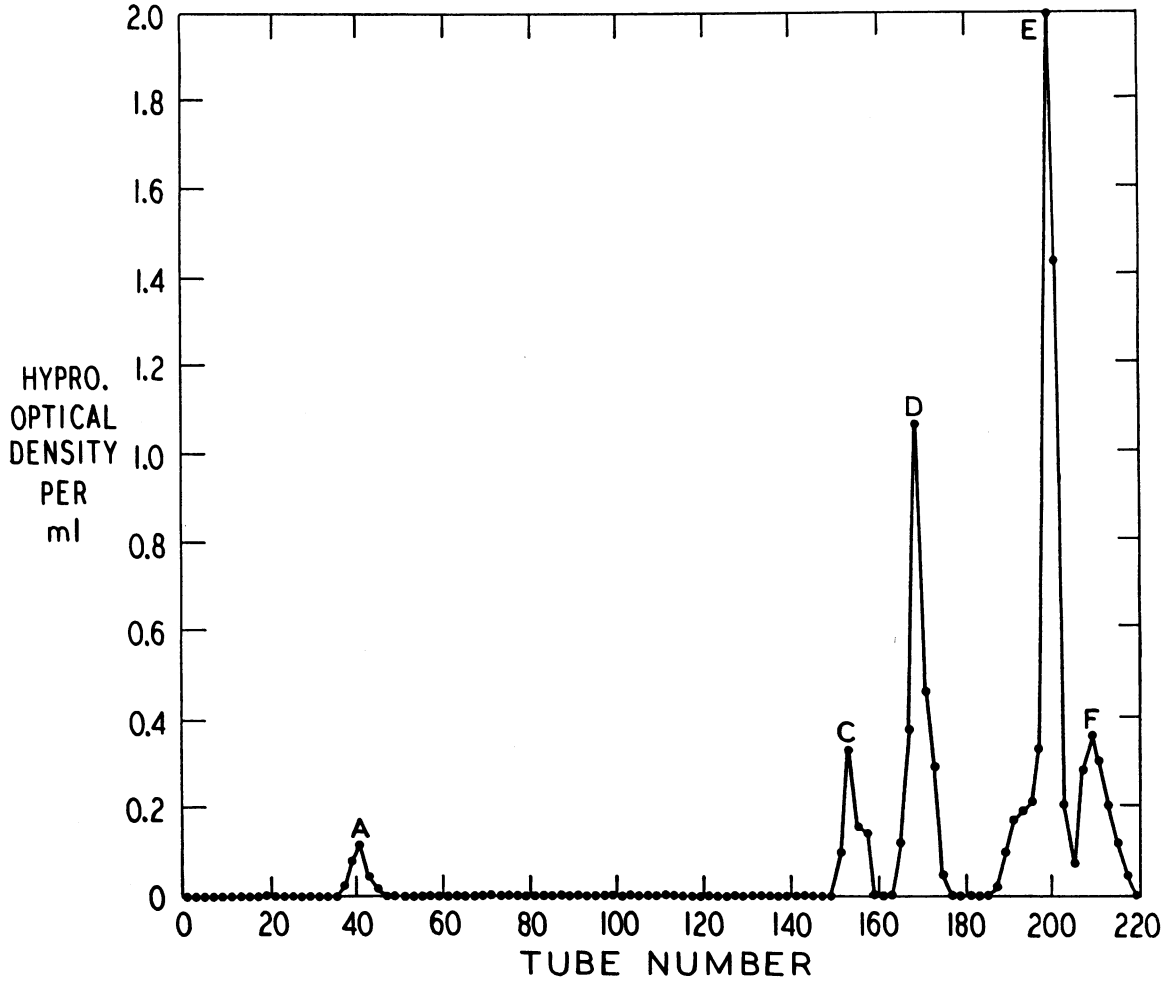

FIG. 3. ELUTION PATTERN OF URINARY HYPRO IN PATIENT WITH MARFAN'S SYNDROME.

tained with all of the specimens that we have studied. With the exception of peak C, which was not detected in a few instances (see Peak C below), we have not found any significant qualitative differences in the hypro peptide elution patterns. There were quantitative variations with respect to the relative amounts of peptide hypro present in the peptide peaks as shown in the last columns of Table I.

Characterization of the hydroxyproline peptides. The hypro peptides eluted from the columns were in each instance contaminated with varying amounts of other peptides and amino acids, or both. Additional procedures were required for their subsequent purification and analysis.

Peak A. The hydroxyproline peptide present in this portion of the column did not react with Ninhydrin, isatin, or dinitrofluorobenzene (DNFB), suggesting absence of a free terminal amino or imino acid. When a portion of the complete fraction was subjected to paper electrophoresis in one dimension, followed by chromatography in the second dimension (solvent A) (fingerprinting), the hypro peptide could be located in an area which gave a positive reaction for peptide when sprayed with the Rydon and Smith reagents, as modified by Pan and Dutcher (17). The peptide behaved as a neutral component under the electrophoretic conditions described above, and migrated with an R_f similar to leucine in solvent A. When the Rydon-Smith positive zone was eluted from unsprayed portions of the papers and hydrolyzed, the hydrolysate was found to account

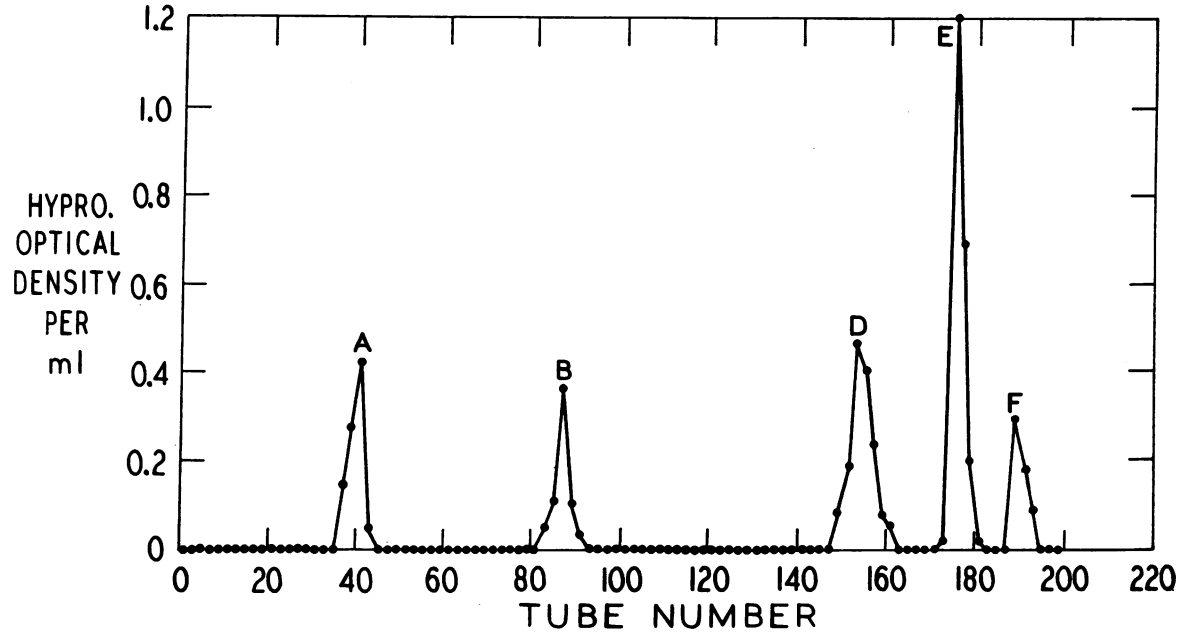

FIG. 4. ELUTION PATTERN OF URINARY HYPRO IN NORMAL FEMALE, THREE DAYS POSTPARTUM, ON HYPRO-FREE DIET.

for all of the hypro present in the original sample and to contain also pro, glycine, and glutamic acid. The latter two amino acids were detected by fingerprinting, whereas the two imino acids were detected by fingerprinting and quantitated by chemical assays. Proline and hypro were present in equimolar concentrations, but the amounts of glycine and glutamic acid appeared to vary with different preparations. Using an electrophoretic system similar to that described by Plaquet and associates (11), we were able to locate all the hypro peptide in a portion of the electrophoregram which did not react with the Rydon and Smith reagents, and none in the two spots which gave a positive Rydon-Smith reaction. The hydrolysate of this fraction contained only proline and hydroxyproline. The component behaved identically when compared electrophoretically and in two

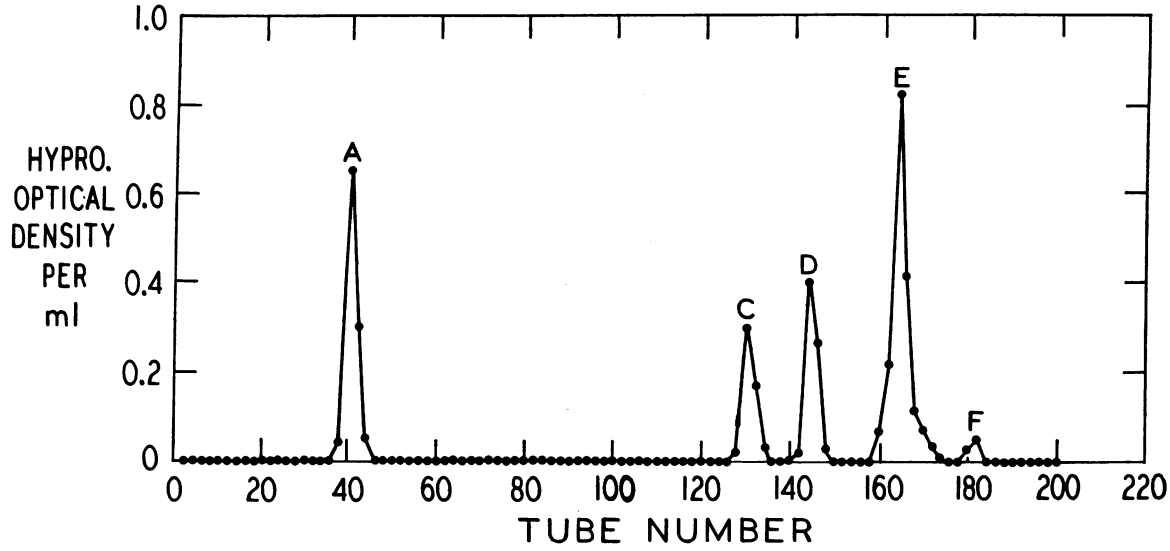

FIG. 5. ELUTION PATTERN OF URINARY HYPRO IN NORMAL FEMALE, FIVE DAYS POSTPARTUM, ON HYPRO-FREE DIET.

chromatographic systems (solvents A and E) with diketopiperazine of pro-hypro, synthesized according to the method of Plaquet and associates (11). We have, therefore, concluded that the hypro component of peak A is the diketopiperazine of pro-hypro.

Peak B. This contained only free hypro.

Peak C. This was a small peak, not observed in every instance. It was absent from the urine in the female with lupus erythematosus, the male (A.D.) with generalized morphea, and on the first day's collection of one of the two postpartum women. It is significant that in Patient A.D. peak C was absent in each of three consecutive 24-hour collections; it was also absent from a 24-hour collection one year later in the same patient.

Rechromatography of urine concentrates was consistent for presence or absence of peak C, so that it is unlikely to be an artifact of the separation process.

Pooled peak C eluates were subjected to electrophoresis and chromatography as above. Heavy contamination with free amino acids and non-hypro peptides occurred, in addition to what appeared to be a single hypro peptide that migrated towards the anode. Free glutamic acid and free proline, not found in the original peak C eluates, appeared after hydrolysis. The presence of glutamic acid could explain the acidic character of the hypro peptide. Further purification is required to clarify the nature of this peptide.

Peak D. This hypro peak coincided with the major Ninhydrin area. Fingerprinting demonstrated the presence of a large number of compounds which were positive for Ninhydrin. Before hydrolysis this peak contained from 11 to 50 times as many amino groups measured as leucine equivalents of Ninhydrin as hypro. Dinitrophenylation of the total peak D products yielded a complex mixture of DNP derivatives which could not be successfully separated by paper chromatography, but three areas containing hypro could be detected in chromatograms developed in solvent B.

Electrophoresis of the peak D peptides (pyridine : acetic acid : water, 4 : 17.5 : 978.5, pH 4, for 3 and ½ hours at 300 volts) separated the hypro peptides into two bands. One band migrated slightly towards the cathode with the neutral amino acids; the second, the major hypro component, showed a more acid character and moved towards the anode between glutamic and the neutral amino acids. By this method the acidic hypro peptide could be freed of most of the contaminating Ninhydrin material. The acidic peptide was eluted from electrophoretograms and the dinitrophenyl derivative prepared. It had an absorption maximum at 355 mμ and behaved very similarly to DNP-glutamic acid in the solvent systems B and C. When eluted from chromatograms by the method of Sanger and Thompson (18) and hydrolyzed by 6 N HCl for 12 hours, it gave DNP-glutamic acid and free hypro, indicating that the glutamic acid was N-terminal. Proline was absent by fingerprinting and by chemical assay in hydrolysates of the original acidic peptide after elution from electrophoretograms.

Because of persistent contamination of other Ninhydrin-positive material, a pooled concentrate of peak D (patient A.D.) was refractionated on a Dowex 50WX2 (200–400 mesh) ion-exchange column with the citrate buffers and column temperature changes prescribed by Moore and Stein (12) for amino acid separation. Three hypro peaks were eluted which were now only slightly contaminated with other Ninhydrin-positive substances, the majority of the Ninhydrin-reacting material having been eluted prior to the hypro peptides. The fractions were desalted (19), and the hypro peptides were purified by paper electrophoresis in pyridine : acetic acid : water (7.5 : 25 : 967.5), pH 3.9, 385 volts for 8 hours, followed by chromatography in solvent A.

The most prominent of the three hypro peaks eluted from the column accounted for 75 per cent of the peptide hypro originally present in peak D and contained the acidic hypro peptide described above. After purification by electrophoresis and chromatography, this peptide was eluted along with appropriate paper blanks and hydrolyzed. It was again found to contain only glutamic acid and hypro. Quantitative Ninhydrin assays before and after hydrolysis and assays for hypro indicated the presence of a tripeptide containing two residues of glutamic acid and one of hypro.

The N-terminal position of glutamic acid was confirmed by *a*) the positive reaction of the peptide with Ninhydrin, *b*) the spectrum of the DNP-peptide, and *c*) the isolation of DNP-glutamic acid after hydrolysis of the DNP-peptide. The

peptide has tentatively been identified, therefore, as glu-(hypro, glu). Two other hypro peptides, obtained by rechromatography on Dowex 50, which account for one-quarter of the hypro originally present in peak D, and which migrate electrophoretically like neutral amino acids, remain to be identified.

Peak E. This peak contained only one hypro peptide which behaved electrophoretically like a neutral component. Its mobility in solvent A was below that of alanine used as a reference compound. The peptide reacted with Ninhydrin to give a yellow zone that turned orange when counterstained with isatin, suggesting an N-terminal imino acid. Workable amounts of the peptide were prepared by streaking the material on paper prior to electrophoresis and chromatography. The peptide thus obtained was hydrolyzed and found to contain only proline and hypro in equimolar proportions. A dinitrophenyl derivative of the peptide was prepared and found to have an absorption in 1 per cent $NaHCO_3$ at 375 mμ, typical of DNP-pro or hypro peptides. When the DNP peptide was hydrolyzed in 6 N HCl, according to the differential hydrolytic procedure described by Schroeder and associates (20), only the yellow artifacts associated with the hydrolytic products of DNP-prolyl peptides (in addition to DNPOH) could be detected on chromatograms of the DNP compounds. The water soluble fractions after hydrolysis were found to contain free hydroxyproline and some free proline. Proline was, therefore, concluded to be N-terminal. From spectrophotometric analysis of the DNP-prolyl peptide and from its hypro assays after hydrolysis, the molar ratio of hypro to DNP peptide was found to be 0.7. As a result of these studies, and since pro and hypro were found to be present in approximately equimolar concentrations in the original peak E and in the purified peptide, we have concluded that this peptide is pro-hypro, and is probably the peptide described by Westall (8). This peptide is usually the predominant hypro peptide in urine (see Table I).

Origin of Peak A. The hypro peptide of peak A was shown above to be the diketopiperazine of pro-hypro. To determine whether peak A could have arisen from pro-hypro, the dipeptide of peak E, during the preparation and separation processes, a sample of pooled peak E was subjected to rechromatography. The sample contained 620 μg of hypro. Like the original urine concentrate, it was treated with perchloric acid to remove potassium and rechromatographed on Dowex 50X2 (200–400 mesh). This resulted in the emergence of 52 μg of hypro in the position usually occupied by peak A, and the remainder as the original peak E. The hypro compound now found in peak A was identical in its chemical and chromatographic characteristics to the diketopiperazine previously described as the original peak A, indicating that peak A (diketopiperazine of pro-hypro) arises from peak E (pro-hypro) during the separation procedures.

Peak F. Only one hypro peptide was present. It behaved as a neutral peptide electrophoretically and migrated between proline and alanine on paper chromatograms developed with solvent A. It gave a yellow spot when sprayed with Ninhydrin which turned a reddish-brown when counterstained with isatin. The peptide was prepared by the streaking methods described for peak E. When hydrolyzed it yielded almost equimolar amounts of pro, hypro, and glycine. Ninhydrin analyses before and after hydrolysis gave a hydrolyzed: unhydrolyzed ratio of 1.2. The dinitrophenyl derivative of the peptide had an absorption maximum at 355 mμ, typical of DNP-aminopeptides. Hydrolysis of this compound yielded DNP-glycine, and free pro and hypro. A slight amount of free glycine (gly) was also detected. That this arose from decomposition of DNP-gly during hydrolysis is suggested by the fact that synthetic DNP-gly-pro also gave small amounts of free glycine after similar hydrolysis. These data, coupled with the demonstration that gly was N-terminal, suggested that the peptide of peak F was gly-(pro-hypro).

Among the products of the reaction mixture of collagenase on gelatin or collagen is the tripeptide gly-pro-hypro (21, 22). A total enzymatic digest of gelatin was fractionated on a Dowex column by the same procedures as the urinary hypro peptides. The tripeptide gly-pro-hypro was eluted from the region corresponding to peak F and further purified by chromatography. Its identity and purity were verified by Ninhydrin assays before and after hydrolysis, quantitative amino acid assay, preparation of DNP derivatives, and mobility in solvent systems A and D. When this tripeptide product

of the gelatin-collagenase reaction was compared with the urinary hypro peptide in peak F, it behaved identically electrophoretically and in two solvent systems (A and D). The DNP derivatives of the two peptides gave the same absorption pattern and migrated identically in the tertiary amyl alcohol system. We conclude, therefore, that peak F contains gly-pro-hypro.

This is the first demonstration in the urine of a tripeptide sequence known to occur in the collagen molecule.

DISCUSSION

The total amounts of hypro in the urine in our studies are comparable to the data of Ziff and colleagues (1) and Prockop and Sjoerdsma (2), and in agreement with these authors, the hypro found is almost all in the peptide form. The highest excretion noted here was in the adult with Marfan's syndrome, as originally noted by Sjoerdsma and colleagues (5). The lowest noted was in a 13-year-old girl with Hurler's syndrome (gargoylism). Her 24-hour collection contained only 26 mg of hypro, which is lower than any of the 16 children studied by Ziff and associates (1).

The two postpartum patients, whose hypro excretion was measured from postpartum days 3 to 5, showed a relatively high but steadily diminishing total amount of hypro peptides, presumably due to involution of the uterus. A decrease in uterine collagen during postpartum involution has been shown in rats by Harkness and Moralee (23) and by Woessner (24) and in excised human uteri by Morrione and Seifter (25). Woessner showed that, in rats, no more than 15 per cent of the degraded collagen would be accounted for by increase in urinary hypro even though there was an increase in free hypro in the uterus and blood. Our data indicate that the qualitative pattern of urinary hypro peptide excretion during the time of involution of the human uterus is normal.

It is of interest that in gargolylism, considered to be a hereditary disorder of connective tissue, Meyer, Grumback, Linker, and Hoffman (26) have reported alteration in mucopolysaccharide excretion. Our studies in one case of gargoylism indicated no alteration of the urinary hypro peptide excretion pattern. Both Marfan's syndrome, with the highest hypro excretion, and Hurler's syndrome, with the lowest, exhibited the same elution pattern of hypro peptide excretion (Table I).

The patient with widespread morphea had very active formation of firm areas of scar tissue involving extensive portions of his skin with little clinical evidence of dissolution of scar tissue once laid down. His total hypro excretion was consistently high compared to our data as well as that of others (1, 2). Except for the persistent absence of peak C, his hypro excretion pattern resembled all others studied. The significance of the absence of peak C from the urine in this patient, as well as in the woman with lupus erythematosus, is unknown.

The amount of urinary hypro has been found to be elevated by increased exogenous intake of gelatin or collagen foods (1, 2). The normal individual studied by us showed the five peaks of hypro peptides when he was on a hypro-free diet as well as on a diet rich in collagen and gelatin. Although the 24-hour excretion rose from 30 to 52 mg of hypro, the percentage distribution and the location of the various elution peaks was unchanged. Thus the hypro end-products of the *in vivo* degradation of collagen appear to be indistinguishable by these methods from the fragments obtained as a result of the degradation of ingested gelatin and collagen.

The method of separation used here indicated five hypro peaks which appear to contain at least seven or eight separate hypro peptides. The hypro peptide fragments thus far identified are *1*) diketopiperazine of pro-hypro, *2*) glu(hypro-glu) from peak D, *3*) pro-hypro (peak E), and *4*) gly-pro-hypro (peak F). The structures of the peptide (or peptides) in peak C, and at least the two other peptides in peak D, remain unidentified. We did not identify either of the two peptides reported by Mechanic and co-workers (9), who used a somewhat different elution system.

This is the first demonstration of the tripeptide sequence gly-pro-hypro in the urine. Of the hypro peptides described here, gly-pro-hypro and pro-hypro are sequences known to occur in the collagen molecule. This is strong supporting evidence that the hypro peptides in urine represent end-products of collagen metabolism. Other evi-

dence for this view has been summarized by Prockop and Sjoerdsma (2).

In view of this evidence, the qualitative similarity of the urinary hypro peptide pattern in a normal male, with and without hypro-free diet, in postpartum women undergoing the normal process of uterine involution, and in patients with a variety of so-called "collagen diseases" is noteworthy. Although these studies give no evidence of structural differences in the collagen molecule in these subjects, it indicates that the end-products of the degradation of collagen are similar in all of them.

SUMMARY AND CONCLUSIONS

1. We have confirmed that urinary hydroxyproline (hypro) exists predominantly in the peptide form, even with large dietary intakes of gelatin or collagen.

2. Urinary hypro peptides may be separated into five peaks by ion-exchange chromatography.

3. The elution pattern of the hypro peptides was similar in a normal subject, patients with rheumatoid arthritis, scleroderma, Marfan's syndrome, and Hurler's syndrome, and in normal postpartum women. In a woman with lupus erythematosus and a male with generalized morphea, the elution pattern differed only in absence of a small peak (peak C).

4. The normal individual excreted the same hypro peptide fragments on a high gelatin intake as on a hypro-free diet, but the total amount was increased.

5. There are seven to eight peptides present in the five peaks of which we have identified *a*) diketopiperazine of proline-hypro, *b*) glu-(hypro-glu), *c*) proline-hypro, and *d*) glycine-proline-hypro.

6. This is the first demonstration of a tripeptide sequence (glycine-proline-hypro) in the urine known to occur in the collagen molecule.

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