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# HIGH MOLECULAR COLLAGEN PEPTIDE FRACTION IN URINE INDICATES DISEASE ACTIVITY IN GENERALIZED SCLERODERMA

Nelly Blumenkrantz and Gustav Asboe-Hansen

*From the Department of Dermatology, University of Copenhagen, Rigshospital, Copenhagen, Denmark*

*Abstract.* Parallel determinations of the collagen metabolites hydroxyproline (Hyp) and hydroxylysine (Hyl) in urine were performed and the values related to age, sex, diet, and disease activity in generalized scleroderma. In normal individuals, the two amino acids followed parellel lines. In the second decade of control individuals, the total urinary output of Hyp and Hyl was augmented, mainly because of an increase of the "1+5" fraction (high molecular peptides precipitated by 5 volumes of acetone, containing all hydroxylysine and part of the hydroxyproline). In other age groups the total Hyp and Hyl and the "1+5" fraction were less than in the second decade. Patients with active generalized scleroderma showed increased urinary excretion of "1+5" Hyp and Hyl. In particular, the variations of the Hyp in the "1+5" fraction, as related to total Hyp, closely followed the clinically established variations in disease activity. Progressive phases can be pin-pointed and improvement assessed under treatment with inhibitors of collagen synthesis. There were no sex differences between the different decade groups; neither were any differences observed in relation to sex or diet in patients with active or non-active scleroderma.

*Key words:* Scleroderma; Urinary indicator of disease activity; 1+5 Acetone-precipitated peptides; Hydroxyproline; Hydroxylysine

Most previous studies on collagen metabolism have been focused on the excretion of hydroxyproline (Hyp; 16, 12). In the past, the lack of an easy, sensitive and reproducible assay has hampered the determination of hydroxylysine (Hyl) parallel with Hyp. However, it is now known that Hyp *per se* does not define collagen metabolism. The possibility exists that, in diseases of connective tissue, the formation of an abnormal collagen or a pathological metabolism of this macromolecule may play a pathogenetic role. This is suggested by the presence of peptides with altered contents of one or both of the two above-mentioned amino acids characteristic of collagen (2, 6, 15). The recent development of quick and specific automated procedures for the detection of Hyl and Hyp (3, 4) enabled us to perform this study, which is based upon analysis of more than one thousand urine samples.

Previous studies have shown that large Hyp-containing peptides present in urine originate from the degradation of newly synthesized collagen, and that only 5–20% of the total urinary Hyp is non-dialysable (11). An increased urinary output of Hyp associated with growth consists of peptides derived from soluble collagen (11).

In generalized scleroderma, periods of activity and progression may alternate with static periods (1). It is difficult to pin-point clinically the phases of progression, as, until now, no quantitative or specific index of disease activity has been known. There is clinical and theoretical evidence, that the drugs hitherto used for scleroderma are preferentially, or exclusively, given a chance to act favourably in the phases of progression.

In order to be able to select the most suitable periods for treatment and to ascertain whether a drug is effective or not or to what extent, the clinician needs a reliable laboratory clue.

## MATERIAL AND METHODS

One thousand and twenty-one urine samples from patients suffering from various skin and connective-tissue diseases were analysed. 103 patients suffering from generalized scleroderma (acrosclerosis or diffuse systemic sclerosis) were evaluated as to disease activity, either

untreated or undergoing treatment with inhibitors of collagen synthesis. In 86, the clinically established degree of activity was correlated with the urinary output of collagen peptides.

The following grades of activity were considered (see Table 1):

I. Regression of dermal sclerosis during treatment.
II. Static, non-progressive, non-regressive.
III. Chronic progressive course, low disease activity.
IV. Moderate disease activity.
V. Fulminant course.

A collagen-free diet was given to hospitalized patients (95% of the total material). A 24-hour urine sample was collected the day before the diet started and another on the very day of the diet. In addition, urines of some non-hospitalized controls (5%) were collected. The latter subjects had an unrestricted food intake. Samples were grouped according to age (decades), sex and diet. The effect of the diet was analysed by comparing the parameters studied on the two 24-hour samples collected from each individual. Analyses of Hyl and Hyp were performed according to the methods of Blumenkrantz & Asboe-Hansen (3, 4).

## *Preparation of Urine Samples for Analysis*

*Total hydroxyproline.* One ml filtered urine was hydrolysed in 6 N HCl at 110°C for 16 hours. After hydrolysis, HCl was evaporated under vacuum at 65°C. Evaporated samples were dissolved in 2 ml phosphate–citrate buffer (3), and 0.5 ml of the solution was diluted with 9.5 ml of the same buffer. The samples were centrifuged and the supernatants used for the assay of "total Hyp".

*Hydroxyproline-containing peptides* were precipitated with different amounts of acetone and, according to the proportions added, called fraction "1+5", "1+9" and "1+19". One ml urine was added to 5, 9 and 19 ml acetone in glass ampoules of 10 and 25 ml. The samples were left at 4°C for 30 min and then centrifuged at 3000 rpm for 15 min. The supernatant was aspirated by vacuum. The small amount of acetone remaining in the pellet was allowed to evaporate and the samples were then hydrolysed, evaporated and dissolved as indicated for total Hyp.

*Hydroxylysine.* One ml filtered urine was precipitated with 5 ml acetone in a 10 ml ampoule. The samples were refrigerated at 4°C for 30 min and then centrifuged at 3000 rpm for 10 min. The supernatant was aspirated by vacuum and the pellet hydrolysed with 6 N HCl at 110°C for 16 hours. Hydrolysis may be performed in 2 N to 6 N HCl under these conditions. After hydrolysis, HCl was evaporated under vacuum at 65°C and the dried sample dissolved in 4 ml of the corresponding phosphate–citrate buffer, pH 7.0 (4). The samples were centrifuged in the same ampoules in which the entire process was performed, and the supernatant was used for Hyl determination.

## *Preliminary Assays*

To establish the conditions for precipitation and hydrolysis of the urine fractions containing Hyp and Hyl, the following experiments were performed.

### *Hydroxyproline fractions*

*Effect of HCl concentration on the yield of hydroxyproline.* Samples of urine as well as "1+5", "1+9" and "1+19" fractions were hydrolysed for 16 h at 110°C in 1, 2, 3, 4, 5, 6 and 8 N HCl.

*Effect of salt concentration on the acetone precipitation of the hydroxyproline-containing fractions.* Samples dialysed for different lengths of time were precipitated with 5, 9 and 19 ml acetone, either direct or after addition of different concentrations of NaCl or $CaCl_2$. The same experiment was performed with 1 ml non-dialysed urine precipitated with the above-indicated volumes of acetone. The precipitated samples were dissolved in distilled water, and 1 mM $CaCl_2$ or 0.15 M NaCl was added. The samples were then reprecipitated with the same amount of acetone as used for the first precipitation. After centrifugation, the samples were hydrolysed, evaporated, dissolved and analysed.

### *Hydroxylysine*

*Effect of different concentrations of HCl.* Samples of urine precipitated with 5 parts of acetone were hydrolysed at 110°C for 16 h with various concentrations of HCl. Maximum hydrolysis yield corresponded to maximum absorbance with the Hyl assay.

*Effect of salt concentration.* The precipitation of the Hyl-containing fraction was assayed after addition of different amounts of NaCl or $CaCl_2$ to the urine samples.

*Effect of pH on the precipitation of Hyl and Hyp containing fractions by acetone.* The same urine sample was brought to pH 2, 5, 6, 7, 8, 9 and 10 and Hyl or Hyp analysed in the "1+5", "1+9" and "1+19" precipitates.

*Effect of previous treatment with 0.0015 M $NaIO_4$ on the assay of Hyl content in "1+5" precipitated fractions.* Three 1 ml aliquots or urine samples were precipitated "1+5". The precipitates were suspended in 1 ml phosphate–citrate buffer, pH 7.0, and 0.5 ml of the suspension was submitted to the following procedure. Tube 1: 0.5 ml suspension +0.5 ml standard containing 5 μg Hyl+1 ml $NaIO_4$; tube 2: 0.5 ml suspension +0.5 ml $H_2O$+1 ml $NaIO_4$; and tube 3: 0.5 ml suspension +1.5 ml $H_2O$. Tubes 1 and 2 were oxidized by $NaIO_4$ for 15 min and the 3 samples were precipitated with 10 ml acetone. The precipitates were washed twice with absolute ethanol. Samples were hydrolysed with 6 N HCl, evaporated and finally dissolved in 2 ml citrate–phosphate buffer, pH 7.0 (4). The solutions were centrifuged and assayed for Hyl.

*Need of hydrolysis for detecting Hyl in urine.* Unhydrolysed as well as hydrolysed, precipitated or non-precipitated, samples were submitted to the automated assay for Hyl (4). Similar aliquots were analysed without previous oxidation with $NaIO_4$—which was replaced by water—to detect the possible presence of interfering substances reacting directly with Ehrlich's reagent used in the Hyl assay (15).

*Effect of dialysis of urine.* Urine samples were dialysed against running tap water for 15, 30 and 120 minutes, and 1 ml of the samples was then precipitated with 5, 9 and 19 ml of acetone. After hydrolysis and evaporation the samples were assayed for Hyp and Hyl. Dissolved "1+5", "1+9" and "1+19" fractions were submitted to

dialysis after being precipitated. Dialysis was performed against running tap water for 15, 30 and 120 minutes. The undialysable material was then hydrolysed and analysed for Hyl and Hyp. The effect of 120 min dialysis on the determination of free glucosyl-galactosyl hydroxylysine (Hyl-Gal-Glu; kindly provided by Drs M. and E. Moczar, Laboratoire de Biochimie du Tissu Conjonctif, Univ. of Paris, France, was assayed. Collagenase Worthington Biochemical Corp., Freehold, N.J., USA) digestion of the precipitated fractions and reprecipitation with different volumes of acetone as well as dialysis of the collagenase-digested peptides were performed. Both procedures were followed by hydrolysis as well as Hyl and Hyp determination.

### *Calculations*

The results were expressed in mg or mM per 24 h for Hyp and Hyl in relation to age expressed as decades of life. In order to establish a relationship between activity of generalized scleroderma and levels of urinary Hyp and Hyl, objective criteria were taken into consideration. The patients were grouped either as controls (not suffering from systemic disease) or as generalized scleroderma. The percentual Hyp content in the "1+5", "1+9" and "1+19" fractions in relation to total Hyp was calculated. The ratios of total Hyp to total Hyl as well as the Hyp to Hyl in the "1+5" precipitates were determined.

Student's *t*-test was used to assess the statistical significance of the differences between the age and activity groups.

## RESULTS

Patients with atopic dermatitis, leg ulcer, psoriasis, contact dermatitis, hirsutism, cutis marmorata, acute systemic and chronic discoid lupus erythematosus showed a normal percentage of Hyp in the "1+5" fraction. Pathological values were found in urines from patients with active generalized and localized scleroderma and in some cases of dermatomyositis. Only the group of generalized scleroderma was included in this study.

Hydrolysed non-precipitated urines gave a much stronger chromogen than the precipitated aliquots. Scanning of the chromogen showed that, while the hydrolysed precipitated samples had only one peak of λ max at 560 nm, indicating Hyl, the non-precipitated material gave a very broad peak with λ max at 450 nm, which extended over that of Hyl at 560 nm. The peak at 450 nm was due to interference of urea (5) and, in consequence, the acetone precipitation was necessary to eliminate this interference. When the assay was performed on non-precipitated material without oxidation with $NaIO_4$, which is a necessary step for Hyl determination, only the peak at 450 nm appeared (5). There was no peak at 450 nm or at 560 nm with the hydrolysed precipitated aliquot under the indicated conditions.

Although the Hyl standard alone did not precipitate with acetone, when the standard was added to urine and then precipitated with acetone, an 80% recovery of the added standard (free Hyl) was obtained after hydrolysis of the precipitate. Unhydrolysed precipitated samples gave a turbidity appearing as a peak, which interfered with the determinations. Thus, precipitation, oxidation, and hydrolysis of the precipitated fraction are necessary in order for the Hyl assay to give optimal results.

When 1 ml aliquots of urine were precipitated with 5, 9 or 19 ml of acetone, similar values of Hyl were found after hydrolysis with 6 N HCl. Duplicate samples of urine, precipitated with the above-mentioned volumes of acetone and hydrolysed under similar conditions, showed increasing values of Hyp. Optimum precipitation of Hyl was obtained when the pH of the urine was within the range 7–10.

Maximum hydrolysis of peptides or glycopeptides containing Hyl were obtained when hydrolysis of the "1+5" precipitate was performed in 2 to 6 N HCl for 16 h at 110°C. The yields of Hyl were lower when the samples were heated under similar conditions in 0.001 to 1.5 N HCl.

The yield of Hyl by hydrolysis at 110°C at 6 N HCl increased from 30 min onward, a plateau being reached after 16 h of hydrolysis.

When the precipitated urine sample was oxidized with $NaIO_4$ and then reprecipitated, hydrolysed, evaporated and analysed for Hyl, it was found that 61% of the total Hyl resisted the periodate oxidation. In consequence, 39% of the Hyl was unglycosylated, this fraction being sensitive to periodate oxidation before hydrolysis.

*Total hydroxyproline.* Maximum hydrolysis yield was obtained in 6 N HCl at 110°C for 16 h. With HCl concentrations higher than 6 N under similar hydrolysis conditions a similar yield was obtained.

*Hydroxyproline-containing peptides.* When 2 N, 4 N, 5 N and 6 N HCl were used to hydrolyse the fractions "1+5", "1+9" and "1+19" at 110°C for 16 h, a similar content of Hyp was found for the same fraction with the different HCl concentrations.

The precipitated fractions were collagenase-sensitive. A low salt concentration gave a low yield of reprecipitated material.

Table I. *Urinary output of collagen metabolites by patients suffering from generalized scleroderma*

Figures represent mean ±standard deviation of the mean

| Age (decades) Grades of activity | No. | Hydroxyproline Total mg | 1+5 mg | 1+5 % of total | Hydroxylysine (mg) | Ratio Total Hyp to total Hyl | 1+5 Hyp to Hyl |
|---|---|---|---|---|---|---|---|
| *1st decade* | | | | | | | |
| Controls | 4 | 51.27± 1.21 | 12.75±2.28 | 20.00±1.19 | 6.80±1.18 | 8.78±1.32 | 1.97±0.14 |
| *2nd decade* | | | | | | | |
| Controls | 6 | 75.50± 9.11 | °°°°34.65±2.02 | °°°°44.58±8.07 | °15.20±2.04 | 4.89±0.32 | 2.27±0.21 |
| Grade I | 9 | 94.06±10.05 | 40.13±5.66 | 39.36±5.11 | 12.96±0.77 | 7.16±0.23 | 2.91±0.32 |
| *3rd decade* | | | | | | | |
| Controls | 5 | 52.20± 4.08 | 13.62±1.50 | °25.71±1.50 | 9.72±0.81 | 6.43±0.60 | 1.27±0.07 |
| Grade I | 4 | 65.97± 2.86 | **22.37±2.03 | *32.84±2.04 | 11.80±1.70 | 5.92±0.61 | ***1.99±0.20 |
| Grade III | 2 | 52.45±10.75 | 13.50±1.11 | *33.33±2.11 | 10.90±1.05 | 6.11±0.41 | *1.58±0.11 |
| Grade IV | 2 | 40.15± 7.21 | 17.22±1.11 | **43.14±6.01 | 10.50±1.61 | 3.81±0.10 | *1.64±0.32 |
| *4th decade* | | | | | | | |
| Controls | 13 | 40.51± 2.93 | 9.80±1.27 | 24.30±1.30 | 7.69±1.21 | 6.11±0.62 | 1.35±0.08 |
| Grade I | 13 | 40.62± 3.05 | *13.42±1.19 | ***33.65±3.05 | 9.95±0.84 | 3.93±0.42 | 1.27±0.01 |
| Grade IV | 5 | 50.74± 5.10 | ****20.70±1.80 | ****40.23±3.02 | 11.74±1.21 | 4.61±0.73 | ***1.79±0.01 |
| Grade V | 1 | 61.60 | 29 | 47.07 | 11.30 | 5.45 | 2.57 |
| *5th decade* | | | | | | | |
| Controls | 11 | 42.96± 6.26 | 10.05±1.56 | 23.00±4.01 | 10.17±1.11 | 4.18±0.31 | 0.95±0.06 |
| Grade I | 6 | *68.53±11.04 | *23.10±2.33 | *33.71±5.20 | *19.20±2.60 | 3.61±0.40 | *1.16±0.18 |
| Grade III | 2 | 21.55± 2.91 | 7.40±0.91 | *34.09±1.11 | 6.72±0.42 | 3.11±0.16 | 1.07±0.04 |
| Grade IV | 2 | 61.45± 2.52 | **26.40±3.40 | **48.96±1.03 | **24.60±1.10 | *2.49±0.11 | 1.06±0.13 |
| Grade V | 6 | *64.97± 6.90 | ****34.07±4.10 | ****55.00±3.02 | ****21.23±2.63 | 3.94±1.42 | **1.93±0.55 |
| *6th decade* | | | | | | | |
| Controls | 16 | 44.56± 2.10 | 10.58±0.84 | 19.40±2.51 | 8.90±0.71 | 5.23±0.82 | 1.17±0.03 |
| Grade I | 9 | 46.80± 3.12 | ****19.90±1.73 | ****40.40±2.10 | ****13.71±0.92 | 3.50±0.28 | ****1.37±0.01 |
| Grade IV | 6 | ***62.00± 7.05 | ****23.05±3.12 | ****42.00±3.14 | ****15.60±2.11 | 4.18±0.57 | ****1.53±0.01 |
| *7th decade* | | | | | | | |
| Controls | 15 | 40.15± 3.81 | 7.81±1.26 | 20.88±0.60 | 7.42±0.63 | 5.62±0.37 | 1.11±0.04 |
| Grade I | 3 | *22.67± 5.19 | 7.99±1.21 | ****33.17±3.08 | 6.30±1.05 | 3.72±0.70 | 1.22±0.01 |
| Grade II | 1 | 23 | 4.38 | 19.04 | 3 | 7.67 | 1.46 |
| Grade III | 2 | 42.80± 3.42 | 12.90±0.49 | ****32.10±2.02 | 8.80±0.42 | **5.07±0.13 | 1.46±0.12 |
| Grade IV | 8 | 44.37± 4.17 | *12.70±1.35 | ****30.00±3.01 | ***9.80±0.77 | ****4.54±0.33 | 1.49±0.02 |
| Grade V | 3 | 36.00± 7.16 | 12.17±2.40 | ****33.00±2.11 | 8.90±2.13 | 4.30±0.72 | 1.47±0.30 |

* significantly different from the controls at the 5%, ** at the 2%, *** at the 1%, and **** at the 0.1% level of probability. °–°°°°: Statistical significance of difference from 1st decade controls.

*Effect of dialysis.* After 120 min dialysis, the Hyp content of the "1+9" and "1+19" equalled that of the "1+5" fraction. There was no difference in the Hyl content of the three mentioned fractions and it was similar to that of the non-dialysed "1+5" precipitate. With shorter dialysis time, fractions "1+9" and "1+19" showed decreasing Hyp contents until equality with the "1+5" fraction was reached. Standard Hyl-Gal-Glu dialysed completely after 120 min.

*Scleroderma*

The results of the analyses of Hyp and Hyl on urines of scleroderma patients and controls are shown in Table 1. There was no significant difference in the contents of total Hyp, the "1+5" Hyp-containing fraction, and Hyl between the urines of control individuals in their first, third, fourth, fifth, sixth and seventh decade. In the second decade, there was an increased urinary output of collagen metabolites, as represented by "1+5"

Table II. *One patient aged 56 years suffering from generalized scleroderma, followed-up by determinations of urinary hydroxyproline and hydroxylysine during his improvement under treatment with* D-*penicillamine*

For comparison, the average values ±standard deviations of 200 normal controls aged 40–60 are given

| Sample | Date (day, month, year) | Hydroxyproline (mg/24 h) | | | | | Hydroxy-lysine (mg/24 h) | Ratios | |
|---|---|---|---|---|---|---|---|---|---|
| | | Total | "1+5" | "1+9" | "1+19" | Non-precipitated | | Total Hyp/Hyl | "1+5" Hyp/Hyl |
| 1 | 2/9/72 | 49.30 | 26.70 | | | | 14.10 | 3.50 | 1.89 |
| 2 | 3/9/72 | 41.40 | 23.80 | | | | 14.30 | 2.90 | 1.66 |
| 3 | 12/10/72 | 52.40 | 24.00 | | | | 14.30 | 3.66 | 1.68 |
| 4 | 13/10/72 | 44.90 | 24.00 | | | | 14.40 | 3.12 | 1.66 |
| 5 | 1/9/73 | 43.00 | 20.24 | | | | 16.40 | 2.62 | 1.23 |
| 6 | 2/9/73 | 37.00 | 14.30 | | | | 13.60 | 2.72 | 1.05 |
| 7 | 24/4/74 | 65.30 | 19.10 | | | | 17.20 | 3.70 | 1.11 |
| 8 | 25/4/74 | 54.80 | 15.80 | | | | 14.80 | 3.60 | 1.06 |
| Controls (200) | | 41.12±1.57 | 8.8±1.08 | 6.97±0.43 | 8.68±0.45 | 18.20±0.64 | 6.87±0.33 | 5.98 | 1.27 |

| | Percentage of Hyp fractions in relation to total Hyp (T) | | | | |
|---|---|---|---|---|---|
| | "1+5" | T–"1+5" | "1+9" | "1+19" | Non-precipitated T–"1+19" |
| 1 | 53.98 | 46.02 | | | |
| 2 | 57.27 | 42.73 | | | |
| 3 | 45.73 | 54.27 | | | |
| 4 | 45.73 | 54.27 | | | |
| 5 | 46.81 | 53.19 | | | |
| 6 | 39.64 | 60.34 | | | |
| 7 | 29.20 | 70.80 | 38 | 11.40 | 21.40 |
| 8 | 28.80 | 71.20 | 29.20 | 17.20 | 24.80 |
| Controls (200) | 22.27±0.38 | 77.73 | 15.76±0.55 | 20.11±0.42 | 48.07±0.48 |

Hyp and Hyl. Analyses of the fractions containing Hyp in 6 normal teen-agers showed that the only fraction which was really increased up to 13 years of age was the "1+5" fraction. No sex differences were observed in the parameters studied, viz. Hyp and Hyl, in the different decade groups.

Patients with generalized scleroderma in an active phase showed increased urinary excretion of "1+5" Hyp and Hyl. Table I shows the increase in the "1+5" fraction which closely correlates with the degree of activity of the generalized scleroderma. This increase is most clearly demonstrated by the percentage of "1+5" Hyp in relation to total Hyp. The ratio total Hyp/Hyl showed a slight decrease in active generalized scleroderma. Table II shows the change from active to non-active generalized scleroderma (clinical improvement) in one patient on treatment with D-penicillamine.

The ratio of Hyp to Hyl in fraction "1+5" was more constant than that of total Hyp to Hyl. Urinary excretion of Hyl followed the same pattern as that of "1+5" Hyp. No statistically significant differences were observed in relation to sex or diet in the patients with generalized scleroderma, active or non-active, belonging to the second decade.

## DISCUSSION

Hyp and Hyl originate from simultaneous hydroxylation of prolyl and lysyl residues of protocollagen, as catalysed by protocollagen–proline and protocollagen–lysine hydroxylases with the require-

ment of similar co-factors. The ratio of these two amino acids characteristic of collagen and related glycoproteins should be constant, unless an abnormal collagen is synthesized, and provided their metabolic rate is similar under normal and pathological conditions.

Our results for total Hyp are in agreement with those of Jasin et al. (8) as to the higher levels of Hyp excreted in the second decade of life. The increased urinary excretion of Hyp and Hyl in puberty may originate from the increased biosynthesis and metabolism of collagen with growth. On the other hand, increased excretion of Hyp found in association with burns, appears to be the result of enhanced catabolism with no compensatory component (7, 9).

Smith et al. (14) did not find any significant alteration in the urinary output of Hyp by patients with generalized scleroderma or morphea. Neither did Ziff et al. (16) find any difference between the urinary output of Hyp in scleroderma patients and normal controls. Korting & Holzmann (10) found an increased hydroxyprolinuria in scleroderma patients with clinical improvement under treatment with progestatives. Rodnan et al. (13) reported on increased total urinary excretion of Hyp in active phases of scleroderma.

Our studies show that "total hydroxyproline" only represents an average sum of the fractions, which themselves do not change in a parallel way. In fact, one may find values of total hydroxyproline within the normal range with an increased or decreased "1+5" fraction. In active phases of generalized scleroderma an increase in collagen metabolism was shown. This was particularly evident from the variation in the percentage of Hyp in the "1+5" fraction.

The increase of the "1+5" fraction in the active phases of generalized scleroderma probably represents an active biosynthesis and metabolism of collagen. According to Krane et al. (11) the high molecular weight material is the most recently synthesized. It is evident that the "1+5" fraction corresponds to the high molecular weight peptides, especially if one considers that, after a 2 h dialysis, the Hyp content of the fractions precipitated with 9 and 19 ml acetone becomes equal to that of the "1+5" fraction.

It is worth noting that Hyl is precipitated *in toto* by addition of 5 vol acetone to urine, while only about 20% of the total Hyp is normally present in that fraction. The collagenase sensitivity of the precipitated fraction confirms their collagen origin. Although, in man, most of the urinary Hyp is present in the form of low molecular weight peptides which are readily dialysable, a small fraction is present in the form of higher molecular weight material (4 500–10 000). The amino acid composition of the latter fraction has shown that not only is Hyp present, but also Hyl (11).

The fact that Glu-Gal-Hyl standard in urine is dialysable while the Hyl content of the "1+5" fraction is practically non-dialysable indicates that free Glu-Gal-Hyl or Gal-Hyl *per se* cannot be taken as representatives of the Hyl excreted in urine.

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G. Asboe-Hansen, M.D.
Department of Dermatology
Rigshospital
Blegdamsvej 9
DK-2100 Copenhagen
Denmark