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POLYMORPHISM OF URINARY 4-HYDROXYPROLINE-CONTAINING POLYPEPTIDES

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SUMMARY

Using molecular sieve chromatography on Bio-Gel P-2 and then on Bio-Gel P-30, hydroxyproline-containing urinary polypeptides (molecular weight $\geq$ 1500 daltons) were separated into eight fractions. The three main fractions were separated further on phosphocellulose giving seven, nineteen and twelve peaks, respectively, each containing 4-hydroxyproline. Hypotheses about the origin of certain polypeptides are proposed, which take into account the sequence of type I collagen. Among these 38 polypeptides only one shows a quantitative variation in Paget's bone disease and was thus purified. It consists of equal amounts of glycine, proline and 4-hydroxyproline. This particular polypeptide may originate from the N-terminal propeptide of type I collagen.

INTRODUCTION

4-Hydroxyproline (4-Hyp)-containing peptides derive mostly from collagen metabolism, except for the small amounts that are present in elastin, the C_1 q fraction of complement and acetylcholine esterase. General improvements in techniques have resulted in a deeper knowledge of peptides from human urine [1—12]. Important progress has been made in the study of collagen [13, 14] and at present five different types have been described: types I, II, III, IV and AB. Several of the major metabolic steps of collagen synthesis have been elucidated and the precursors preprocollagen and procollagen have been isolated. The amino acid sequence of the N-terminal type I collagen propeptide has been established, showing the presence of five residues of 4-Hyp [15]. These results led us to re-investigate the urinary polypeptides in order to reveal whether or not some of the peptide population

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originates from the pro-peptides and if they can be distinguished from the remainder derived from collagen metabolism.

We describe in this paper experiments showing the number of hydroxyproline-containing polypeptides and initial results concerning the structure and origin of one of them.

EXPERIMENTAL

All reagents were purchased from Merck (Darmstadt, G.F.R.), except for *p*-dimethylaminobenzaldehyde (Carlo Erba, Milan, Italy) and Chloramine T (Prolabo, Paris, France). Any other sources are mentioned later.

In order to obtain sufficient amount of peptides and to simplify the problems of separation we used urinary polypeptides pooled from five patients suffering from Paget's bone disease. The 4-Hyp urinary output consisted of between 3120 and 6350 μmol per 24 h. A 500-ml volume of urine was

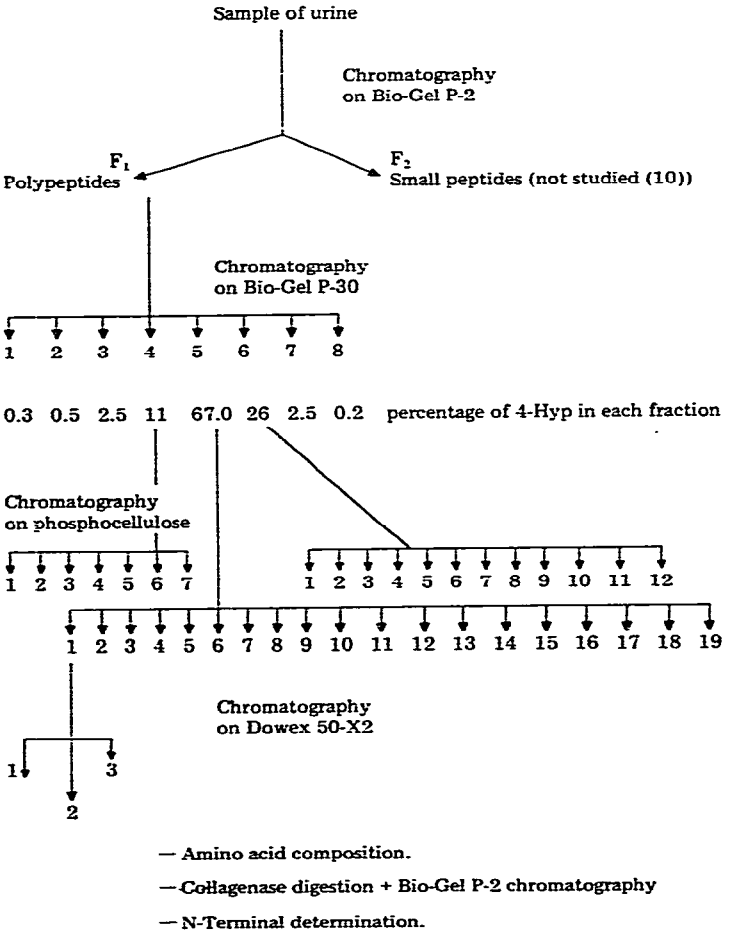

Fig. 1. Analytical procedure for the study of 4-hydroxyproline-containing urinary polypeptides.

used for the study. Previous work had shown a reproducible pattern obtained with different subjects [10, 16]. The experimental procedure is outlined in Fig. 1. The urine sample was lyophilized and dissolved in 50 ml of 0.1 mol/l acetic acid. Aliquots of 5 ml were layered on to a 90 × 2.6 cm column of Bio-Gel P-2 (50—100 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.) and eluted with the same solution at a flow-rate of 60 ml/h. The effluent was collected in 5-ml fractions with a Gilson automatic collector. 4-Hyp and all amino acids were measured in each tube after alkaline hydrolysis using an automatic system [16]. The material eluted in the void volume and recovered from the Bio-Gel column was lyophilized and dissolved in 10 ml of 0.1 mol/l acetic acid and layered on to a 90 × 2.6 cm column of Bio-Gel P-30 (100—200 mesh) (Bio-Rad Labs.). The analytical procedure was similar to that described above. The main fractions recovered were lyophilized and dissolved in an acetate buffer (0.01 mol/l, pH 3.8) and analysed on a 30 × 1.6 cm column of phospho-cellulose P11 (Whatman, Maidstone, Great Britain) [17]. The column was eluted with a solution of increasing ionic strength obtained with a four-chamber Varigrad (Boskamp, Strasbourg, France). The first three compartments contained buffered solutions of sodium acetate (0.01 mol/l, pH 3.8) and the last contained in addition 1 mol/l sodium chloride. The total volume of solution was 800 ml.

The peptides were eluted at a flow-rate of 60 ml/h and the eluate was collected with an automatic collector in 5-ml fractions. Some of the peptides were purified by chromatography on a 30 × 1.6 cm column of Dowex 50-X2 (200—325 mesh) (Bio-Rad Labs.) and eluted with 0.5 mol/l acetic acid. Clostridium collagenase (Worthington, Freehold, NJ, U.S.A.) was used under the conditions described by Krane et al. [2]. The reaction products were separated on Bio-Gel P-2 as described above.

The amino acid compositions were determined for peptides hydrolysed with 5.6 mol/l hydrochloric acid at 105°C for 24 h. The procedure involved the use of a Beckman Multichrom B analyser as described previously [18]. The determination of N-terminal amino acids were made according to the procedure of Gray and Hartlay [19] using silica gel thin-layer plates (Merck, Darmstadt, G.F.R.). 4-Hydroxyproline was also determined as described previously [20]. The percentage of 4-Hyp in each peak was deduced from the area calculated on the chromatogram.

RESULTS

The behaviour of urinary polypeptides on Bio-Gel P-2 is illustrated in Fig. 2. We term polypeptides the molecules eluted in the void volume on the Bio-Gel P-2 column corresponding to an apparent molecular weight of 1500 daltons. The separation of the polypeptides (fraction F1) on Bio-Gel P-30 is shown in Fig. 3. The peaks were numbered from P30-1 to P30-8. Most (95%) of the total 4-Hyp is present in the three peaks P30-4, -5 and -6. These fractions were submitted to complementary fractionation procedures.

Each of the three peaks were analysed by chromatography on phospho-cellulose and showed different patterns, as illustrated in Fig. 4a, b and c, respectively. The amount of hydroxyproline in each peak was calculated

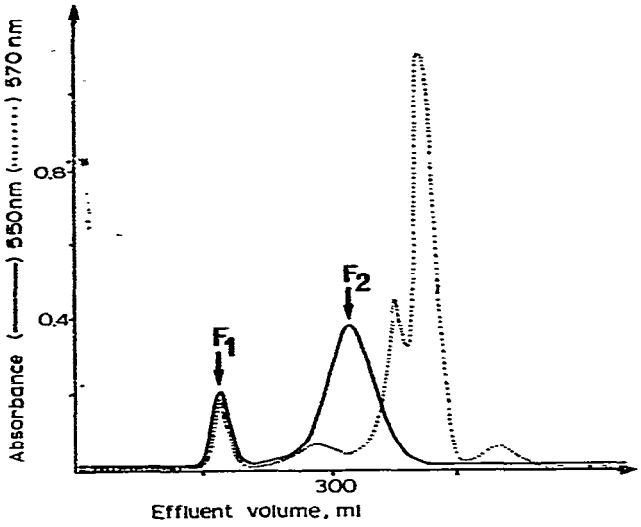

Fig. 2. Chromatography on Bio-Gel P-2 of one sample of urine. Solid line, automatic detection of 4-Hyp in each fraction; broken line, automatic detection of all peptidic molecules with ninhydrin. The first fraction, F_1, is then fractionated on Bio-Gel P-30.

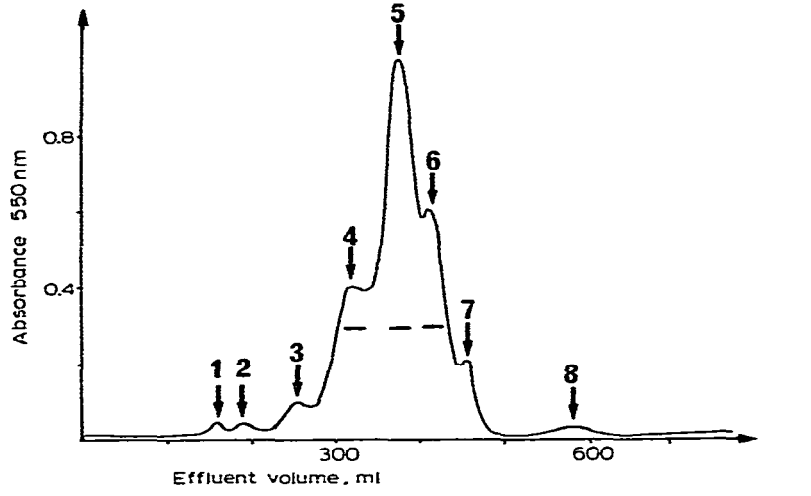

Fig. 3. Chromatography on Bio-Gel P-30 of the polypeptide recovered from the Bio-Gel P-2 column. 4-Hyp is detected in each fraction with an automatic device. Peaks 4, 5 and 6 are separated by chromatography on phosphocellulose.

as a percentage of total polypeptide 4-Hyp [10]. Between normal subjects and patients with Paget's bone disease, only one such fraction showed quantitative variations and was identified in this system as the fraction P30-5-1. This particular fraction contained high levels of glucosamine and galactosamine in addition to amino acids and was purified by chromatography on Dowex 50-X2 (Fig. 5). The purified peptide was digested with bacterial collagenase and its amino acid composition is given in Table II. The amino acid compositions of all peptides are given in Tables I—III.

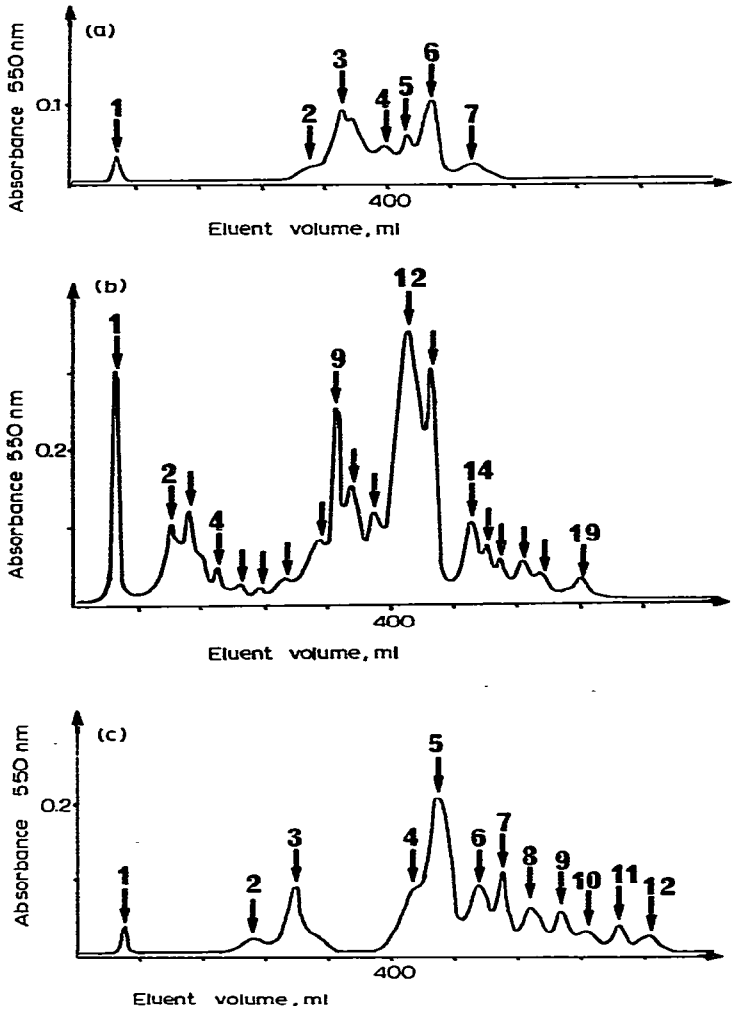

Fig. 4. (a) Elution on phosphocellulose of polypeptides contained in fraction P30-4. 4-Hyp is detected in each fraction. (b) Elution on phosphocelullose of polypeptides contained in fraction P30-5. (c) Elution on phosphocellulose of polypeptides contained in fraction P30-6.

DISCUSSION

About one fifth of the total urinary 4-Hyp is present in polypeptide sequences [21]. The technique we have used permits the separation of at least 38 fractions of large-sized polypeptides. In fact, we have not demonstrated the homogeneity of each fraction and it is possible that some of them may contain several different molecules. As a result of the large number of different fractions, each peptide represents only a small percentage (0.1—8%) of the total polypeptide 4-Hyp. These quantitative results may explain why previously only a small number of the polypeptidic fractions have been isolated, viz., ten by Krane et al. [2] and more recently only six by Dubovsky

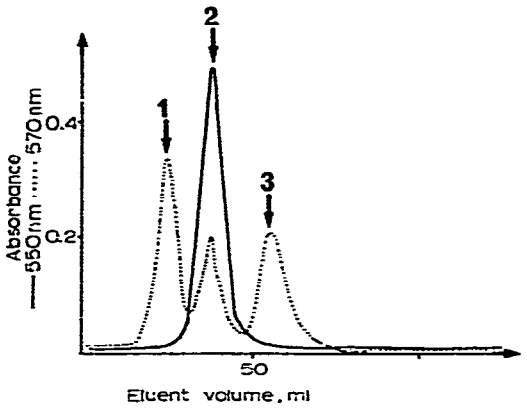

Fig. 5. Separation of the peak P30-5-1 on Dowex 50-X2. Solid line, specific detection of 4-Hyp; broken line, detection of all molecules with ninhydrin after alkaline hydrolysis. Peak 2 contains the polypeptide $(Gly—Pro—4\text{-}Hyp)_n$.

TABLE I

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-4 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4a)

The fraction P30-4 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak						
	1	2	3	4	5	6	7
4-Hyp	2.6	9.9	8.5	7.8	7.8	8.8	5.0
Asp	14.2	9.3	7.0	5.9	6.4	8.7	7.5
Thr	11.9	7.3	3.7	4.1	3.5	4.0	4.4
Ser	8.3	5.7	3.9	3.7	4.1	5.9	6.5
Glu	9.1	8.9	7.7	8.9	9.3	10.1	12.0
Pro	14.1	9.8	12.0	10.0	9.8	7.7	8.1
Gly	10.2	21.9	27.7	29.6	30.1	28.3	27.4
Ala	8.6	8.7	12.2	13.3	11.9	9.3	7.5
Val	7.6	3.7	2.7	2.8	2.4	1.9	1.8
Met	—	—	—	—	—	—	—
Ile	2.2	1.7	1.1	0.8	0.6	0.6	0.4
Leu	5.8	3.1	1.9	1.9	1.6	1.5	1.9
Tyr	1.3	0.5	—	0.8	0.7	—	—
Phe	3.1	1.5	0.7	0.9	0.9	0.8	1.2
Hyl	—	0.4	0.6	—	—	0.7	0.6
Lys	—	4.5	6.9	4.6	5.5	4.2	6.7
His	0.5	1.4	0.9	1.2	0.9	1.8	1.8
Arg	0.8	1.6	2.2	3.7	4.7	5.8	7.4
Glc-NH_2	48.7	15.1	26	3.5	1.5	0.9	—
Gal-NH_2	16.1	2.5	—	—	—	—	—

TABLE II

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-5 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4b)

The fraction P30-5 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak																			
	1	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
4-Hyp	13.3	32.4	13.3	19.6	17.2	11.1	9.7	7.8	9.2	10.3	10.6	11.7	9.3	11.0	9.5	9.0	8.0	9.4	8.5	8.8
Asp	11.6	1.0	10.7	4.8	5.5	6.2	7.6	8.6	6.9	10.8	11.4	9.1	9.8	9.5	7.7	7.5	6.9	8.7	8.5	8.8
Thr	7.0	0.6	4.4	3.5	5.4	9.1	7.3	5.8	4.2	2.5	3.0	3.1	2.1	3.2	3.1	2.9	3.0	3.0	3.5	3.8
Ser	6.3	1.2	9.1	6.5	5.1	4.5	5.5	5.1	4.5	3.5	3.6	3.3	3.1	4.7	5.0	4.8	4.7	4.3	4.8	4.2
Glu	6.6	—	5.0	5.0	5.4	7.1	9.2	9.2	9.0	6.1	6.7	8.4	8.8	10.1	10.5	10.5	10.6	10.8	11.1	11.4
Pro	21.3	31.2	10.7	27.1	21.6	20.3	14.4	12.8	12.4	4.9	8.1	9.8	10.1	8.4	8.0	9.0	9.6	6.7	5.1	4.8
Gly	18.3	33.6	26.2	26.8	28.9	24.0	24.4	24.4	26.3	31.1	29.9	29.4	31.6	29.9	31.3	29.9	29.1	29.1	27.7	25.7
Ala	3.8	—	8.6	2.1	5.8	6.6	8.0	9.9	10.9	18.2	9.6	12.5	14.2	8.6	8.6	9.2	9.8	6.9	6.4	2.5
Val	3.9	—	4.3	0.7	1.9	2.1	2.5	3.3	3.4	0.8	1.9	2.0	1.8	2.3	1.3	1.5	1.6	1.4	2.2	1.3
Met	0.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ile	0.8	—	1.2	0.2	0.4	0.6	0.8	1.1	1.0	0.5	0.6	0.4	0.6	0.6	0.5	0.5	0.4	0.6	0.9	1.0
Leu	2.5	—	2.7	1.2	1.9	4.0	3.2	3.1	3.0	1.7	1.6	0.9	0.6	1.2	0.9	0.8	0.9	1.5	2.1	1.9
Tyr	0.7	—	1.2	—	—	—	0.6	0.7	0.4	0.3	0.2	0.2	—	—	—	—	—	—	—	—
Phe	1.4	—	2.4	—	0.4	0.6	0.9	1.1	1.3	0.5	0.5	0.4	—	—	—	—	—	—	0.9	—
Hyl	—	—	—	—	—	—	0.6	—	0.4	0.5	0.7	0.6	0.6	0.8	0.9	1.2	1.5	2.1	2.2	1.1
Lys	1.3	—	—	2.3	0.4	3.0	3.3	4.2	4.9	5.8	9.4	5.1	6.9	5.5	5.7	5.7	5.7	6.7	6.8	12.3
His	0.2	—	—	—	—	—	0.6	0.8	0.6	0.4	0.6	0.6	0.6	0.8	1.1	1.1	1.1	1.9	2.4	3.6
Arg	0.3	—	—	—	0.4	0.8	1.1	2.0	1.6	2.0	1.5	2.4	0.7	3.3	5.9	6.4	7.2	6.9	6.8	8.8
Glc-NH_2	34		32																	
Gal-NH_2	9.4		—																	

*Amino acid composition of the fraction P30-5-1 purified on Dowex 50-X2.

TABLE III

AMINO ACID COMPOSITIONS OF POLYPEPTIDES CONTAINED IN FRACTION P30-6 AND SEPARATED BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE (FIG. 4)

The fraction P30-6 was obtained during Bio-Gel P-30 chromatography (Fig. 3). Results are given as residues per 100 residues.

Amino acid	Peak											
	1	2	3	4	5	6	7	8	9	10	11	12
4-Hyp	13.2	9.3	14.9	11.9	16.1	9.3	9.5	9.0	9.9	10.7	8.7	5.7
Asp	10.9	10.7	7.5	11.4	9.0	9.4	12.0	9.6	9.4	11.1	11.8	9.7
Thr	4.8	6.4	3.1	3.1	2.4	2.1	3.1	3.6	3.3	2.7	2.8	3.3
Ser	8.8	5.7	3.6	4.1	3.5	8.2	5.5	5.6	6.4	4.7	5.0	5.2
Glu	17.7	14.0	9.7	11.0	9.5	12.8	11.1	11.6	12.7	11.7	13.0	12.4
Pro	4.7	8.5	14.7	8.0	11.3	7.0	4.7	7.7	5.8	4.0	3.4	3.2
Gly	25.7	24.0	31.4	26.8	29.0	29.8	27.6	26.9	25.0	24.1	23.0	24.0
Ala	6.3	6.7	5.0	6.5	8.4	4.1	8.0	6.8	7.6	7.9	5.9	7.4
Val	1.4	3.4	1.2	2.7	1.0	1.1	2.0	1.5	1.4	2.2	1.7	1.8
Met	—	—	—	—	—	—	—	—	—	—	—	—
Ile	0.7	1.0	0.4	0.7	—	0.4	0.5	0.4	—	—	1.3	1.3
Leu	2.5	4.0	1.9	2.8	1.0	1.2	1.5	1.2	1.7	2.7	3.1	3.2
Tyr	1.2	—	—	1.0	—	—	—	—	—	—	—	—
Phe	0.7	1.0	—	0.5	—	—	—	—	—	—	—	—
Hyl	—	—	0.3	0.5	0.8	1.6	1.4	1.3	1.2	1.5	1.1	1.7
Lys	1.3	3.3	2.6	5.8	3.8	9.3	7.0	6.7	6.3	8.0	9.1	9.5
His	—	—	—	0.3	—	0.5	1.1	1.1	1.3	2.4	2.1	2.8
Arg	—	1.6	3.7	2.5	4.2	3.1	5.1	7.0	7.9	6.4	8.0	8.8
Glc-NH_2	3.5											
Gal-NH_2	3.5											

and Meyer [22]. None of them have been either purified or characterized. The techniques we used here are not adequate for the rapid or complete isolation of these components.

Amino acid analyses of fractions P30-5-3 and P30-5-4 showed high levels of 4-Hyp, Pro and Gly. Fractions P30-5-9 and P30-5-12 are characterized by their content of 4-Hyp, Pro, Gly and Ala. These results correspond to several amino acid sequences in types I and III collagen. Half of the fractions contain a small amount (less than 1.7%) of Hyl and none contain 3-Hyp. It is probably for this reason that basement membrane collagen metabolism results in very few polypeptides. The separation patterns we have obtained for the polypeptides from different pathological situations were identical [12], except for those from patients suffering from Paget's bone disease. In these instances we found that the fraction labelled P30-5-1 increased significantly. It contains 4.1 ± 0.2% (n = 12) of the polypeptidic 4-Hyp. In normal subjects (n = 15) this fraction contains only 2.3 ± 0.3% of the polypeptidic 4-Hyp. This fraction was purified by chromatography on Dowex 50-X2 and separated into three peaks as shown in Fig. 5. The second peak eluted contained essentially three amino acids, Gly, Pro and 4-Hyp, corresponding

to the probable sequence $(Gly—Pro—4\text{-}Hyp)_n$. This polypeptide was digested with bacterial collagenase. No N-terminal amino acid could be identified. The molecular weight and amino acid composition of this component plus the quantitative variation associated with Paget's bone disease supported the concept that it may originate from the amino terminal extension of procollagen.

Further studies using labelled proline and antibodies might provide further information about the metabolic pathway and the physiopathological regulation mechanism of the different types of collagen.

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REFERENCES

1 J.D. Smiley and M. Ziff, Physiol. Rev., 44 (1964) 30.
2 S.M. Krane, A.J. Munoz and E.J.R. Harris, J. Clin. Invest., 49 (1970) 716.
3 R. Bourillon and J.L. Vernay, Biochim. Biophys. Acta, 117 (1966) 319.
4 M.G. Cherian and A.N. Radhakrishnan, Biochim. Biophys. Acta, 101 (1965) 291.
5 K. Kivirikko, in C.A. Hall and D.S. Jackson (Editors), International Review of Connective Tissue Research, Academic Press, New York, 1971, Vol. 5, p. 235.
6 E.M. Wagner, B.R. Kaplean and S.L. Shepard, Biochim. Med., 13 (1975) 343.
7 S. Ansorge and H. Hanson, Hoppe-Seyler's Z. Physiol. Chem., 348 (1967) 334.
8 R. Cahill, R. Smith and R.G. Westall, Clin. Sci., 38 (1970) 519.
9 N. Steiner and A. Niederwieser, Clin. Chim. Acta, 92 (1979) 431.
10 A.G. Szymanowicz, A. Malgras, A. Randoux and J.P. Borel, Biochim. Biophys. Acta, 576 (1979) 253.
11 E. Svojtková, K. Macek, Z. Deyl and M. Adam, J. Chromatogr., 183 (1980) 221.
12 G. Laurain, C. Behar and A.G. Szymanowicz, Clin. Chim. Acta, 109 (1981) 45.
13 D.J. Prockop, K.I. Kivirikko, L. Tuderman and N.A. Guzman, N. Engl. J. Med., 301 (1979) 13.
14 D.J. Prockop, K.I. Kivirikko, L. Tuderman and N.A. Guzman, N. Engl. J. Med., 301 (1979) 77.
15 D. Horlein, P.P. Fietzek, E. Wachter, C.M. Lapiere and K. Kühn, Eur. J. Biochim., 99 (1979) 31.
16 A.G. Szymanowicz, Ph.D. Thesis, University of Reims, 1979.
17 K.A. Piez, in G.N. Ramachandran and A.H. Reddi (Editors), Biochemistry of Collagen, Plenum Press, New York, 1976, p. 1.
18 A.G. Szymanowicz, G. Poulin, N. Fontaine, J.P. Werquin and J.P. Borel, J. Chromatogr., 190 (1980) 457.
19 N.R. Gray and B.S. Hartlay, Biochem. J., 89 (1963) 59.
20 A.G. Szymanowicz, A. Randoux and J.P. Borel, Ann. Biol. Clin., 37 (1979) 113.
21 A.G. Szymanowicz, P.M. Joubert, A. Garbe and J.P. Borel, Clin. Chim. Acta, 100 (1980) 155.
22 J. Dubovsky and R.D. Meyer, Clin. Chim. Acta, 62 (1975) 277.