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Composition of Fractions of Denatured Collagen Resolved on Amberlite CG-50

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The nature of the fragments of denatured collagen fractionated [1] on Amberlite CG-50 depends on the conditions of degradation: the proportion of the fragments eluted by sodium hydroxide decreases and the gradient buffer-eluted fraction increases with rising temperature. Only the gradient buffer-eluted fraction of collagens denatured below or at + 65°C yields distinct α-components on gel electrophoresis. The fractions which emerge from the column differ in their contents of polar amino acids and in the ratio of hydroxyproline to proline, but their contents of hydroxy amino acids are equal.

Heat-degraded insoluble collagen can be fractionated on an Amberlite CG-50 column by eluting first with a buffer of pH 5.5, then with a solution of progressively increasing pH and finally with a sodium hydroxide solution.[1] The number of collagen fragments is large and we have usually pooled the eluates into the three fractions. The separation of the fragments into these fractions may be of physiological interest: the first fraction eluted or the "pH 5.5-buffer-eluted fraction", is large when the collagen is either soluble or derived from a young animal.[2] These findings prompted a study of the compositions of the fractions.

EXPERIMENTAL

Preparation of the collagen fractions. Fresh depilated skins of growing guinea pigs or calves were homogenized and extracted three times with a 2- to 10-fold volume (v/w) of 0.45 M sodium chloride with agitation.[3] The neutral salt-soluble collagen in the combined extracts was purified according to Gross.[4]

The residue was extracted four times with a 0.15 M citrate buffer of pH 3.7.[5] The citrate-soluble collagen was purified by precipitation with sodium chloride (added up to 15 %, w/v) and by repeated extraction with citrate buffer and finally precipitated by dialysis against 0.02 M disodium phosphate. All manipulations were carried out at + 4°C.

The precipitate was suspended in a 0.01 M sodium acetate buffer of pH 4.8 and extracted repeatedly with this solution until no more protein dissolved. The insoluble collagen was gelatinized by increasing the temperature stepwise as follows: 15 min at + 40°C, 120 min at + 65°C, 120 min at + 90°C, and finally 120 min at + 120°C.

Chromatography on an Amberlite CG-50 column. Amberlite CG-50 (Type I, 100—200 mesh, from British Drug House Ltd., Poole, England) was prepared as described by Moore, Spackman and Stein,[6] equilibrated with 0.1 M McIlvaine's buffer of pH 5.50 ± 0.02 and allowed to settle to a column of 30 × 2 cm in a water-jacketed tube held at + 38°C.

When the denatured sample (50—100 mg of gelatinized collagen in 0.1 M McIlvaine's buffer of pH 4.0) had drained into the column, it was eluted with 500 ml of the above-mentioned buffer of pH 5.50. Gradient elution was arranged by pumping 1300 ml of 0.5 M disodium phosphate solution to a closed mixing vessel which contained 200 ml of McIlvaine's buffer (pH 5.50) covered with liquid paraffin. The column was then eluted with 800 ml of 0.1 N sodium hydroxide solution and finally with 500 ml of 1.0 N sodium hydroxide solution. All the eluting solvents had been deaerated by heating. The flow rate was about 130—150 ml/h. The eluate was collected in a thermostated siphon in 10 ml aliquots which were pooled. The fractions were located by a modified biuret reaction,[7] lyophilized to reduce the volume, desalted with a Sephadex G-25 column, lyophilized again, and stored frozen.

Amino acid analyses were carried out with an automatic amino acid analyser built according to Spackman, Stein and Moore.[8] The hydrolysis was carried out in 5.7 N hydrochloric acid under nitrogen in sealed tubes at + 110°C overnight.

The collagen components were analysed by *starch-gel electrophoresis* as described elsewhere.[9]

RESULTS AND DISCUSSION

Effect of temperature during the gelatinization. When the temperature of gelatinization is raised to + 120°C both fractions eluted by sodium hydroxide solution almost disappear and are mainly found in the gradient buffer-eluted fraction (Fig. 1).

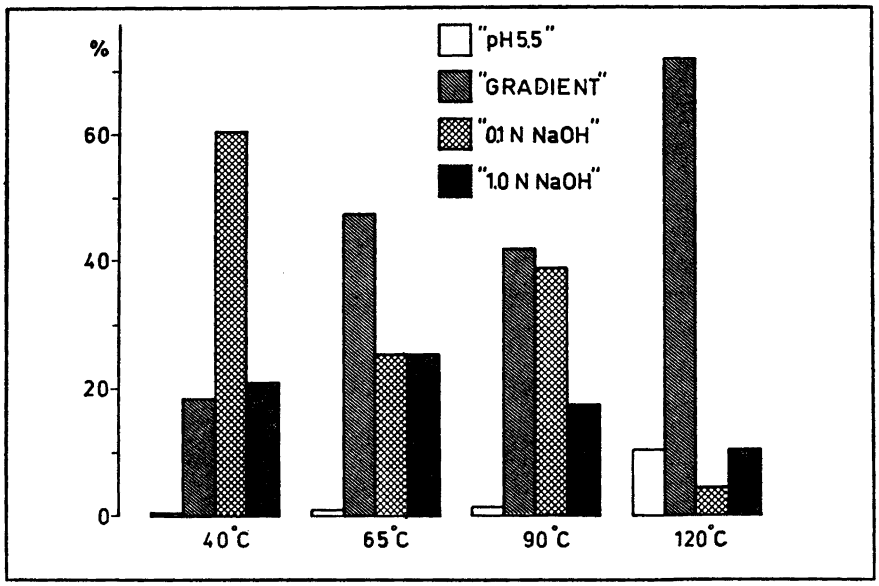

Fig. 1. Effect of temperature during gelatinization on the weight distribution of fractions of insoluble collagen of calf skin resolved on Amberlite CG-50. The ordinate indicates the percentage of each fraction.

Collagen heated above + 65°C had degraded so far that no distinct components could be detected by gel electrophoresis. This agrees with our earlier results.[10] Both fractions eluted by sodium hydroxide from collagens degraded either at + 40°C or at + 65°C did not contain any well-defined electrophoretic components and the pH 5.5-eluted fractions were too small to be studied. However, the gradient buffer-eluted fraction yielded distinct α2- and α1-components. Because the gradient buffer-eluted fraction increases at + 120°C at the expense of the fractions eluted by sodium hydroxide, it thus contains materials of two kinds: the original gradient buffer-eluted α-components (+ 40°C to + 65°C) and fragments of the fractions eluted previously with sodium hydroxide (+ 90°C to + 120°C).

Other studies have shown that the collagen decomposes on heating it above + 65°C in a 0.01 M acetate buffer of pH 4.8, and even at + 40°C if the pH is alkaline. Gelatinization of insoluble calf skin collagen in comparable conditions in 0.01 M hydrochloric acid at + 40°C liberates much more material

Table 1. Amino acid composition of fragments of denatured collagen resolved on Amberlite CG-50. The samples were prepared from the various skin collagens of growing guinea pig. The amino acid values are expressed as moles/1000 residues and the number of analyses is given in parentheses. The values for serine and threonine have not been corrected for the decomposition during hydrolysis and chromatography.

Amino acid	Eluted by buffer of pH 5.5 (3)	Gradient buffer-eluted (4)	Eluted by NaOH (2)
Hydroxyproline	109	106	108
Aspartic acid	54	47	49
Threonine	18	18	18
Serine	32	35	38
Glutamic acid	75	65	71
Proline	113	115	132
Glycine	350	349	359
Alanine	122	118	109
Cysteine	0	0	0
Valine	17	19	24
Methionine	3	5	2
Isoleucine	7	11	12
Leucine	21	22	25
Tyrosine	1	4	0
Phenylalanine	9	12	6
Hydroxylysine	2	3	0
Ornithine	1	0	0
Lysine	28	24	15
Histidine	0	4	0
Arginine	39	47	36
Acidic residues	128	112	120
Basic residues	69	78	51
Excess of acidic residues	59	34	69
Hydroxyproline/proline	0.97	0.91	0.82
Total imino acids	222	220	240
Hydroxyamino acids	162	165	164

than gelatinization in 0.01 M sodium bicarbonate solution (pH 10.5) or in 0.01 M sodium hydroxide.

It was observed repeatedly that the denatured citrate-soluble collagen precipitated on its application to the column unless it had been gelatinized at + 120°C for 120 min.

Amino acid compositions. The mean amino acid compositions of fractions resolved on Amberlite CG-50 are presented in Table 1. The pH 5.5-buffer-eluted fraction had high contents of aspartic acid and glutamic acid but also of lysine, and therefore this fraction is the most polar. The number of proline residues is smaller in the pH 5.5-buffer-eluted and the gradient buffer-eluted fractions than in the fraction eluted by sodium hydroxide but the fractions all contain similar amounts of hydroxyproline. The total number of hydroxyl-bearing residues is the same in all the fractions, which corroborates the hypothesis that hydroxy amino acid content of collagen is constant.[11]

Insoluble collagens and all collagens from aged individuals yield gelatins which contain high proportions of fractions eluted by sodium hydroxide.[2] Because the amino acid composition must be constant in all collagens of the same species, it remains to be investigated whether more heat-resistant fragments are formed in the less polar regions of the collagen molecule on aging.

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REFERENCES

1. Kulonen, E., Virtanen, U. K., Viljanto, J. and Seppälä, P. *Acta Chem. Scand.* **16** (1962) 695.
2. Kulonen, E., Pikkarainen, J., Näntö, V. and Majaniemi, T. *Proteinanalytisches Symposium,* Göttingen, Germany 1963.
3. Gross, J., Highberger, J. H. and Schmitt, F. O. *Proc. Natl. Acad. Sci. U.S.* **41** (1955) 1.
4. Gross, J. *J. Exptl. Med.* **107** (1958) 247.
5. Gallop, P. M. *Arch. Biochem. Biophys.* **54** (1955) 501.
6. Moore, S., Spackman, D. H. and Stein, W. H. *Anal. Chem.* **30** (1958) 1185.
7. Pikkarainen, J. and Kulonen, E. *Suomen Kemistilehti* **B 38** (1965) 226.
8. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
9. Näntö, V., Pikkarainen, J. and Kulonen, E. *J. Am. Leather Chemists' Assoc.* **60** (1965) 63.
10. Pikkarainen, J. and Kulonen, E. *Acta Chem. Scand.* **18** (1964) 1667.
11. Pikkarainen, J. *Acta Physiol. Scand. Suppl.* **309** (1968).

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