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Amino Acid Composition of Dermal-Collagen Fractions in Rats of Different Ages

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González Cadavid, Denduchis & Mancini (1963) showed that aging has two effects on rat-skin collagen: the relative proportion of this protein in the tissue increases, and there is a decrease in the amount of its soluble fractions. The latter phenomenon seems to be quite general, since it occurs in rat's tendon, aorta, uterus, lung, muscle, heart, liver, kidney and spleen (McGavack & Kao, 1960), in rat's bone and cartilage (Kao, Hitt, Dawson & McGavack, 1962) and in the development of skin granuloma in guinea pigs (Jackson, 1957).

To explain the low solubility of older collagen the most favoured hypothesis is based on the aggregation of soluble precursors to form the insoluble adult collagen fibres (Gross, 1959). The amino acid composition of collagen during the aging process has received relatively little attention.

Chvapil & Kobrle (1961) determined proline and hydroxyproline in collagen from lungs and tendons obtained from rats. Other investigations reporting complete amino acid analysis of collagen or gelatin used single samples from various organs of different species, in all cases unrelated to the age of the animal (Eastoe, 1955, 1961; Piez, Weiss & Lewis, 1960; Piez & Gross, 1960; Harding, 1963; Piez, Eigner & Lewis, 1963; see also review by Harrington & Hippel, 1961).

In the present work we determined the amino acid composition of different fractions of rat-skin collagen obtained from newborn, young, adult and senile animals.

MATERIAL AND METHODS

Male Wistar rats were used. The different ages are defined as (*a*) 'newborn': 1 or 2 days old, 6 g. average weight; (*b*) 'young': 2 months old, 220 g. average weight; (*c*) 'adult': 1 year old, 450 g. average weight; (*d*) 'senile': 2 years old, 550 g. average weight. The numbers of rats used were 40 newborn, 4 young, 4 adult and 2 senile. Uniform conditions of living and diet were maintained throughout the whole experiment.

The animals were killed with coal gas and the hair was removed with an electric hair-cutter, followed by a hand razor. After this treatment the skin from the dorsal and ventral areas of the body was removed. Care was taken to avoid all contamination with foreign tissues. The skin pools thus obtained were extracted and purified according to Jackson, Leach & Jacobs (1958) with minor variations (González Cadavid *et al.* 1963). The following collagen fractions were prepared: (*a*) neutral-salt-soluble fraction. This fraction was purified by precipitation with 17% (w/v) sodium chloride. The precipitates were dissolved in sodium chloride solution (*I* 0·50). In the last step this solution was clarified by centrifugation (100 000*g*, 1 hr.), dialysed against distilled water and freeze-dried; (*b*) citrate-soluble fraction. This fraction was purified exactly according to Jackson (1957), and freeze-dried; (*c*) insoluble fraction. The residue from the extractions was transformed into gelatin (Jackson, 1957). The supernatant from the precipitation of this solution with 5% trichloroacetic acid was dialysed against distilled water, filtered and freeze-dried.

Before analysis each sample was dried *in vacuo* over phosphorus pentoxide at 105° for 24 hr. Total hydrolysis of the samples was carried out in 6N-hydrochloric acid thrice distilled in glass. The acid solutions (5 mg./ml.) were heated in vacuum-sealed glass ampoules for 48 hr. at 100°, then taken to dryness in a rotatory evaporator and kept overnight *in vacuo* over sodium hydroxide. The residues were dissolved in 0·2N-sodium acetate buffer, pH 2·2 (Moore & Stein, 1954), to a final concentration of approx. 1 mg. of original protein/ml., filtered and kept at −20° until analysed.

The amino acid analyses were performed by the chromatographic procedure of Moore, Spackman & Stein (1958), combined with the colorimetric method of Rosen (1957), for the estimation of the amino acids in the effluent. This method was applied without modification on the 2 ml. samples, but the diluting mixture used by Rosen was replaced by 50% ethanol. The colour yields thus obtained agreed within ±3%, or better, with those given by Rosen except for glycine, lysine and ammonia, which were 0·95, 0·99 and 0·77 respectively. For proline, hydroxyproline and hydroxylysine the values were 0·22 (at 440 mμ), 0·095 (at 440 mμ) and 1·05 (at 570 mμ) respectively.

Recovery experiments with known mixtures of amino acids were done periodically during the course of the work. In all instances the values obtained were the expected ones with a mean deviation not exceeding 3%, for each amino acid, with the exception of methionine (94±4·1%) and proline (99·3±3·9%).

Owing to its low colour-yield proline was measured in separate portions of the samples by the method of Chinard (1952). Interfering amino acids were eliminated by a previous treatment with Permutit (Troll & Lindsley, 1955). In the chromatographic method of Moore *et al.* (1958) the hydroxyproline and aspartic acid peaks partially overlap.

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For this reason and also because of its low colour-yield, hydroxyproline was measured in separate portions of the samples by the method of Neuman & Logan (1950), as modified by Martin & Axelrod (1953). When the amount of hydroxyproline in each sample was known, it was possible to derive a suitable factor to correct the values obtained for aspartic acid in the chromatographic run. Control experiments with known mixtures of both amino acids gave aspartic acid estimates consistently within $\pm 3\%$ of the theoretical values.

Glycine and alanine are present in such high proportions in collagen that the effluent after the ninhydrin reaction must be diluted several times to fall within the useful range of the spectrophotometer. Small deviations from Lambert–Beer's law occur under these conditions, and suitable corrections were applied.

The methionine content was calculated by combining values for the methionine and the methionine sulphoxide peaks; to this sum a further 6% was added to compensate for low recovery, as found in the control experiments.

Eastoe (1955) and Piez *et al.* (1960) studied the losses occurring during collagen and gelatin total hydrolysis, with similar results. Since our experimental conditions were very similar to those used by Piez *et al.* (1960), we applied correction factors derived from their work. Accordingly the values found for serine, threonine, methionine and tyrosine were increased by 9·8, 4·8, 18 and 25% respectively. The incomplete liberation of valine was compensated by a +4·8% correction and the total ammonia was decreased by 10·6%. Carbohydrates were determined by the thymol–sulphuric acid method of Gómez & Gris (1954). The hexosamine content was determined by the Boas (1953) procedure on samples hydrolysed in 4N-hydrochloric acid at 100° for 15 hr.

Nitrogen was determined according to Shepherd *et al.* (1954).

RESULTS AND DISCUSSION

Table 1 shows the amino acid composition found for the different fractions of collagen analysed.

Since Cobbet, Kenchington & Ward (1963) demonstrated that approximately 1 residue of tyrosine/1000 amino acid residues is present in specially purified collagen, any amount in excess of that value is an index of contaminating non-collagen proteins in the sample. By this criterion the fractions analysed are reasonably purified and are comparable with corresponding fractions prepared by Jackson *et al.* (1958) and Piez *et al.* (1963) (see Table 2), with the exception only of the gelatin from insoluble collagen, especially that from newborn animals. This fraction had 1·5–4 times as much tyrosine as the others.

In the neutral-salt-soluble collagen of newborn animals the total weight recovered in the form of amino acids was only 79·3% of the total. This fact, together with a Kjeldahl nitrogen value significantly higher than the one calculated from the amino acid composition, points to the existence of nitrogenous

Table 1. *Amino acid composition of dermal-collagen fractions in rats of different ages*

Values are given as number of amino acid residues/1000 total residues and are the average of independent analyses carried out on two separately hydrolysed samples. The samples from 1-year-old rats for the insoluble fractions were accidentally lost.

	Neutral-salt-soluble				Citrate-soluble				Insoluble		
	1 day	2 months	1 year	2 years	1 day	2 months	1 year	2 years	1 day	2 months	2 years
Ala	98·8	102·2	100·1	105·5	102·6	104·7	103·8	102·6	90·7	101·1	105·8
Arg	50·8	51·1	48·5	47·9	48·1	50·4	48·5	50·8	43·9	48·2	48·0
Asp	48·0	45·8	45·5	45·0	43·8	42·7	44·3	44·4	48·4	46·6	47·3
Glu	72·8	72·5	73·3	71·5	70·4	70·3	70·6	67·0	81·1	73·3	70·0
Gly	316·9	331·0	333·7	328·2	335·7	336·7	336·1	329·4	328·2	329·2	330·0
His	5·5	3·3	5·8	5·7	5·1	5·0	5·4	4·1	5·1	5·6	3·0
Hylys	6·2	5·8	5·7	6·3	6·9	7·0	5·8	6·2	7·1	5·6	4·7
Hypro	106·7	89·2	86·6	91·4	99·6	87·8	91·5	96·0	88·8	95·5	92·5
Ileu	14·2	13·7	12·8	12·0	11·8	13·7	11·4	11·7	13·7	12·6	10·8
Leu	24·9	26·3	27·8	24·9	23·8	27·3	24·8	23·8	26·8	23·4	24·0
Lys	28·1	32·3	32·4	28·3	29·4	32·4	34·9	30·1	29·9	33·7	29·6
Met	8·3	6·9	7·9	7·1	5·8	7·7	7·4	4·8	8·2	7·2	4·8
Phe	11·4	12·0	13·2	11·8	12·3	11·2	10·6	11·3	11·8	12·8	13·3
Pro	115·1	117·9	113·6	121·0	113·5	115·4	120·4	130·2	102·5	125·8	127·4
Ser	43·3	42·3	41·7	42·6	42·7	37·6	39·4	41·5	59·2	36·5	41·3
Thr	19·9	19·8	19·7	18·6	20·4	18·3	18·7	18·4	22·0	15·5	18·1
Tyr	3·1	3·1	4·7	3·8	2·3	2·9	2·3	1·8	7·3	4·0	4·4
Val	24·2	24·8	27·0	28·6	25·8	26·8	27·5	26·2	25·3	24·1	25·2
Amide N	(56·6)	(49·0)	(47·8)	(54·7)	(49·4)	(42·1)	(50·4)	(58·8)	(47·4)	(45·8)	(42·6)
Kjeldahl N (%)	17·0	17·2	18·5	17·3	18·9	17·7	18·1	19·2	—	17·6	18·5
g. of N/100 g. of sample*	14·8	17·0	18·1	17·6	18·1	18·1	18·4	18·4	17·0	17·8	18·0
Wt. recovery (%)†	79·3	91·3	96·9	94·7	97·3	97·0	98·5	98·0	92·9	96·2	97·2
Mean residue wt.	99·1	92·8	92·4	92·3	92·0	92·0	92·1	92·2	99·3	92·6	92·0

* Sum of amino acid and amide nitrogen.

† Calc. from the amino acids plus amide values found.

non-protein components in this fraction different from hexosamines, since the value found for these substances amounted only to 0·29 %. For all the other fractions and ages the hexosamine content was below the limit of detection of the method applied (0·10 %).

The carbohydrate content in the different collagen fractions, expressed as hexose per cent, was as follows: neutral-salt-soluble, 1 day: 1·70; 2 months: 2·12; 1 year: 0·72; citrate-soluble, 1 day: 1·33; 2 months: 1·00; 1 year: 0·69; 2 years: 1·30; insoluble, 2 months: 0·66; 1 year: 0·49; 2 years: 0·74.

To appraise critically the amino acid values collected in Table 1 it must be remembered that the methods applied to measure the amino acids have an accuracy of approximately ± 5 % in the optimum range of concentrations, although for the amino acids in very low proportion in collagen (tyrosine, methionine, histidine and hydroxylysine) it is presumably lower. The serine, threonine, methionine and tyrosine values are necessarily obtained with the application of approximate correction factors. If, for the reasons given above, the irregular variations found in histidine, hydroxylysine, serine, threonine, methionine and tyrosine are discarded as not significant, the rest of the amino acids remain fairly constant during the aging process. However, the ratio proline/hydroxyproline is regularly lower in newborn animals than in the other ages, namely 1·08 compared with 1·32 (av.) in neutral-salt-soluble, 1·14 compared with 1·33 (av.) in citrate-soluble, and 1·16 compared with 1·35 (av.) in the insoluble collagen. The respective averages and standard deviations for all fractions (duplicate analyses) within each age group were as follows: 1 day, 1·12 ± 0·05 (insoluble collagen not included); 2 months, 1·32 ± 0·04; 1 year, 1·31 ± 0·04; 2 years, 1·35 ± 0·04.

The Neuman & Logan method for the estimation of hydroxyproline can be affected by interfering substances of carbohydrate origin, e.g. pyrrole-2-carboxylic acid (Deasy, 1961). For this reason with each sample analysed it was established that no colour was produced when water was added instead of hydrogen peroxide. Further, the proline/hydroxyproline ratios of the neutral-salt-soluble and citrate-soluble collagens from newborn and from 2-year-old animals were also measured by a chromatographic procedure with the Amino Acid AutoAnalyzer (Technicon Corp., Chauncey, New York). The values thus obtained confirmed those calculated from Table 1. The averages and standard deviations for the sums of proline and hydroxyproline in all fractions (duplicate analyses) within each age group were as follows: 1 day, 216·6 ± 6·6 (insoluble collagen not included); 2 months, 210·5 ± 8·1; 1 year, 206·1 ± 4·6; 2 years, 219·5 ± 4·9.

Table 2 shows the molar amino acid composition of the collagen fractions studied, expressed as averages of the values found for all ages within each fraction. The insoluble collagen from newborn rats was not included since its purity, judged by its tyrosine content, was not comparable with that of

Table 2. *Composition of dermal-collagen fractions from rat and rabbit skin*

Values are given as number of amino acid residues/1000 total residues. The values obtained are averages of the amino acid values found for all ages within each fraction, with the exception of the insoluble collagen from newborn rats (see text).

	Rat skin (present study)			Rabbit skin (Jackson, Leach & Jacobs, 1958)			Rat skin (Piez, Eigner & Lewis, 1963)
	Neutral-salt-soluble	Citrate-soluble	Insoluble	Neutral-salt-soluble	Citrate-soluble	Insoluble	Acid-soluble
Ala	101·6	103·4	103·4	100·6	112·1	102·3	106
Arg	49·6	49·5	48·1	47·5	48·4	45·6	51
Asp	46·1	43·8	46·9	54·8	47·5	50·2	46
Glu	72·5	69·6	71·6	75·7	70·4	69·2	71
Gly	327·5	334·5	329·6	295·2	317·4	309·0	331
His	5·1	4·9	4·3	7·2	4·4	5·6	4·9
Hylys	6·0	6·5	5·2	4·8	4·7	5·0	5·7
Hypro	93·4	93·7	94·0	96·9	100·7	104·3	92·9
Ileu	13·2	12·2	11·7	17·0	11·2	12·1	10·8
Leu	26·0	24·9	23·7	29·7	25·5	23·3	23·8
Lys	30·3	31·7	31·6	31·9	28·5	27·5	28·1
Met	7·6	6·5	6·0	6·5	4·6	8·7	7·8
Phe	12·1	11·4	13·0	15·1	12·7	12·4	11·3
Pro	116·9	119·9	126·6	111·3	122·7	142·7	121
Ser	42·5	40·3	38·9	44·3	38·6	40·3	43
Thr	19·5	19·0	16·8	27·4	20·9	19·8	19·6
Tyr	3·7	2·3	4·2	5·6	1·9	2·0	2·4
Val	26·2	26·6	24·7	26·9	25·4	21·7	24·0
Amide N	(52·0)	(50·2)	(44·2)	(50·7)	(40·6)	(50·0)	(41)

the other fractions. The results obtained by Jackson *et al.* (1958) with rabbit skin, and by Piez *et al.* (1963) with acid-soluble collagen of rat skin, are included for comparison. Most of the values collected in this Table agree with each other within experimental error.

The results obtained in the present work (Table 1) do not agree with the observations of Chvapil & Kobrle (1961) on the proline and hydroxyproline content of lung and tendon collagen from rats during the aging process. These authors found that, though the proline values remained almost constant, the hydroxyproline increased approximately two-fold from birth up to the age of 250 days. One explanation for this discrepancy may be connected with the different locations of the collagens analysed in both investigations; but, since Chvapil & Kobrle did not purify the extracted collagen, foreign proteins, present in different amounts according to the age of the animal (see González Cadavid *et al.* 1963), may be responsible for the variation that they reported.

The results given in this paper support the conclusion that the physicochemical changes brought about by age on dermal collagen are not related to a parallel gross change in amino acid composition.

SUMMARY

1. Purified neutral-salt-soluble, citrate-soluble and insoluble fractions of rat-skin collagen were obtained from newborn, young, adult and senile animals, and the amino acid compositions determined.

2. The amino acid composition of the different collagen fractions remained fairly constant during the aging process.

3. The ratio proline/hydroxyproline was lower in newborn animals than in those of other ages.

4. The average amino acid values obtained are in good agreement with previously published analyses.

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