Variations in Some Physicochemical Properties of Collagen with Age (34021)

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(Introduced by J. Seifter)

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The physicochemical changes produced in collagen during aging are probably due to an increase in the number of intra- and intermolecular crosslinkages which gradually transform the soluble molecules of tropocollagen into insoluble fibers. The increase in intermolecular crosslinks would also explain the decrease in the amount of neutral salt- and acid-extractable collagen which can be extracted from tissues with advancing age.

Verzar (1) showed that the isotonic thermal contraction increases with age of the host, and explained these results as being due to increased crosslinking during aging.

However, Bakerman (2) found no change in the ratio of the α to β components of human skin collagen obtained from patients of different ages, suggesting that there is no alteration of intramolecular bonding during chronological aging. On the other hand, Heikkinen *et al.* (3), working with collagens from rat skin and tail tendon, obtained evidence that the amount of β components increased with age while the amount of α components decreased. Butzow and Eichhorn (4) found a marked decrease with age in α components in the acid-soluble collagen from rat tail tendon.

A decrease with age of host has been reported for both the tyrosine (5) and the carbohydrate (6) contents of various collagen fractions.

In this paper, to obtain further insight into the nature of the aging process, some physicochemical properties and chemical compositions of soluble collagen fractions from skins of 1-day-old and 2-year-old rats respectively are compared.

Methods. Preparation of collagen. Rat skin was obtained from 1-day-old and 2-year-old Wistar rats. The 2-year-old rats were previously sheared. Skins were freed of gross fat, washed with water, homogenized in a Virtis homogenizer with 0.15 *M* NaCl in 0.2 *M* Tris buffer (pH 7.4), and extracted for 24 hr. The homogenate was centrifuged, the supernatant fraction removed, and the residue again extracted with the same buffer for 48 hr. The supernatnat fractions from a given homogenate were pooled and clarified by filtration through a celite filter cake on a Büchner funnel. Solid NaCl was added to the filtrate to a final concentration of 20%, the solution allowed to stand in the cold for 24 hr and then centrifuged at 15,000 rpm/ for 30 min. The precipitate was dissolved in another portion of buffer, and precipitation with NaCl repeated. The collagen was redissolved and precipitated a third time by dialyzing against 0.02 *M* disodium hydrogen phosphate. The resulting precipitate was removed by centrifugation and dissolved in 0.1 *M* acetic acid. The solution was filtered with suction through celite and, after exhaustive dialysis against 0.01 *M* acetic acid, was lyophilized. The residue obtained after extraction with 0.15 *M* NaCl was re-extracted with 1.0 *M* NaCl in 0.2 *M* Tris buffer (pH 7.4), following the same procedure described above. After the salt extractions and prior extraction with citrate buffer, the tissue paste was washed twice with distilled water to remove salt. It was suspended in 0.2 *M* citrate buffer (pH 4.3), and the extraction was carried out for 24 hr. After centrifugation, the residue was extracted with the same buffer for 48 hr. The supernatant fractions were pooled and

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filtered through celite. The filtrate was dialyzed against 0.02 *M* Na_2HPO_4; the collagen fibers which formed were removed by centrifugation, and dissolved in 0.1 *M* acetic acid by stirring for 24 hr. The solution was dialyzed exhaustively against 0.01 *M* acetic acid and then lyophilized. The residue from the citrate extraction was then extracted with 0.5 *M* acetic acid following a procedure similar to that described for the fraction extracted with citrate.

Analytical methods. Viscometric measurements were made on samples dissolved in 0.15 *M* citrate buffer (pH 3.5), by constant stirring for 24 hr. The solutions were then centrifuged at 40,000 rpm for 45 min at 4°. The clear supernatant fractions was then studied at 20° ± 0.01 in an Ostwald viscometer with flow time for water of 112 sec.

Denaturation curves were obtained by viscosity measurements of samples equilibrated at different temperatures.

Sedimentation velocity was performed in a Spinco Model E ultracentrifuge. The samples were dissolved in sodium formate buffer, pH 3.8, 1 = 0.15 and heated at 45° for 10 min. The runs were performed at 40° and at 42,040 rpm.

Chromatography on carboxymethylcellulose was performed as described previously (7) using CM cellulose from Bio Rad, Lot No. 1310, exchange capacity 0.54 mEq/g. The collagen, suspended in distilled water and stirred overnight at 4°, was dialyzed for 24 hr against acetate buffer, pH 4.8, 1 = 0.06. The sample was denatured by warming to 45° for 30 min, and insoluble material removed by filtration. The neutral salt-soluble collagen fractions were centrifuged at 45,000 rpm for 1 hr before they were denatured by heating.

Acrylamide gel electrophoresis was performed according to the method of Nagai *et al.* (8) with a 7.5% running gel.

Chemical determinations. The samples for the chemical determinations were prepared by suspending the dry material in water and heating at 60–65° for 15 min. The warm gelatin solutions were clarified by centrifugation. Protein concentration was determined by the micro-Kjeldahl procedure and direct nesslerization on duplicate samples. The nitrogen contents of the various collagens was assumed to be 18.2%.

TABLE I. Physicochemical Properties of Rat Skin Collagen.

Extraction media	Age	$[\eta]$	T_m	$S°20,_w$
0.15 *M* NaCl	1 day	12.5	33°8	3
0.15 *M* NaCl	2 years	12.1	33°8	2.8
0.2 *M* Citrate (pH 4.3)	2 years	13.5	33°8	—

Ester-like bonds were determined by methods previously described (9, 10).

Hexose determinations were carried out by the method of Seifter *et al.* (11) with glucose as standard.

Aldehyde groups were determined by a method described by Paz *et al.* (12) with glyceraldehyde as standard.

Amino groups were determined by the ninhydrin reaction according to the method of Rosen (13).

Amino acid analyses were performed on samples hydrolyzed in 6 *N* HCl at 106°, *in vacuo*; an automatic amino acid analyzer (14) was used.

Hydroxyproline determinations were by the method of Blomfield *et al.* (15).

Proline was determined by the method of Chinard (16).

Tyrosine determination was performed according to the method of Marenzi *et al.* (17).

Results. The values for the intrinsic vicosity, denaturation temperature (Tm), and sedimentation velocity for the 0.15 *M* NaCl- and citate-soluble collagen fractions are shown in Table I. It can be seen that the salt-extracted collagens of 1-day-old and 2-year-old animals have similar intrinsic viscosity values; whereas the acid-extracted collagen from the older animals has a somewhat higher value than does the salt-extracted collagen. These findings agree generally with those reported by Bornstein *et al.* (18) who compared acid-extracted with salt-extracted collagens.

The denaturation temperature (Tm) (see Fig. 1) was equal for the different collagen fractions, and the value of 33.8° obtained was similar to that found by von Hippel *et al.* (19) for calfskin collagen dissolved in 0.15 *M* citrate buffer (pH 3.7).

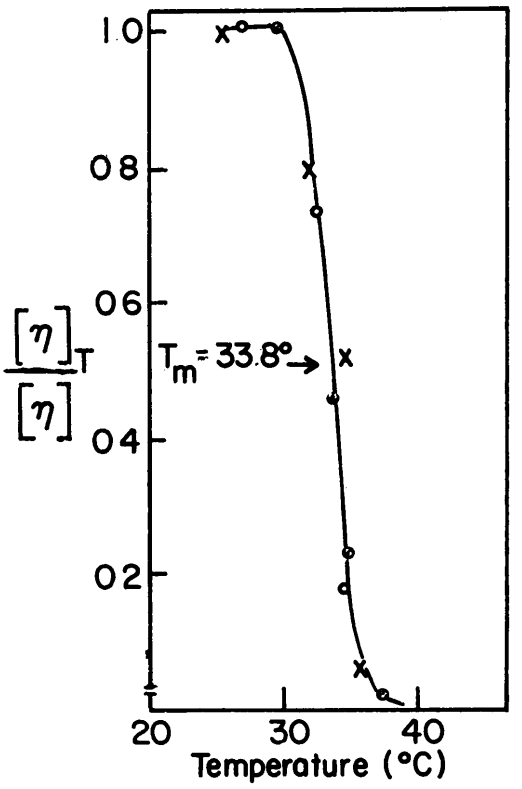

FIG. 1. Thermal denaturation of citrate-extracted (O—O) and 0.15 *M* NaCl-extracted (O—O) collagen from 2-year-old rats and 0.15 *M* NaCl-extracted (×—×) collagen from 1-day-old rats. The ordinate shows the relation between the intrinsic viscosity at T (°c) and the intrinsic viscosity of native collagen.

The sedimentation velocity constants obtained were similar for the various collagen fractions studied.

Chromatography on carboxymethylcellulose allowed the separation of four components identified by gel electrophoresis of small samples (0.2 ml.). Peaks corresponding to $\alpha 1$, $\alpha 2$, β_{11}, and β_{12} subunits were observed. Representative chromatograms of acid-extracted collagens are illustrated in Figs. 2 and 3. The approximate relative proportions of subunits were observed. Representative chromatograms of acid-extracted collagens are illustrated in Figs. 2 and 3. The approximate relative proportions of subunits in the denatured collagen were calculated from the areas under the peaks on the chromatograms. These data and the distribution of collagen structures are shown in Table II. It is apparent that the proportion of double chain β-components increases as the extracting solvent changes from salt to acid, indicating that the citrate buffer and the acetic acid are more effective in extracting crosslinked material. All solvents used extracted larger amounts of β components from the skins of the 2-year-old animals as compared to 1-day-old animals. The increase in β components, especially in β_{12}, indicates an alteration in the ratio of the α to β components during chronological aging. As obtained by chromatography, the β_{12} component is always contaminated with γ-component which never exceeds 10%.

Table III shows the results obtained in the determination of hexoses, aldehyde groups, amino groups, and ester-like bonds. The hexose content was found to be significantly increased in all the collagen fractions from 1-day-old animals as compared to the same fractions from 2-year-old animals. The neutral salt- and citrate-soluble collagens from 1-day-old animals showed a greater amount of aldehyde groups than did the corresponding fractions from 2-year-old rats. This difference is very large between the two 0.15 *M* NaCl-soluble fractions. Also, the 0.15 *M* NaCl fraction from the 2-year-old animals had a higher aldehyde content than did the other fractions from the same-aged animals.

Determination of amino groups was performed to determine whether splitting of pep-

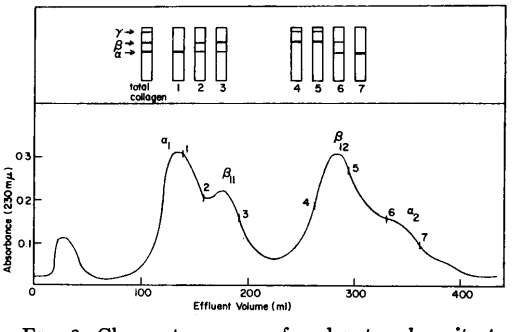

FIG. 2. Chromatogram of denatured citrate-extracted collagen from 1-day-old rats on carboxymethylcellulose. The acrylamide gel electrophoresis patterns show the composition of samples taken from the effluent at the numbered points.

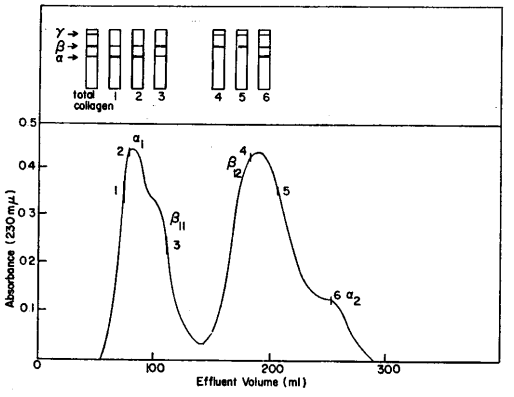

FIG. 3. Chromatogram of denatured citrate-extracted collagen from 2-year-old rats on carboxymethylcellulose. The acrylamide gel electrophoresis patterns show the composition of samples taken from the effluent at the numbered points.

tide bonds had occurred as a consequence of any degradative process. The values obtained are similar to those reported for different collagens and the variations are not significant.

The number of ester-like bonds were similar in all the fractions regardless of age. The tyrosine contents in the collagen fractions from the 1-day-old animals were higher when compared to the corresponding fractions from the 2-year-old group. The other amino acids show no significant differences (Table IV).

Discussion. From the experimental data one may infer that the different soluble collagen fractions exhibit physicochemical differences in relation to the age of the host animal.

The denaturation temperature, T_m, which represents the transition of native collagen to gelatin, is the same for the different collagen fractions from 1-day-old and 2-year-old animals. Covalent crosslinkages between the individual strands seem to be relatively unimportant for stabilization of the collagen superhelix, since the increase in amount of β components in various soluble collagens shown in this paper is not correlated with T_m. This confirms previous results (7) which demonstrated that the stability of the collagen macromolecule depends on the total

TABLE II. Approximate Distribution of α, β, and γ Components in Denatured Ratskin Collagen and the Calculated Possible Structures in the Native Sample with Age.

Extraction media	Age 1 day							Age 2 years						
	Collagen components (%)[a]				Collagen structures[b]			Collagen components (%)[a]				Collagen structures[b]		
	$\alpha 1$	β_{11}	β_{12}	$\alpha 2$	γ	$(\alpha 1)_2\alpha 2$	$\alpha 1\beta_{12}$	$\alpha 2\beta_{11}$	$\alpha 1$	β_{11}	β_{12}	$\alpha 2$	γ	$(\alpha 1)_2\alpha 2$	$\alpha 1\beta_{12}$	$\alpha 2\beta_{11}$
0.15 *M* NaCl	45	10	22	22	1	51	33	15	43	10	28	18	1	42	42	15
1 *M* NaCl	40	12	28	19	2	39	42	18	36	14	29	18	3	33	44	21
0.2 *M* Citrate (pH 4.3)	33	14	30	16	7	27	45	21	22	18	44	9	7	0	66	27
0.5 *M* Acetic acid	31	16	35	14	4	19	53	24	22	17	44	7	9	0	66	25

[a] Calculated from the areas under the peaks on the chromatograms.
[b] Calculated from the components distribution.

TABLE III. Content of Aldehydes, Ester-Like Bonds, Hexoses, and Amino Groups in Rat Skin Collagen.

	Residues per 1000 total residues							
	1 Day				2 Years			
	0.15 *M* NaCl	1 *M* NaCl	0.2 *M* Citrate (pH 4.3)	0.5 *M* Acetic acid	0.15 *M* NaCl	1 *M* NaCl	0.2 *M* Citrate (pH 4.3)	0.5 *M* Acetic acid
Aldehydes	7.12	1.92	2.72	0.82	2.76	0.78	0.62	1.39
Ester bonds	5.6	6.25	5.9	5.57	6.15	5.7	6.0	6.7
Hexoses	8.95	5.58	5.0	6.80	4.25	2.38	2.37	2.56
Amino groups	42	31.5	36	35	37.6	36.5	36	36

imino acid content and is independent of degree of intramolecular crosslinkage.

The higher intrinsic vicosity of acid-extracted as compared with salt-extracted collagen is probably due to the presence of a greater proportion of high molecular weight aggregates in the former preparation. Bornstein *et al.* (18) showed that the differences disappear after treatment with chymotrypsin, and Drake *et al.* (20) reached similar conclusions.

It was shown that the amount of soluble collagens decreases with age with a concomitant increase in the insoluble collagen due to the increase in the degree of intra- and intermolecular crosslinkage. Heikkinen *et al.* (3), applying starch gel electrophoresis to tendon and rat skin collagens, demonstrated an increase in amount of β chains. Bakeman (2) on the other hand stated that there is a constant α/β ratio in corresponding collagen fractions from human skin regardless of age of host. The experimental data presented here show a relative increase in amount of β components as compared to α components for collagens obtained from older as compared

TABLE IV. Amino Acid Composition of Rat Skin Collagen Fractions from 1-Day-Old and 2-Year-Old Animals.

	Residues per 1000 total residues			
	1 Day		2 Years	
Amino acid	0.15 *M* NaCl	0.5 *M* Acetic acid	1 *M* NaCl	0.5 *M* Acetic acid
Hydroxyproline	92.0	83	90	87
Aspartic acid	46	42	45.5	40
Threonine	22	21.6	20	20
Serine	35	39.5	32.0	36
Glutamic acid	78	79	75	78
Proline	122	118	126	120
Glycine	321	325	320	328
Alanine	114	114	110	114
Valine	20.5	23	23	22
Methionine	8	7	7	7
Isoleucine	12	13	12	11
Leucine	24	28	26	25
Tyrosine	4.5	6.4	2.5	3
Phenylalanine	13	12.8	13.6	12
Hydroxylysine	6	7.0	7.0	6
Lysine	33	35	34.5	33
Histidine	6	5	5	4.5
Arginine	43.5	42	51	54

with younger animals and for collagens extractable with media of higher as compared with lower ionic strengths.

The decrease in the ratio of the α to β components is remarkable for the citrate- and acetic acid-soluble fractions from 2-year-old animals, and one may assume from the subunit distribution that no uncrosslinked molecules were present in the samples. If one assumes that β_{11} and β_{12} derive largely from intra- rather than intermolecularly crosslinked structures, one may conclude that there is an increase of intramolecular bonding during aging.

The amino acid composition in all cases was typical of mammalian collagens and similar to that presented by Piez *et al.* (21) for rat skin collagen. It is felt that the larger tyrosine content observed in the collagen fractions from 1-day-old animals is not due to contamination of the preparations with other proteins since the proportions of proline, hydroxyproline, and glycine were typical of purified collagens. The ratio of proline/hydroxyproline was constant for all the soluble collagens regardless of age of animal, and the high content of tyrosine was always found in the collagen fractions from 1-day-old rats.

La Bella *et al.* (5) described a decrease in the tyrosine residues of solubilized collagen with age and suggested their transformation into crosslinking quinoid components. Dabbous (22) also suggested a role for tyrosine in the intramolecular and intermolecular interactions. Tyrosine residues occur in near-terminal crosslinking regions of α and β chains of rat skin collagen [Bornstein *et al.* (23) and Kang *et al.* (24)]. Rojkind *et al.* (25) in fact isolated a peptide which contained both an aldehyde component and tyrosine. However, the exact role of the tyrosine in the crosslinking region and the decrease in tyrosine content with age is not yet apparent.

The number of ester-like linkages mentioned (10, 26) as possible participants in intra- and intermolecular crosslinkage were similar among the different collagen fractions from 1-day-old and 2-year-old animals.

The higher hexose content in the collagen fractions from the 1-day-old animals compared to 2-year-old animals may be related to different degrees of interaction between mucopolysaccharides of the connective tissue and collagen with age. This was shown in histochemical studies of the evolution of the cutaneous connective tissue with age (27). This role of hexose is also suggested by the work of Spiro on collagen of kidney basement membrane (28). The presence of collagenous reticulum rich in carbohydrates could explain the higher content of hexoses in soluble collagens from 1-day-old animals (29).

The increase in intermolecular crosslinkages with aging may be responsible for the decrease in the amount of soluble collagen. This is accompanied by a concomitant increase in the intramolecular bonding and may lead to the higher content of the β components in relation to the α components in old animals. A decrease in tyrosine, aldehydes, and hexose content which have been pointed out as possible direct participants in intra- and intermolecular crosslinkages is also shown.

Summary. By use of extraction with neutral salt solutions and acidic solutions of graded ionic strength, purified soluble collagens were obtained from the skins of 1-day-old and 2-year-old rats. The extracted collagens of 1-day-old rats did not differ significantly from those of 2-year-old rats with respect to denaturation temperature, viscosity, or sedimentation velocity. However, collagens obtained from the older as compared with the younger animals did have lesser contents of tyrosine, hexoses, and aldehydes. Contents of ester-like bonds and amino acid composition did not differ in the corresponding collagens of 1-day-old and 2-year-old rats. Significantly, there was a difference in degree of intramolecular crosslinkage between corresponding collagens of young and older rats as demonstrated by the larger contents of β components in the collagens of the 2-year-old animals.

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