Chemical Changes Associated with Aging of Collagen *in vivo* and *in vitro*

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Collagen extracted from rat skin by neutral-salt solutions contains less aldehydes than the more insoluble collagen fractions. The concentration of aldehydes in collagen is directly related to its capacity to form stable cross-linked gels, which do not redissolve on cooling and become more insoluble in a variety of reagents. Whereas the absorption spectrum of neutral-salt-soluble collagen treated with *N*-methylbenzothiazolone hydrazone resembles that of acetaldehyde, the more insoluble collagen fractions show increasing amounts of a component that behaves like an $\alpha\beta$ -unsaturated aldehyde. The ratio between α - and β -sub-units present in a particular fraction of soluble collagen seems to be constant and independent of the age of the animal. Neutral-salt-soluble collagen, which has a low concentration of β -components, will generate intramolecular bonds if gelled at 37°. These intramolecular bonds seem to precede the formation of stable intermolecular cross-links, since these gels can redissolve when cooled to yield a soluble collagen with a higher content of β -components of intramolecular origin.

Collagen is a fibrous protein that exhibits age-dependent variability in its physical and chemical characteristics (Jackson & Bentley, 1960). The recently synthesized macromolecular aggregates are readily dispersed by dilute solutions of neutral salts, but, with time, fibres are formed which become so insoluble that they remain unaffected in concentrated solutions of urea or guanidine. These marked changes, which are accompanied by increased mechanical (Fry, Harkness & Harkness, 1964) and thermal stability (Verzar, 1964), are attributed to the formation of new covalent bonds that stabilize the closely packed structure. There exists no clear evidence for any chemical change, other than that involving solubility, which can support these findings, nor any indication that the functional groups on the molecule have undergone transformation that can be directly correlated with the aging process.

In the present work we have attempted to investigate further the participation of aldehyde residues in cross-linking, in an attempt to correlate their nature and concentration with some measurable parameters, such as solubility in various solutions and the degree of intramolecular cross-linking of the tropocollagen sub-units.

METHODS

Extraction and purification of neutral-salt-soluble collagen and cysteamine-soluble collagen from dorsal skin of male albino rats was carried out as described previously

(Deshmukh & Nimni, 1968; Nimni, 1966). The final preparations were centrifuged at 105 000*g* for 90 min. The concentration of collagen was estimated by determining hydroxyproline content of the acid hydrolysates of these supernatants by the Stegemann (1958) procedure adapted to the Technicon AutoAnalyzer (Grant, 1964).

Thermal aggregation. Neutral-salt-soluble collagen and cysteamine-soluble collagen (2.5 mg./ml.) were incubated at 37° for different times. At the end of each time-interval the samples were rapidly cooled to 4°. The change in turbidity during aggregation and redissolution was followed by measuring the extinction at 530 nm.

Aging of collagen *in vitro*. Several sets of gels were prepared by incubating neutral-salt-soluble collagen and cysteamine-soluble collagen at 37°. At various time-intervals the gels were taken out of the incubator and kept shaking overnight at 4° with: (a) 0.45 M-NaCl in 0.02 M-sodium phosphate buffer, pH 7.0; (b) 0.2 M-cysteamine in 0.45 M-NaCl, pH 7.0; (c) 0.2 M-acetic acid. The samples were centrifuged at 105 000*g* for 1 hr. and the collagen contents of supernatants and residues determined.

Thermal shrinkage. Gels of neutral-salt-soluble collagen and cysteamine-soluble collagen, aged at 37° for various times, were heated from 37° to 60° at a constant rate (1°/min.) and the change in nature of both types of gels was observed. The moment at which the gel underwent contraction and shrinkage could be clearly detected. This occurred over a very short temperature span and this was recorded as the *T_s* (shrinkage temperature).

Extraction of collagen from skins of rats of different ages. Procedure 1. Collagen fractions were obtained from dorsal skins of rats of different ages by repeated extractions with various solvents such as 0.15 M-NaCl, 0.45 M-NaCl, pH 7.0, 0.5 M-sodium citrate buffer, pH 3.6, and 0.2 M-cysteamine (Nimni, Deshmukh & Bavetta, 1967). The residual mass was

dialysed against water to remove excess of cysteamine and was gelatinized by autoclaving at 121° for 4 hr.

Procedure 2. Dorsal skin (0.5 g.) was washed with 0.15 M-NaCl and extracted with 0.45 M-NaCl, pH 7.0, and 0.5 M-sodium citrate buffer, pH 3.6, as described before. The residue was then extracted with 8 M-urea for 48 hr. and the remaining skin mass was gelatinized after dialysis against water.

Collagen extracted with 0.45 M-NaCl, 0.5 M-sodium citrate buffer and 0.2 M-cysteamine was dialysed against 0.02 M- Na_2HPO_4 . The precipitated material was dissolved in 0.45 M-NaCl, 0.5 M-acetic acid and 0.45 M-NaCl respectively. The denatured collagen extracted with 8 M-urea was not precipitated by dialysis against water. A sample of each collagen fraction was hydrolysed and used for hydroxyproline determination.

Determination of aldehydes. Samples of each collagen fraction were dialysed against 0.1 M-glycine-HCl buffer, pH 4.0, overnight and gelatinized by heating at 60° for 30 min. Aldehydes were determined by the method of Paz *et al.* (1965).

Sub-unit studies. Concentrations of all collagen fractions were adjusted to 1–1.5 mg./ml. after dialysis against 0.05 M-acetic acid. The samples were denatured at 40° for 10–15 min. and polyacrylamide-gel electrophoresis was carried out either by the method reported by Fessler & Bailey (1966), to separate α - from β -sub-units, or by the method of Veis & Anesey (1965) to resolve the various types of α - and β -sub-units. The concentrations of the components were measured with a Canalco model F Microdensitometer.

Solubility of insoluble collagen in cysteamine and acetic acid. Two pieces of dorsal skin of rats weighing 80–100 g. were extracted with 0.15 M-NaCl, 0.45 M-NaCl, pH 7.0, and 0.5 M-sodium citrate buffer, pH 3.6, as described earlier. The residue was kept shaking at 4° with (1) 0.2 M-cysteamine in 0.45 M-NaCl, pH 7.0, and (2) 0.2 M-acetic acid over a period of 7 days. Samples of the supernatant solutions after centrifugation at 40000g for 1 hr. were taken out daily, dialysed, hydrolysed and used for hydroxyproline determination. Aldehyde content of the final supernatants was determined as described above.

Fractionation of salt-soluble collagen by thermal precipitation. Neutral-salt-soluble collagen (2.45 mg./ml.) was incubated at 37° overnight and cooled and kept at 4° for 1 hr. The material was then centrifuged at 40000g for 1 hr. The precipitate was dissolved in 0.1 M-acetic acid and the supernatant was aggregated again at 37° overnight. The procedure was repeated four times. The time-periods for cooling were set arbitrarily so as to obtain a fairly good amount of precipitate for further studies. Samples of the supernatants were preserved at each stage of this procedure.

The amount of collagen in each precipitate and supernatant fraction was determined. The aldehyde content was measured as described by Paz *et al.* (1965) and the α -sub-unit/ β -sub-unit ratios of the supernatant fractions, after polyacrylamide disc electrophoresis (Fessler & Bailey, 1966), were obtained with a Canalco model F Microdensitometer.

RESULTS

Collagen extracted from the skin of young rats by 0.45 M-sodium chloride and 0.2 M-cysteamine was gelled by heating at 37°. The rate of gel formation,

Fig. 1. Solutions of neutral-salt-soluble collagen (a) and cysteamine-soluble collagen (b), containing 2.5 mg./ml., in 0.45 M-NaCl, pH 7.0, were heated at 37°. After incubation at this temperature for various times, they were cooled and the changes in gel structure were monitored by measuring the extinction.

measured by an increase in turbidity of the solution, was faster for material extracted by cysteamine (Figs. 1a and 1b). The stability of these gels was monitored by cooling and observing the reversibility of this process. The gels originating from the cysteamine-soluble collagen tended to become insoluble much faster than those made from the salt-soluble collagen, reflecting a greater capacity of the former to generate stable cross-links.

Some of the chemical changes that accompany this process of stabilization are shown in Table 1. All the gels become progressively insoluble in cold 0.45 M-sodium chloride, 0.2 M-cysteamine or 0.2 M-acetic acid with time. Those gels originating from cysteamine-soluble collagen become insoluble in all these solvents at a faster rate than those made with 0.45 M-sodium chloride-extracted collagen.

The tendency of collagen solutions to aggregate when the solute is present in moderately high concentration (in this case 3 mg. of collagen/ml.) is reflected by the fact that dialysis of the original

Table 1. *Percentage dissolution of gels of neutral-salt-soluble collagen and cysteamine-soluble collagen in various solvents as influenced by period of incubation at 37°*

Collagen samples dissolved in 0.45 M-NaCl, pH 7.0, were incubated at 37° for various times, then redissolved by shaking with different solvents at 4° for 24 hr. and centrifuged at 105 000g for 1 hr. The material in the supernatant was analysed for hydroxyproline. Values for zero time refer to percentage of collagen remaining in solution after the samples of initial collagen solutions (3 mg./ml.) were dialysed for 48 hr. at 4° against the solvents indicated and centrifuged at 105 000g for 1 hr.

Type of collagen gelled	Age of gel at 37°	Collagen (% dissolved at 4°)		
		In 0.45 M-NaCl, pH 7.0	In 0.2 M-cysteamine pH 7.0	In 0.2 M-acetic acid
0.45 M-NaCl-soluble	0	90.0	85.0	66.0
	1 day	40.0	43.6	60.5
	3 days	25.0	28.7	51.8
	1 week	12.1	15.0	42.0
	2 weeks	8.7	13.0	29.8
	3 weeks	1.7	2.4	16.6
	4 weeks	1.0	2.7	7.9
0.2 M-Cysteamine-soluble	0	90.0	87.0	68.0
	1 day	23.2	29.8	42.6
	3 days	15.4	19.7	35.4
	1 week	10.1	14.5	18.3
	2 weeks	6.8	7.3	11.2
	3 weeks	1.2	3.1	9.6
	4 weeks	1.1	2.5	9.3

Fig. 2. Collagen gels before and after heating at 58° for 5 min. The cysteamine-soluble collagen (a) was incubated for 3 days at 37° before heat denaturation. The 0.45 M-NaCl-soluble collagen (b) was incubated for 1 week at 37° before heat denaturation.

solution in the cold against its own solvent for 48 hr. causes 10% of this material to be precipitated when centrifuged at 105 000g for 1 hr. (zero-time value, Table 1). Dialysis against 0.2 M-cysteamine or acetic acid caused even larger amounts to become insoluble.

Another parameter that yields valuable information on the nature of the cross-links formed during gelation is the dissolution (gelatinization) or contraction of the fibrous network when heated above the denaturation range. Some of the changes observed are illustrated in Fig. 2.

The gels originating from the cysteamine-extracted collagen exhibited the phenomenon of contraction even after short periods of stabilization (2 days). On the other hand the gels made from 0.45 M-sodium chloride-soluble collagen did not show this effect unless aged *in vitro* for at least 2 weeks. Before this time these gels became gelatinized without exhibiting retraction. Shrinkage in both cases occurred between 56° and 57°.

We have recently reported that the collagen solubilized by cysteamine contained a larger amount of aldehydes than the more readily extractable neutral-salt-soluble material (Deshmukh & Nimni, 1968), and we have further extended this investigation to material extractable by 8 M-urea as well as the residual insoluble collagen.

The aldehydes present in all these fractions obtained from rats of different ages are shown in Table 2.

Table 2. Aldehyde content of different collagen fractions extracted from the skin of rats of various ages

Details are given in the text.

Acetaldehyde content of collagen fractions (μ moles/100 mg. of collagen)

Age of animal (months)	Procedure 1				Procedure 2	
	0.45 M-NaCl	0.5 M-Citrate, 0.2 M-Cysteamine pH 3.6	0.2 M-Cysteamine (72 hr.)	Gelatinized (120 hr.) residue	8 M-Urea	Gelatinized residue
1	0.83	1.25	2.53	2.72	3.30	2.68
2	0.76	1.13	2.16	2.55	3.20	2.46
4	0.70	1.02	1.92	2.50	2.97	2.39
6	0.63	0.97	1.62	2.41	2.15	2.09
18	0.61	0.91	1.47	2.34	1.89	1.80

Fig. 3. Absorption spectra of *N*-methylbenzothiazolone hydrazone derivatives of: (a) acetaldehyde, crotonaldehyde and an equimolar mixture of both compounds; (b) neutral-salt-soluble, cysteamine-soluble and acetic acid-soluble collagen; (c) acetic acid-soluble collagen before and after incubation with 0.2 M-cysteamine; (d) denatured collagen extracted by 8 M-urea from insoluble skin residue of young and old rat skin.

The more soluble collagen fractions contained less aldehydes than the more insoluble ones, but within a specific fraction the values observed show a relative decline as the animals aged. In procedure 1 the minced skin was extracted successively with 0.45 M-sodium chloride, pH 7.0, 0.5 M-citrate, pH 3.6, and 0.2 M-cysteamine, pH 7.0 (twice). The residue was gelatinized by autoclaving with water. In procedure 2, the exposure to cysteamine was omitted since the possibility existed that the high aldehyde content seen in this fraction may reflect an 'unmasking' of aldehydes by this reagent. Nevertheless the collagen solubilized by 8 M-urea, as well as the heat-gelatinized residue, still exhibits a similar high aldehyde titre.

Since the spectrum of azine products obtained by treating *N*-methylbenzothiazolone hydrazone with aldehydes gives an indication of the nature of the aldehydes present (Paz *et al.* 1965), this was investigated further.

Fig. 3(a) shows the spectral pattern of a simple aldehyde (acetaldehyde), an $\alpha\beta$ -unsaturated aldehyde (crotonaldehyde) and a mixture of both.

Fig. 3(b) shows the spectral characteristics of the aldehydes in different collagen fractions. The 0.45 M-sodium chloride and 0.2 M-cysteamine-soluble collagens show a peak at 310 nm, whereas the 0.5 M-acetic acid-extracted collagen shows a peak at 320 nm. In addition, the acetic acid-soluble collagen shows a marked shoulder at 355 nm., which is not present in the other forms of collagen. It should be mentioned that the total amounts of aldehydes present in acid-soluble collagen were in the same range as found in other forms of more insoluble collagen (2.5–2.7 μ moles/100 mg. of collagen).

Incubation of this acid-soluble collagen with cysteamine and subsequent dialysis to eliminate the excess of thiol causes the shoulder to disappear, but the absorption maximum remains at 320 nm. (Fig. 3c).

In the most insoluble collagen fractions, those extracted by 8 M-urea and the gelatinized residues, it became apparent that with increasing age there appeared an increasing amount of aldehyde, which causes a shoulder at 355 nm. The extreme experimental findings for the 8 M-urea-extracted collagen

Fig. 4. Extinction (integration units) of bands corresponding to α - and β -sub-units of denatured soluble collagen separated by polyacrylamide-gel disc electrophoresis and stained with Amido Black. Concentrations of collagen ranged between 10 and 60 μ g. dissolved in volumes less than 50 μ l. of 0.05 M-acetic acid. The method of Fessler & Bailey (1966) was used.

Table 3. Sub-unit composition of neutral-salt-soluble and cysteamine-soluble collagen from the skin of rats of various ages

Sub-units of thermally denatured 0.45 M-NaCl-soluble collagen and 0.2 M-cysteamine-soluble collagen from the skin of rats of various ages were separated by polyacrylamide-gel disc electrophoresis and scanned with the microdensitometer (25 μ g. of collagen/polyacrylamide gel).

Type of collagen	Age of animal (months)	Sub-unit distribution (%)		α -Sub-unit/ β -sub-unit ratio
		α	β	
0.45 M-NaCl (pH 7.0)-soluble	1	77	23	3.3
	2	78	22	3.6
	4	76	24	3.1
	6	77	23	3.3
	18	77	23	3.3
0.2 M-Cysteamine-soluble	1	50	44	1.10
	2	46	54	0.85
	4	47	53	0.90
	6	42	56	0.70
	18	44	56	0.77

fractions, those from rats 1 month and 1½ years old, are shown in Fig. 3(d).

To investigate the variation in intramolecular cross-linking occurring in the different collagen fractions obtained from animals of various ages, the α -sub-unit/ β -sub-unit ratio was measured after electrophoresis of the denatured collagens in polyacrylamide gel.

Fig. 4 shows that the method used for evaluation of α -sub-unit/ β -sub-unit ratios holds adequately over a large span of collagen concentrations (10–50 μ g. of collagen/polyacrylamide gel). Samples of 0.45 M-sodium chloride-soluble collagen and 0.2 M-cysteamine-soluble collagen dissolved in 0.05 M-acetic acid were used in this assay.

The α -sub-unit/ β -sub-unit ratios calculated for the 0.45 M-sodium chloride- and 0.2 M-cysteamine-soluble collagen fractions obtained from animals of different ages are summarized in Table 3.

The salt-soluble collagen showed a marked consistency in sub-unit composition. The 0.2 M-cysteamine-extracted collagen reflects a larger span of values, but failed to show any real significant changes that could be attributed to age. The variations observed in this fraction can be most likely attributed to contamination with more soluble collagen, especially with the very young animals, where the amount of soluble collagen is very large.

It is a common observation that acetic acid will extract considerable amounts of collagen from rat skin after it has been exhaustively washed with neutral-salt solutions and citrate buffer. The efficacy of acetic acid and cysteamine to solubilize the citrate-insoluble residue from rat skin was compared and the results of this experiment are summarized in Fig. 5.

The solubilizing capacities of both solvents are comparable, acetic acid being slightly more effective. Cysteamine at a neutral pH causes very little swelling of the skin residue, whereas acetic acid

shows a very marked swelling effect. This could not be measured effectively with young rat skin, since practically all the collagen became soluble, but can be evidenced when calf skin or older rat skin is exposed to these agents.

Because the amount of aldehydes present in soluble collagen may be related to its capacity to form fibrils an attempt was made to fractionate this soluble collagen on this basis. Fessler (1960a,b) has described the fractionation of salt-soluble collagen into various fractions with different capacities to form fibres, and Wood (1962) has further investigated the heterogeneity of this material.

The concentration of aldehydes present in the neutral-salt-soluble collagen before precipitation, as well as that found in the different fractions isolated by repeated thermal precipitations and cold redissolutions, is shown in Table 4. The amounts of collagen separated at each step are also listed in Table 4. The concentration of aldehydes in the collagen remaining in the supernatant

decreases after every precipitation. The first precipitate seems to contain the collagen with the highest aldehyde content, and subsequent precipitates seem to reflect the amount of aldehydes present in the soluble collagen precursor.

When the supernatants were gelled by incubation for 24 hr. at 37°, the amount of cooling required partially to redissolve the gel varied at each interval. After the first precipitation the gel had to be cooled for 1 hr. to obtain a significant redissolution. After the fourth precipitation significant dissolution occurred after less than 5 min. of cooling.

The changes in the intramolecular bonding that accompany the thermal precipitation of salt-soluble collagen are also shown in Table 4. Whereas the original salt-soluble collagen contains mostly α -chains, after thermal aggregation there was a marked increase in β -components of intramolecular origin. This formation of intramolecular cross-links during gelation supports the view that intramolecular and intermolecular cross-links may occur simultaneously (Veis & Anesey, 1965), although the intramolecular cross-links seem to acquire chemical stability faster than the intermolecular ones. Fig. 6 shows that the sub-units present before and after thermal precipitation are similar and both are β -components (β_{11} and β_{12}), presumably of intramolecular origin.

Fig. 5. Rates of dissolution of insoluble collagen from the skin of a 40-day-old rat in 0.2M-acetic acid and 0.2M-cysteamine in 0.45M-NaCl, pH 7.0.

DISCUSSION

The reconstitution of fibrils from collagen in solution has generated interest because under the appropriate conditions it may mimic the process of fibrillogenesis *in vivo*. The kinetics of this process have been studied and reviewed in detail by Wood (1962, 1964). The initial process of nucleation and subsequent growth into fibres are rather distinct

Table 4. Aldehyde content and sub-unit composition of neutral-salt-soluble collagen and its various fractions separated during the process of repeated thermal precipitation and redissolution at 4°

Neutral-salt-soluble collagen was incubated at 37° overnight, cooled at 4° and centrifuged. The procedure was repeated four times. All precipitates and supernatants at every step were analysed for their aldehyde content and sub-unit composition (α -sub-unit/ β -sub-unit ratio).

No. of thermal precipitations	Total collagen (mg.)		Acetaldehyde content (μ moles/100 mg. of collagen)		Supernatant collagen α -sub-unit/ β -sub-unit ratio
	Supernatant	Precipitate	Supernatant	Precipitate	
Original neutral-salt-soluble collagen	12.2	—	0.84	—	4.0
First precipitation	9.61	2.55	0.75	0.96	1.77
Second precipitation	8.53	1.14	0.71	0.77	1.81
Third precipitation	6.34	2.17	0.60	0.68	1.80
Fourth precipitation	4.58	1.66	0.53	0.59	1.82

Fig. 6. Polyacrylamide-gel disc electrophoresis patterns of neutral-salt-soluble collagen (a) before and (b) after thermal aggregation at 37° and redissolution at 4°. The method of Veis & Anesey (1965) was used.

processes undergone by different fractions of a heterogeneous collagen population. Wood (1964) has shown that salt-soluble collagen forms fibrils at a slower rate than acid-soluble collagen. In this respect the collagen extracted by cysteamine at neutral pH behaves like that extracted by acetic acid. The reason for the greater nucleating capacity of certain types of collagen is not clear. One difference that seems to be of significance is that, whereas the more mature forms of soluble collagen contain approximately equal amounts of α - and β -components, the more recently synthesized collagen, that soluble in neutral-salt solutions, contains mostly α -components (Piez, Lewis, Martin & Gross, 1961; Orekhovitch, Shpikiter, Mazourov & Kounina, 1960). Even though these various types of soluble collagen are remarkably alike in chemical composition and physical properties, some subtle differences, which may be related to the presence of tightly bound peptides or glycopeptides (Schmitt *et al.* 1964), may account for their differences in solubility (Davidson & Cooper, 1968) and electrophoretic mobility (Clark & Veis, 1968). Although acetic acid extracts the neutral-salt-soluble fraction along with the more insoluble collagen, it has been our consistent observation that salt-soluble collagen, at moderately high concentrations (2–3 mg./ml.), is partially precipitated when exposed to acetic acid.

We have recently reported that the collagen extractable by cysteamine contains a larger amount of reactive aldehydes than does salt-soluble collagen (Deshmukh & Nimni, 1968). These experiments were performed with a 40000g supernatant of a collagen solution. In the present studies we used

the supernatants after centrifuging for 90 min. at 105000g. Beyond 90 min. at this speed no further precipitate separates. These solutions, which behave as monodisperse systems during sedimentation (Nimni *et al.* 1967), exhibit the same properties as the 40000g supernatant. The finding that the collagen extracted by cysteamine forms stable fibres at a faster rate and becomes more readily insoluble in various solutions tested than the salt-soluble collagen can be explained in terms of its high aldehyde content.

The thermal shrinkage of the collagenous network, which is manifested by a retraction of the collagen gel, can be explained on the basis of the 'melting' of the crystalline arrangement of the intermolecularly cross-linked polypeptide chains. It differs from the collagen-to-gelatin transition in a monodisperse solution, where this transformation represents the 'melting' process stripped of intermolecular attachments.

The cysteamine-soluble collagen very rapidly generates a sufficient number of heat-stable intermolecular cross-links at 37°. Neutral-salt-soluble collagen needs longer incubation period at 37° to generate enough intermolecular cross-links to form a continuous fibrous network. Once such stable cross-links are formed, both types of gels show retraction when heated beyond their denaturation temperature.

When collagen was fractionated according to solubility, the less readily soluble fractions contained progressively larger amounts of detectable aldehydes. In addition, within each specific fraction, the amount of aldehydes present showed a slight decline with increasing age of the animals. Denaturation of the collagen did not seem to be an important factor, since fractions extracted from the insoluble collagen by either cysteamine or acetic acid (native collagen), or by 8M-urea or by autoclaving (gelatin), contained approximately the same high aldehyde titres.

The spectrum obtained during the reaction of various aldehydes with *N*-methylbenzothiazolone hydrazone varies according to the nature of the aldehyde involved (Paz *et al.* 1965). The spectral changes observed among the various fractions and ages seem to support the postulate that an aldol-type condensation may occur at some stage of the formation of intramolecular bonds (Rojkind, Blumenfeld & Gallop, 1964, 1966; Rojkind, Rhi & Aguirre, 1968; Bornstein, Kang & Piez, 1966; Bornstein & Piez, 1966). The more stable collagen, that which requires more drastic procedures for solubilization (acetic acid, cysteamine or urea), shows a shoulder peak at 355 nm., which may be an indication of the presence of $\alpha\beta$ -unsaturated aldehydes originating from the dehydration of an aldol-type reaction.

The acetic acid-extracted collagen has the largest shoulder at the wavelength of maximum absorption of an $\alpha\beta$ -unsaturated aldehyde. The shoulder can be suppressed by incubating this collagen with cysteamine and removing the excess of cysteamine by dialysis. This could be explained either by the fact that aminothiols may bind more tightly to such unsaturated aldehydes as does thiosemicarbazide (Rojkind *et al.* 1968) or that an addition of the thiol to the olefinic double bond changes the spectral characteristics of this aldehyde.

In all the fractions investigated, except that extracted by 0.45 M-sodium chloride, there seemed to be a gradual increase in aldehydes, which give rise to a shoulder at 355 nm., with increasing age of the animals.

Butzow & Eichhorn (1968) have reported that, as rats aged from 1 to 24 months, the acid-soluble collagen extracted from their tail tendons became gradually enriched in β -components. Since 0.5 M-acetic acid will solubilize almost completely the collagenous soft tissue of the young rat, this fraction represents a heterogeneous mixture of newly synthesized and older collagen. Heikkinen & Kulonen (1964) have previously shown that rat tail-tendon fibres extracted with an acetate buffer at pH 4.5 and at 40° yielded a denatured collagen containing more β -components if the tissues originated from older animals. Since the amount of collagen that can be extracted by neutral salts is much greater in young animals than in older ones (Gross, 1958; Nimni *et al.* 1967), and since acetic acid extracts both the salt-soluble fraction as well as the more insoluble fraction, the proportion of α - and β -components in an acetic acid extract of tissue, not previously washed with neutral salts, should be expected to reflect the proportion in which these fractions are present.

In the present experiments we find that within a particular collagen fraction the ratio of α - to β -components in the collagen solubilized is constant and independent of the age of the animal. The findings by Butzow & Eichhorn (1968) may therefore be explained by the fact that neutral-salt-soluble collagen, which is very rich in α -components, decreases markedly with age, and therefore contributes to a smaller extent to the material solubilized by acetic acid as the animal ages. Although the fraction extracted from skin by neutral cysteamine from a 1-month-old rat contains a slightly higher amount of α -sub-units, this could reflect a contamination of this fraction by the 0.45 M-sodium chloride-soluble collagen, which is present in such large amounts at this early stage of development.

Variation among species has been a major problem in understanding the solubility characteristics of collagen and the age-related changes.

Although 0.5 M-acetic acid and 0.2 M-cysteamine, pH 7.0, solubilize about 90% of the insoluble collagen present in the skin of a young rat, they will solubilize only about 15% and 25% respectively from the skin of foetal calf 2 months before birth (K. Deshmukh & M. E. Nimni, unpublished work). Therefore, in comparing different species, gestation period may be an important factor to consider, since cross-linking may be a phenomenon that depends to a great extent on the length of time during which the molecules have been in close proximity. The aging of collagen gels *in vitro* may offer a suitable model for studying this event.

When solutions of salt-soluble collagen are heated at 37° the material that becomes more readily cross-linked and does not redissolve when cooled is that containing the largest amount of aldehydes. The remaining supernatant retains a collagen that has been depleted of the aldehyde-rich fraction. By repeating this process of thermal aggregation and redissolution by cooling, the aldehyde content of the remaining collagen can be depleted to the values normally found in lathyrin collagen, which is close to 0.5 μ mole of aldehydes/100 mg. of collagen.

The collagen that returns to solution after being gelled shows a significant increase in β -components. It seems that incubation of neutral-salt-soluble collagen at 37° gives rise to stable intramolecular cross-links, which may not have yet participated in the formation of covalent intermolecular bonds. This latter process may require longer periods of molecular contact.

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