Age-Dependent Changes on Insoluble Collagen of the Skin of Rats

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Age-Dependent Changes on Insoluble Collagen of the Skin of Rats

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Summary

1. Age-dependent changes of the properties and of the behavior of "labile" collagen and insoluble collagen in gel filtration were investigated in thermal contraction fluid of rat skin.

2. Free hydroxyproline was not detected in the solution obtained after thermal contraction of the insoluble fraction but hydroxyproline as a constituent of the complex was found in large amounts in young rat skin when compared with the adult.

3. It was shown in gel filtration that the properties of the "labile" collagen which was released after thermal contraction of the insoluble fraction were different between young and adult, as in the insoluble collagen.

4. The resistance to thermal contraction of the insoluble fractions increased with the addition of formaldehyde, and the tendency was more definite in adult skin. Solubilization of adult skin was unaltered by the addition of urea, but in young skin the addition introduced a marked increase.

Verzár (1), who studied collagen fibers from the tendon of rat's tail, found that the thermal contraction at 58° becomes stronger with increasing age. Furthermore, using the skin of cattle of different ages, he observed that hydroxyproline is liberated into the surrounding fluid at this temperature and that very young animals seem to have a higher hydroxyproline content (2, 3). It is necessary to establish the preparation of native and of pure insoluble collagen in order to investigate the physical and the chemical properties of insoluble collagen. Since "insoluble collagen" is a fraction which extracted by autocleave twice at a pressure of 15 lb./in^2., the fraction is not more native collagen. Therefore, in these studies it was used for native insoluble collagen which remains after the extraction of soluble collagens from defatted skin with 0.2M NaCl as a first solvent and then secondly with 0.2M sodium citrate buffer, pH 3.5. The present paper describes some age-dependent changes of the properties and behavior of substances in the contraction fluid in gel filtration of insoluble collagen of the skin of rats.

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Materials and Methods

Animals and treatment of the skin

The rat collagen preparations used in all experiments were obtained from Wister strain male albino rats fed on commercial diet (NMF; Product of Oriental Yeast Co., Ltd.), maintained in our laboratry. The animals were provided with food and water *ad lib.*. Immediately after decapitation, pieces of skin cleaned of hair, subcutaneous fat and muscle, were miced in small sections with scissors. The skin sections were defatted three times with an excess of acetone at 4° for 24 hours.

Fractionation of collagen

The defatted skin sections prepared as described above were lyophilized and stored in a deep freezer prior to the extraction of collagen. Fractionation and extraction of collagen from these connective tissue fractions were carried out according to the procedure described by Jackson (4). Insoluble fractions on the 0.2M sodium citrate buffer (pH 3.5) treatment were used in this experiment for "native insoluble collagen".

Thermal contraction

100 mg of insoluble fractions were permeated to 4° in 5 ml of Ringer solution for 10 minutes and then 5 ml of the same solution which had been maintained to 65° were added. After standing at 65° for 10 minutes the suspension was then filtrated (5).

Sephadex gel filtration

A Sephadex G-75 (Pharmacia) column (1.5×84 cm) maintained at 37° with an over pipe, was used for the gel filtration. The solution of labile collagen, *i.e.*, that dissolved in 10 minutes at 65°, which will hereafter be referred to as "T.C. fraction", was applied to the top of the column and eluted with deionized water. Fractions of each 6.1 ml were collected at the flow rate of 13 ml per hour.

Other analytical methods

The protein content was determined by the Cupper-Folin method (6) using gelatin as the standard. Qualitative analysis of hydroxyproline in several collagen fractions and in T.C.fractions either with or without hydrolysis in 6N HCl at 110° for 24 hours was measured by modification (7) of the Neumann-Logan method (8).

Results

Collagen composition of the defatted skin in young and old rats.

Table 1 shows the hydroxyproline contents of several collagen fractions obtained from the defatted skin of young and old rats. In young skin soluble

TABLE 1. *Hydroxyproline Contents of Soluble and Insoluble Collagen.*

Age of rats (month)	Hydroxyproline (μg/mg defatted skin)			
	Insoluble fraction[a)]	Insoluble collagen	Acid soluble collagen	Neutral salt soluble collagen
2	57.1±4.8[b)] (6)	53.3±2.4 (5)	5.8±1.5 (10)	1.1±0.4 (10)
20	75.0±6.5 (6)	67.8±2.6 (4)	1.9±0.5 (7)	0.1±0.0 (5)

a) Native insoluble fraction which remains after extraction of defatted skin with 0.2 M sodium citrate buffer, pH 3.5.
b) Standard deviation. Numbers in parenthesis indicate numbers of samples in the groups.

collagens were three to nine times that of old rat skin, but the insoluble collagen increased as the aging advanced. This tendency is consistent with well known evidence (9).

Liberation of hydroxyproline during thermal contraction.

Table 2 shows the amount of hydroxyproline of both free and complex forms released during thermal contraction. It seems that the free hydroxyproline is liberated into the surrounding fluid under this thermic condition. But with the amino acid fraction which was adsorbed on Dowex-50 resin, the "free" hydroxyproline was not detected. Most of the hydroxyproline was observed after hydrolysis in complex form, while the thermal contraction hydroxyproline as the complex form was released in large amounts in young and smaller amounts in adult skins. As shown in Fig. 1-a and Fig. 1-b, solubilization set in at above 37° for both insoluble fractions and became marked between 40° and 60°. The protein and

TABLE 2. *Hydroxyproline Contents of Free and Complex Form Released during Thermic Contraction*

Age of rats (month)	Hydroxyproline (μg/100 mg insoluble fraction)		
	Free form		Complex form
	Direct	Dowex-50 adsorbed	
2(4)[a)]	97.2±10.3[b)]	2.0±0.5	4036±683
20(4)	40.5±11.0	0.8±0.5	1931±494

a) Number of rat, b) Standard deviation.
100 mg of insoluble fraction of young and adult skins were heated to 65° in 10 ml Ringer solution for 10 minutes. The filtrate was divided into three equal parts, the first part was used directly for the estimation of hydroxyproline, the second part was applied to a Dowex-50×8 column (0.8×3.0), after washing the column with distilled water amino acid fraction adsorbed on this regin was eluted from the column with 2M NH_4OH, the other was hydrolyzed in 6N HCl at 110° for 24 hours.

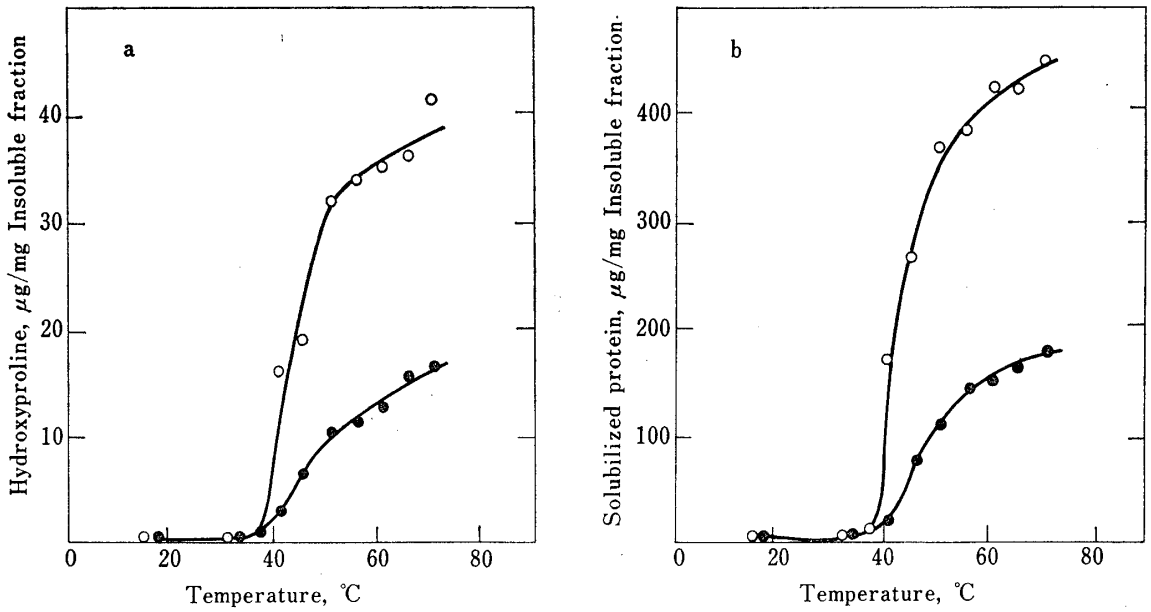

FIG. 1. Protein and Hydroxyproline Released from Insoluble Fraction of Young and Adult Skin during Thermic Contraction.

Change of solubilization were plotted versus incubation temperature. Open and closed circles represent the amounts of protein (b) and hydroxyproline (a) released from the skins of young and adult rats, respectively.

hydroxyproline released from the insoluble young fractions into the sorrounding fluid at these temperatures were about two times as much as for the adult fractions.

The behavior of the solubilized fractions and insoluble collagen in gel filtration.

In the above experiment it was recognized that a quantitative difference exists between the T.C.fraction of young and adult skins. Therefore, we made a rough estimate of the size of the molecules which contribute to the labile collagen by Sephadex gel filtration. Fig. 2 shows the elution profiles of proteins by gel filtration using T.C.fraction of young and adult skins. In young skin a relatively high molecular protein was eluted initially, followed by in fraction number 64 and 68. In adult skin, however, only one peak which had a shoulder in fraction number 64 was obtained in the position in the effluent corresponding to the last peak of the young skin. Fig. 3 shows the elution pattern of insoluble collagen by gel filtration of young and adult skin. The insoluble collagen of young skin was eluted with a symmetrical one peak in fraction number 76. Contract to the collagen, in insoluble collagen of adult skin, various molecular size proteins were eluted at first and then the main fraction corresponded to the young insoluble collagen was eluted.

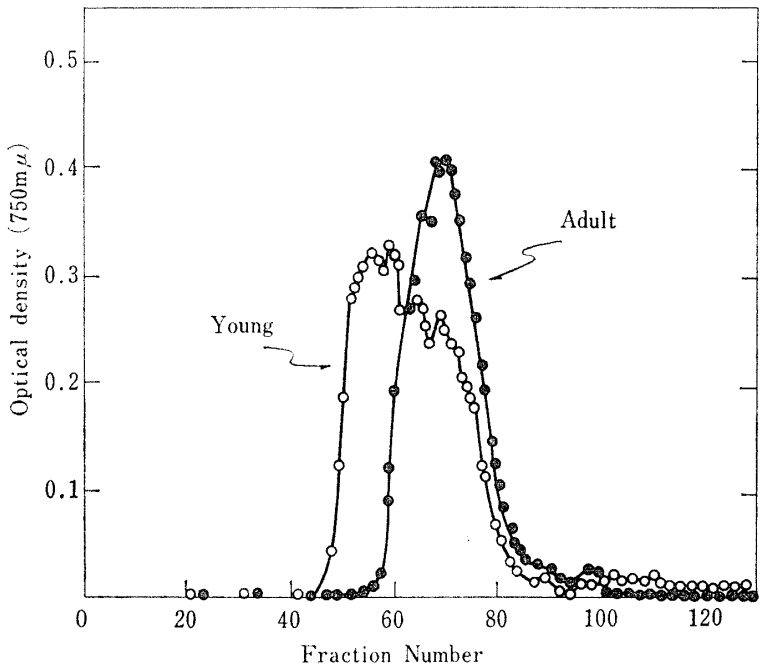

FIG. 2. Elution Profiles of Solubilized Matter in Thermic Contraction of Insoluble Fraction of Young and Adult Skin by Gel Filtration.

About 110 mg and 230 mg of insoluble fraction of young and adult skin, respectively, were heated with the method described in the text. The filtrate was added to the column of Sephadex G-75 with a bed volume of approximately 0.57 liters. Absorbancy at 750 mμ of each fraction (6.1 ml per tube) were plotted versus fraction numbers. Two elution patterns obtained from the separate chromatographies were exactly superimposed, and were represented in this figure.

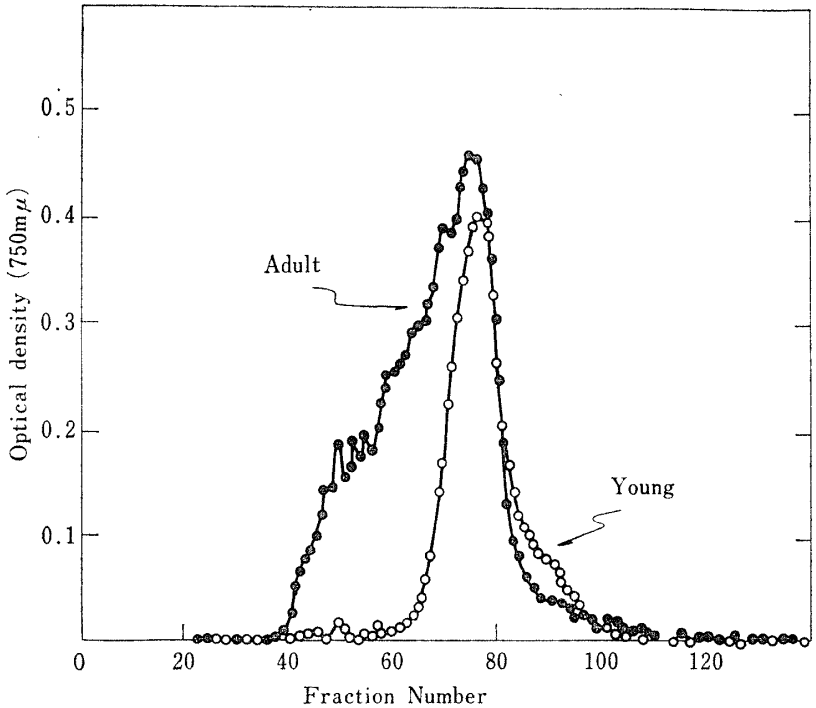

FIG. 3. Elution Profiles of Insoluble Collagen of Young and Adult Skin by Gel Filtration.

About 20 mg and 40 mg of insoluble collagen of young and adult skin, respectively, were chromatographed in the similar manner to the case of the soluble fraction obtained from the thermic contraction (Fig. 2). Two elution patterns obtained from the separate chromatographies were exactly superimposed, and were represented in this figure.

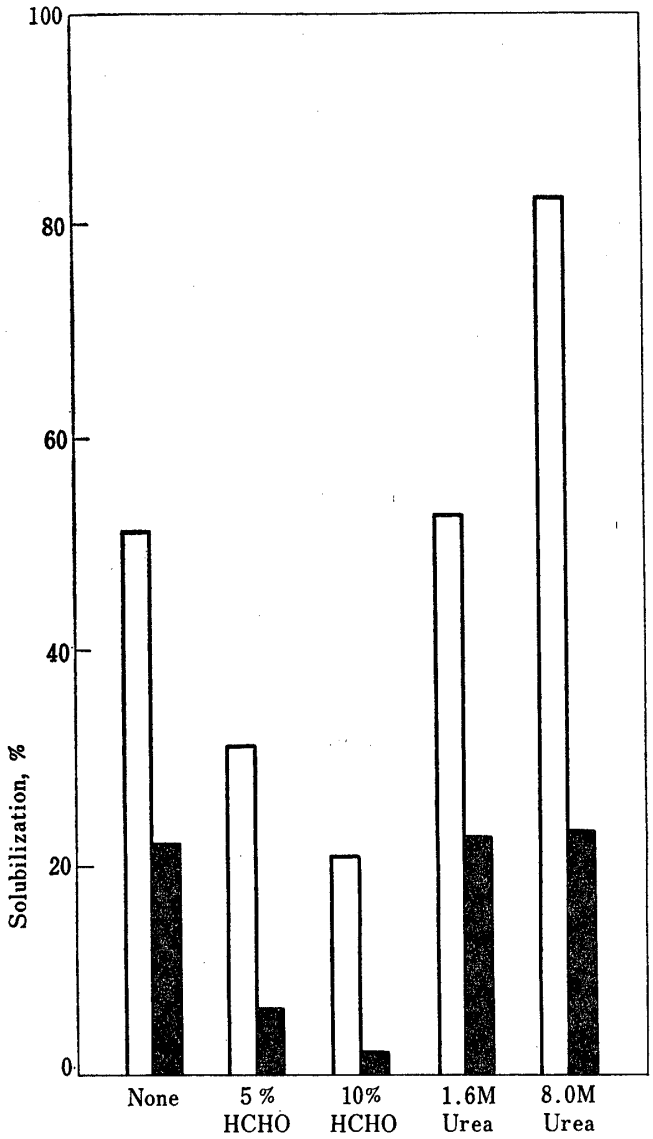

FIG. 4. Influence of Formaldehyde and Urea on Thermic Contraction of Young and Adult Skin. □ Young and ■ Adult.

Effect of formaldehyde and urea on solubilization of insoluble fraction.

Fig. 4 shows the influence of formaldehyde and urea on the solubility of the insoluble fractions during the thermal contraction of young and adult skins. The solubilization of the insoluble fraction reduced with increasing concentrations of formaldehyde. This tendency was clearly observable when the insoluble fraction of adult skin was treated with 10% formaldehyde. On the other hand, when the insoluble fraction of adult skin was treated with urea, further solubilization was not observed. However in that of young skin 83 per cent of the insoluble fraction was solubilized with treatment of thermal contraction in the presence of 8M urea.

Discussion

The age-dependent changes in physical properties of collagen are probably due to increased hydrogen bonding and altered collagen-mucopolysaccaride inter-relationships. Ohori *et al* (10) of our laboratory showed that the total mucopoly-succharide increased from 3 month old to 6 month old rats and that the chondroi-tin sulfate and heparin content in the defatted skin of rat decreased while that of hyaluronic acid and heparitin sulfate increased as the aging advanced. Verzár (1) has shown using the skin of cattle of different ages, that the thermal contraction at 65° becomes greater with increasing age. In the same experiment he found that with thermal contraction the free hydroxyproline was liberated into the sorrounding fluid, and he suggested that it is possible to use the liberation of hydroxyproline at 65° as a test for the biological age of collagen. The present study using skin of rats, however, has shown that in direct determination of hydroxyproline released into the surrounding fluid during the thermal contraction, the quantity (2 months; 100 μg/100 mg, 20 months; 40 μg/100 mg) was consistent with the values reported by Verzár (2). But as shown in table 1 significant free hydroxyproline was not detected. As these results show, it is impossible to think that free hydroxyproline is released from the insoluble collagen molecules. In regard to the apparent hydroxyproline values in direct determination of hydroxy-proline, a possible explanation might be that some contaminants *e.g.* "labile" collagen in sorrounding fluid distorted the readings. As shown in Fig. 2 and Fig. 3, the T.C.fraction of young skin appeared in the population with two or three different proteins. However, when the insoluble collagen of the young skin was chromatographed, these protein peaks disappeared and a single peak, like that of a lower molecular weight protein peak, was observed. Contrary to the young skin, in the T.C.fraction of the adult skin, only one peak which had a shoulder was obtained. Also in the gel filtration of insoluble collagen of adult skin various molecular weight fractions were observed and a peak like that of the smallest molecular weight was eluted in a position corresponding to the young insoluble collagen. From these results, it may be suggested that the thermal stability of insoluble collagen becomes stronger with increasing age. Therefore, the largest molecular weight protein peak which was observed only in the T.C.fraction of young skin was also observed in the insoluble collagen of adult skin, being obtain with a high temperature treatment as autocleave. Furtheremore, it might be thought that all insoluble collagen of the young skin was cleaved in low molecular weight fractions during the autocleave treatment. It has been recognized that formaldehyde is the classic example of a tanning substance and urea is a kind of denaturant which cleaves the intermolecular hydrogen bonds of the protein. Verzár and Huber (11) observed that the temperature of incipient contraction of rat tail tendon fiber in 10% urea is lower in young than old. As shown in Fig. 4, the solubilization of adult skin was reduced to one-tenth of the control by the

addition of formaldehyde and unaltered by the addition of urea. But in young skin the addition of urea resulting in a marked increase in the solubilization. From these data, it may be thought that, (I) because the formaldehyde is powerfully combined to the amino methylol group on the collagen molecules, cross-linking methylene bridges are produced on the collagen molecules (12), the resistance to thermal contraction becomes stronger in the insoluble collagen of adult skin, (II) hydrogen bond persists as a predominant form of intermolecular crosslink in the young insoluble collagen, and other cross-linkings, except for the hydrogen bond *e.g.* Schiff base (13–15), aldol condensation (16), dihydroxylysinonorleucine (17), etc, increase as aging advances.

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