The Relationship between Reversibility of Fibril Formation and Subunit Composition of Rat Skin Collagen

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1. Salt-soluble rat skin collagen was precipitated from solution at neutral pH and 37°. On cooling, a portion of the collagen returned into solution. The fractions were separated, the supernatant was concentrated and the precipitate was redissolved in dilute acetic acid. 2. Solutions of supernatant and precipitate were subjected to the same fractionation procedure, giving four fractions. 3. Each fraction was examined by starch-gel electrophoresis and a relationship between subunit composition and the fractionation procedure was noted. The collagen that redissolved on cooling contained less of the more highly cross-linked components than did either the fraction remaining in the precipitate or the starting material.

The collagen molecule is approximately 14 Å in diameter and 3000 Å long (Boedtker & Doty, 1956). It is believed to consist of three subunits of similar molecular weight called α -chains (Orekhovitch & Shpikiter, 1955), one of which, the α_2 -chain, is known to have a slightly different amino acid composition from the other two, the α_1 -chains (Piez, Lewis, Martin & Gross, 1961). During maturation *in vivo* covalent cross-links are formed between α -chains within molecules, giving rise to β -chains (two α -chains) and γ -chains (three α -chains). Covalent cross-links are also formed between the α -chains of different molecules. Since a solution of collagen contains molecules at various stages of maturation, when denatured either thermally or with urea it may give rise to a complex mixture of subunits. Veis & Anesey (1965) tentatively demonstrated the existence of all the theoretically possible combinations, namely α_1 -, α_2 -, β_{11} -, β_{12} - and γ_{112} -chains formed by intramolecular cross-linkage and β_{22} -, γ_{111} -, γ_{122} - and γ_{222} -chains formed by intermolecular cross-linkage.

Under suitable conditions collagen has the ability to form fibrils *in vitro*, apparently identical with those found *in vivo* (Gross, Highberger & Schmitt, 1955; Jackson & Fessler, 1955), and it has been shown by Wood (1962) that highly cross-linked collagen is precipitated from solution more rapidly than is the less cross-linked protein. Connel & Wood (1964) showed that treatment of soluble collagen with molluscan enzymes leads to a decrease in the

ease of fibril formation, associated with which there is a conversion of β -chains into α -chains. Similar results have also been reported after treatment with proteolytic enzymes (Rubin, Pfahl, Speakman, Davison & Schmitt, 1963).

It therefore appears that highly cross-linked collagen forms fibrils more readily than does less cross-linked material, and it may be expected that a solution that contains a mixture of cross-linked and non-cross-linked molecules will produce fibrils in which the 'inner core' contains the bulk of cross-linked collagen. Fibril formation *in vitro* is partially reversible (Gross, 1958; Fessler, 1960*a,b*), and consequently any collagen subsequently returning into solution may be expected to arise from the outer layers of the fibril and hence to contain less of the cross-linked collagen than does that remaining in fibrillar form.

Starch-gel electrophoresis is a technically simple method of separating soluble collagen subunits. The purpose of the present work has been to adapt this technique to semi-quantitative assessment by reflectance densitometry, to gain more detailed information on the fractionation of solutions of collagen.

MATERIALS AND METHODS

Preparation of soluble collagen. Collagen was extracted from the skins of male Wistar rats, wt. 150–200g. The skins, which were stored at -10° until required, were soaked in a solution of 5% (w/v) NH_4Cl –1% (w/v) NaCl –0.01% thiomersal at room temperature for 5 days (Gross & Kirk, 1958), to facilitate removal of epidermis and subcutaneous muscle. The minced skins were defatted with

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Scheme 1. Procedure used to obtain six fractions from salt-soluble rat skin collagen.

chloroform-methanol (2:1, v/v), and extracted three times (for 3 days each) with 10% (w/v) NaCl at 4-6°. Acid-soluble collagen was extracted from the washed residue with 3% (v/v) acetic acid by the same procedure.

Pooled extracts of the salt-soluble and acid-soluble collagen were purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Cooper & Davidson, 1965) followed by repeated precipitation with NaCl (Jackson & Fessler, 1955) and finally precipitation by exhaustive dialysis against 0.02 M- Na_2HPO_4 . The purified collagens were stored in 3% (v/v) acetic acid at 4-6°.

Hydroxyproline and protein concentration determinations. Hydroxyproline was determined by the method of Prockop & Udenfriend (1960). Protein concentrations were determined by the micro biuret method of Goa (1953). The latter technique was calibrated with stock salt-soluble collagen,

the concentration of which had been determined on a dry weight basis.

Fractionation procedure. Stock salt-soluble collagen (100-200 ml.) was dialysed against two changes (2 l. each) of 0.1 M- Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.0, and gelled by warming to 37° for 2 hr. The gel was then cooled to 4-6° for 24 hr. and the precipitate (F1) and supernatant (F2) fractions were separated by centrifugation at 78000g for 1 hr. in a Spinco model L preparative ultracentrifuge. The supernatant was concentrated against Carbowax at 4-6° and dialysed exhaustively against 3% (v/v) acetic acid. The precipitate was redissolved in 3% (v/v) acetic acid and dialysed against the dilute acid to remove excess of Na^+ and phosphate ions. The fractionation procedure was then repeated with the supernatant and precipitate fractions. On this occasion, however, the supernatant was centrifuged

immediately after gelation, giving a precipitate and a supernatant containing unprecipitated collagen. This resulted in four fractions (F3–F6) and the relationship between them is shown in Scheme 1.

Starch-gel electrophoresis of collagen subunits. Samples of collagen were denatured at 40° for 15 min. and were analysed for their subunit composition by starch-gel electrophoresis by the method of Nääntö, Pikkariainen & Kulonen (1965), with the following modifications. A Perspex trough (150 mm. × 70 mm. × 6 mm.) was used. It was water-jacketed and provided with a cover (130 mm. × 70 mm.), also water-jacketed. During a run the temperature was held at 35°. The electrode compartments contained acetate buffer, pH 4.7 (Nääntö *et al.* 1965) and 1.0 M, and connexion to the gel was made by wicks consisting of several layers of cheese-cloth soaked in the same buffer. A current of 40 mA was applied for 6 hr., giving a voltage gradient across the gel of 4–5 v/cm. The gels were stained with 0.2% Nigrosine in methanol–acetic acid–water (5:1:5, by vol.) for 5 min., and excess of stain was removed by several changes of solvent. The percentage composition was obtained by using a Joyce, Loebl and Co. Ltd. (Gateshead, Co. Durham) double-beam recording and integrating densitometer. Six distinct bands were revealed by this technique, four of which were identified by Nääntö, Maatela & Kulonen (1963) as α_2 -, α_1 -, β_{12} - and β_{11} -chains, in order of decreasing mobility. Two further bands of lower mobility, termed X₁ and X₂, were also demonstrated, and these appeared to be larger aggregates (Hollmén & Kulonen, 1964). Although visibly distinct, the β -chains were not sufficiently well separated for individual determination and were therefore treated as a single component.

All experiments were carried out at a protein concentration of 0.16–0.20%.

RESULTS AND DISCUSSION

Stock salt-soluble collagen contained 17.5% of nitrogen and 12.9% of hydroxyproline on hydrolysis. Values for acid-soluble collagen were 19.2% of nitrogen and 14.0% of hydroxyproline.

The percentage compositions of salt- and acid-soluble collagens and the fractions derived from salt-soluble collagen are shown in Table 1.

Salt-soluble collagen contains a higher percentage of single chains than does acid-soluble collagen, 47.7% compared with 34.1%. The overall pattern, however, is reasonably similar and is in agreement with the finding of Nääntö *et al.* (1963). Fraction F1 showed the predicted alteration in subunit composition, there being less of the α -chains and slightly less of the β -chains, but an increase in both X₁ and X₂ fractions, compared with the original material; this last difference was significant ($P < 0.005$). Fraction F2 showed a significant fall in the content of β -chains ($P < 0.0005$) compared with salt-soluble collagen, but a slight though not significant increase in both X₁ and X₂ fractions. This finding suggests that cross-linking might have occurred during incubation of the gel at 37°. The four fractions resulting from the second fractionation cycle again illustrate the alteration in subunit composition very clearly. Fraction F3, the most highly cross-linked, had significantly less α_1 -chain ($P < 0.005$) and α_2 -chain ($P < 0.005$), similar amounts of β -chains and X₁ fraction, and significantly more X₂ fraction ($P < 0.005$), compared with the initial salt-soluble collagen. Fraction F6, which was expected to be the least highly cross-linked fraction, had significantly more α_1 - and α_2 -chains ($P < 0.005$ and < 0.01 respectively) and no detectable X₂ fraction compared with salt-soluble collagen.

The results indicate that collagen that is not highly cross-linked returned into solution after fibril formation more readily than did cross-linked material. This finding was consistent with the results reported by Gross (1964), who demonstrated increasing irreversibility of fibril formation with increasing length of incubation at 37° and who interpreted the phenomenon as being due to the formation of cross-links. The cross-links are believed to involve a lysine-derived aldehyde which is present in the α -chains (Piez, Martin, Kang & Bornstein, 1966). This aldehyde has been blocked

Table 1. Subunit composition of fractions of salt-soluble rat skin collagen

Collagen samples were denatured at 40° for 15 min. For details of fractionation procedure see the Materials and Methods section. The results are expressed as means $\pm$ s.d.

Collagen	No. of observations	Percentage of subunit in fraction				
		α_2	α_1	β	X ₁	X ₂
Salt-soluble	4	17.3 $\pm$ 1.69	30.4 $\pm$ 1.52	36.3 $\pm$ 0.94	10.7 $\pm$ 2.80	6.0 $\pm$ 3.19
Acid-soluble	6	11.9 $\pm$ 0.36	22.2 $\pm$ 0.53	37.8 $\pm$ 0.94	12.2 $\pm$ 0.50	15.8 $\pm$ 1.10
F1	4	13.9 $\pm$ 1.32	27.4 $\pm$ 3.42	32.2 $\pm$ 1.42	13.3 $\pm$ 2.88	13.3 $\pm$ 2.63
F2	4	19.5 $\pm$ 4.52	30.2 $\pm$ 2.86	28.7 $\pm$ 2.43	12.4 $\pm$ 2.93	9.0 $\pm$ 2.34
F3	4	10.9 $\pm$ 1.96	24.0 $\pm$ 1.30	33.3 $\pm$ 5.28	13.9 $\pm$ 3.08	17.8 $\pm$ 4.43
F4	4	17.7 $\pm$ 2.55	29.9 $\pm$ 1.78	38.9 $\pm$ 1.64	10.7 $\pm$ 2.41	2.8 $\pm$ 3.27
F5	4	15.3 $\pm$ 2.13	27.4 $\pm$ 2.94	31.5 $\pm$ 2.49	15.9 $\pm$ 2.15	9.9 $\pm$ 3.48
F6	4	21.1 $\pm$ 0.98	40.3 $\pm$ 2.59	29.2 $\pm$ 2.05	9.5 $\pm$ 2.14	0

by thiosemicarbazide (Tanzer, Monroe & Gross, 1966), and under these circumstances fibrils produced *in vitro* remain completely reversibly soluble.

Fessler (1960*a,b*) has fractionated soluble collagen and obtained a fraction similar to the fraction F6 described in the present work. This collagen was incapable of forming fibrils *in vitro*. The reasons for this behaviour are unknown but might include any of the following: (i) a subtle alteration of the molecules, not amounting to complete denaturation, that destroys the ability to form fibrils (Piez, 1967); (ii) a loss of lysine-derived aldehyde, owing to its accumulation in other fractions (Piez, 1967); (iii) the presence, as an impurity, of a small tightly-bound inhibitor that accumulates in this fraction.

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