The Induction of Cross-links in Soluble Rat Skin Collagen *in vitro*

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1. Starch-gel electrophoresis was used to investigate the subunit composition of salt-soluble and acid-soluble rat skin collagen. 2. Cross-linkage of collagen subunits *in vitro* was performed (a) when in fibrillar form and (b) when in solution. In the former case the increase in number of cross-links appeared to be predominantly intermolecular and in the latter case predominantly intramolecular.

Collagen can be extracted from rat skin by using neutral salt or dilute acid solutions. The protein so obtained is known as tropocollagen and consists of thin rod-like molecules approx. 3000 Å long and 14 Å in diameter (Boedtker & Doty, 1956). Soluble collagen may be denatured under mild conditions, for example by heating at 40° at an acid pH, or by treatment with urea. Under these conditions three classes of subunit are observed with molecular weights about one-third, two-thirds and unity when compared with that of the undenatured tropo-collagen molecule (Engel & Beier, 1963; Piez, 1965). The components of the smallest class are polypeptides called α -chains and there are believed to be three/tropocollagen molecule. The amino acid composition of these chains has been studied extensively and the results demonstrate that two of the three chains/molecule are probably identical in rat skin (Piez, Eigner & Lewis, 1963) and dogfish skin (Lewis & Piez, 1964a), but that all three are different in codfish skin (Piez, 1964).

Denaturation of soluble rat skin collagen gives a solution containing mostly α_1 -, α_2 -, β_{11} - and β_{12} -chains (the β -subunits consist of two α -chains covalently linked) and a small amount of γ_{112} -chain (all three α -chains covalently linked but not in the native-type triple-helix configuration). The proportions of α -, β - and γ -chains derived from a collagen solution depend to a large extent on the nature of the extracting solvent. Acid-soluble collagen contains more of the β - and γ -chains than does salt-soluble collagen. The subunits mentioned above are thought to arise from intramolecular cross-link formation, but other subunits have been detected in solutions of denatured rat tail-tendon collagen and tentatively identified as β_{22} -, γ_{111} -, γ_{122} - and γ_{222} -chains (Veis & Anesey, 1965). These subunits are presumed to arise from intermolecular cross-linkage.

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The technique of starch-gel electrophoresis has been applied to the separation of this mixture of subunits (Näntö, Maatela & Kulonen, 1963; Hollmén & Kulonen, 1964; Näntö, Pikkarainen & Kulonen, 1965) and resolution of the α_1 -, α_2 -, β_{11} - and β_{12} -chains achieved. Also, two further bands were revealed which migrate more slowly than those mentioned previously and which are thought to consist of larger aggregates of subunits.

The changes occurring during fractionation of soluble collagen solutions have been studied by using starch-gel electrophoresis semi-quantitatively (Bannister, 1969), and the results suggest that this method is suitable for the study of other factors that may influence the subunit composition of collagen. In the present paper work is presented showing changes in the electrophoretic pattern of rat skin collagen on the induction of cross-links *in vitro*.

METHODS AND MATERIALS

Preparation of collagen. Collagen was extracted from the skins of male Wistar rats, wt. 150–200 g. Preparation of the skins and extraction of salt- and acid-soluble collagens was performed as described by Bannister (1969). In some experiments the minced skin was defatted by soaking in chloroform-methanol (2:1, v/v) and purified collagens from this material are subsequently termed 'defatted'.

The stock salt-soluble collagen contained 17.5% of N and 13.4% (w/w) of hydroxyproline. The values for stock acid-soluble collagen were 18.2% of N and 14.0% (w/w) of hydroxyproline. There was little difference between normal and 'defatted' collagens. Hydroxyproline was determined by the method of Prockop & Udenfriend (1960).

Starch-gel electrophoresis. Electrophoresis was performed by the method of Näntö *et al.* (1965) as modified by Bannister (1969). The samples of collagen [concn. 0.5–1.0% (w/v) in 3% (v/v) acetic acid] were denatured either by maintenance at the appropriate temperature for 15 min. or by mixing with an equal volume of 10M-urea.

Induction of cross-links in vitro. Samples of salt-soluble collagen (0.16%, w/v) in 0.1M- Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.0, were incubated at 37° for 48 hr. The control consisted only of collagen and buffer and the experimental

sample contained, in addition, 0.05 M-D-glucose. At the end of the incubation period the gels were dialysed against 3% (v/v) acetic acid at 4–6° to redissolve the collagen.

In a further series of experiments a solution of salt-soluble collagen (0.16%, w/v) was incubated at 30° for 3 hr. in 87.5 mM-acetate buffer, pH 4.8, containing either formaldehyde (1.43 mg./ml.) or quinol (1.43 mg./ml.). Controls were incubated in buffer alone. At the end of the experiment the samples were concentrated by dialysis against Carbowax at 4–6° and then dialysed against 3% (v/v) acetic acid.

RESULTS

Under the conditions of electrophoresis employed the subunits migrated from the anode. On staining six bands were revealed, which corresponded to the α_2 -, α_1 -, β_{12} -, β_{11} -, X_1 - and X_2 -bands identified by Nantö *et al.* (1963). The β -bands, although clearly distinct, were not adequately resolved for separate measurement and were therefore treated as a single component.

Effect of temperature of denaturation on subunit composition. Samples of 'defatted' acid-soluble collagen were denatured at temperatures between 37.5° and 100°, and their percentage subunit compositions determined by starch-gel electrophoresis.

The results are shown in Table 1. Little change occurred between 37.5° and 45°. At 60°, however, a discrete band migrating faster than the α_2 -band was observed, and in addition the quality of resolution deteriorated because background staining became noticeable. Both these features were enhanced at 65° and 70°, and heating at 100° destroyed all bands.

Effect of extraction with chloroform-methanol on subunit composition. The percentage subunit compositions of salt-soluble and acid-soluble collagens extracted from 'defatted' and untreated skin minces and denatured with urea are shown in Table 2. There was no difference between the salt-soluble collagens, but 'defatted' acid-soluble collagen contained fewer α -chains and significantly more β -chains ($P < 0.01$) than did the acid-soluble collagen from untreated skins.

Induction of cross-links in vitro. The results of incubation of collagen in the presence of glucose, formaldehyde and quinol on the percentage subunit composition are shown in Table 3. Incubation at 37° of the gelled collagen alone produced a decrease in the percentage of total α -chains from 46.7% to 38.8%. The decreases for the individual chains were

Table 1. *Effect of temperature of denaturation on the subunit composition of acid-soluble rat skin collagen*

The material used was 'defatted' acid-soluble collagen and was denatured at the temperature given for 15 min. It was then subjected to starch-gel electrophoresis by the method of Nantö *et al.* (1965) as modified by Bannister (1969). The percentage composition is expressed as mean $\pm$ S.D.

Temp.	No. of observations	Percentage of subunit in sample					
		Pre- α	α_2	α_1	β	X_1	X_2
37.5°	3	—	11.7 $\pm$ 0.8	23.3 $\pm$ 2.2	38.9 $\pm$ 2.0	11.3 $\pm$ 0.9	14.8 $\pm$ 3.5
40	6	—	11.9 $\pm$ 0.9	22.2 $\pm$ 1.2	37.8 $\pm$ 2.3	12.2 $\pm$ 1.2	15.8 $\pm$ 2.7
45	3	—	11.8 $\pm$ 1.1	24.4 $\pm$ 2.9	38.3 $\pm$ 1.2	11.0 $\pm$ 1.4	14.5 $\pm$ 3.7
60	3	3.3 $\pm$ 1.4	12.5 $\pm$ 1.9	24.3 $\pm$ 2.3	35.8 $\pm$ 0.1	10.2 $\pm$ 0.9	13.9 $\pm$ 4.9
65	4	4.4 $\pm$ 0.5	12.3 $\pm$ 0.7	23.2 $\pm$ 2.2	33.3 $\pm$ 0.4	10.3 $\pm$ 0.6	16.4 $\pm$ 2.5
70	4	5.3 $\pm$ 0.9	14.5 $\pm$ 1.4	29.7 $\pm$ 2.4	35.5 $\pm$ 1.4	8.2 $\pm$ 2.2	6.8 $\pm$ 1.7

Table 2. *Percentage subunit compositions of salt- and acid-soluble rat skin collagen*

The samples of collagen were denatured with urea as described in the Methods and Materials section. 'Defatted' collagens were extracted from minced skins after extraction of fat-soluble material with chloroform-methanol (2:1, v/v). The normal collagens were not treated in this way. The samples were then subjected to starch-gel electrophoresis by the method of Nantö *et al.* (1965) as modified by Bannister (1969). The percentage composition is expressed as mean $\pm$ S.D.

Collagen sample	No. of observations	Percentage of subunit in sample				
		α_2	α_1	β	X_1	X_2
Salt-soluble						
Normal	4	17.3 $\pm$ 0.9	29.4 $\pm$ 1.5	36.0 $\pm$ 0.9	11.1 $\pm$ 1.1	6.4 $\pm$ 2.5
'Defatted'	4	17.3 $\pm$ 1.7	30.4 $\pm$ 1.5	36.3 $\pm$ 0.9	10.7 $\pm$ 2.8	6.0 $\pm$ 3.2
Acid-soluble						
Normal	4	13.5 $\pm$ 0.7	27.5 $\pm$ 1.5	30.7 $\pm$ 2.9	11.8 $\pm$ 0.1	16.5 $\pm$ 4.0
'Defatted'	6	12.5 $\pm$ 1.5	22.2 $\pm$ 1.2	36.7 $\pm$ 2.7	11.1 $\pm$ 1.1	17.5 $\pm$ 3.3

Table 3. *Induction of cross-links in salt-soluble rat skin collagen in vitro*

The protein used was salt-soluble rat skin collagen that was denatured with urea as described in the Methods and Materials section. The samples were subjected to starch-gel electrophoresis by the method of Nääntö *et al.* (1965) as modified by Bannister (1969). The percentage composition is expressed as mean $\pm$ s.d.

Experiment	No. of observations	Percentage of subunit in sample				
		α_2	α_1	β	X ₁	X ₂
Initial salt-soluble collagen	4	17.3 $\pm$ 0.9	29.4 $\pm$ 1.5	36.0 $\pm$ 0.9	11.1 $\pm$ 1.1	6.4 $\pm$ 2.6
Glucose experiment*						
No glucose	4	13.0 $\pm$ 1.4	25.8 $\pm$ 0.9	30.4 $\pm$ 2.1	15.6 $\pm$ 1.9	15.2 $\pm$ 4.3
+ glucose (0.05 M)	4	11.7 $\pm$ 1.1	25.2 $\pm$ 1.1	25.1 $\pm$ 2.8	18.6 $\pm$ 0.6	19.3 $\pm$ 2.1
Acetate-buffer experiment†						
Control	4	16.3 $\pm$ 4.4	30.0 $\pm$ 3.5	34.9 $\pm$ 2.2	12.0 $\pm$ 3.4	6.9 $\pm$ 4.6
+ formaldehyde (1.43 mg./ml.)	4	12.5 $\pm$ 2.9	24.2 $\pm$ 2.5	30.2 $\pm$ 2.8	20.7 $\pm$ 2.5	12.3 $\pm$ 6.1
+ quinol (1.43 mg./ml.)	4	18.9 $\pm$ 2.3	30.3 $\pm$ 1.0	36.1 $\pm$ 2.2	11.1 $\pm$ 0.9	3.6 $\pm$ 0.8

* The sample of collagen was maintained at 37° for 48 hr. in 0.1 M-phosphate buffer, pH 7.0.

† The samples of collagen were maintained at 30° for 3 hr. in 87.5 mM-acetate buffer, pH 4.8.

significant, α_2 -chain from 17.3% to 13.0% ($P < 0.02$) and α_1 -chain from 29.4% to 25.8% ($P < 0.05$). There was also a decrease of 5.4% in the amount of β -chains, which was significant ($P < 0.05$). These decreases were offset by increases of 4.5% in the amount of X₁- and 8.8% of the X₂-band. The latter increases were statistically significant ($P < 0.05$ and < 0.05 respectively). The inclusion of glucose enhanced the changes in subunit composition that were observed in the control, although analysis of subunit composition revealed no statistically significant differences from the control.

In the experiments carried out to induce cross-linking in acetate buffer, the control sample, and that with quinol, showed no significant changes in composition on incubation compared with the initial salt-soluble collagen. The sample incubated with formaldehyde, however, showed qualitative changes similar to those observed earlier, namely decreases in the contents of α - and β -chains and increases of bands X₁ and X₂ though only the increase in content of band X₁ from 11.1% to 20.7% was significant ($P < 0.005$).

DISCUSSION

Effect of temperature of denaturation on subunit composition. The instability of collagen subunits was studied at acid pH values and 40° by Engel & Beier (1963), who demonstrated the loss of β -chains in the ultracentrifuge over a period of about 2 days. Studies of a similar type were made by Lewis & Piez (1964b), though at a less acid pH (4.8 compared with 3.7), in which appreciable amounts of β_{12} -chain were lost. The products, however, were not α -chains, which suggests that cleavage of labile bonds within chains occurred rather than splitting

of the covalent cross-links between chains. This hypothesis could explain the appearance of a new component, that migrated faster than the α_2 -chain, on heating at temperatures of 60° and above. Heating at 100° presumably brought about large-scale and non-specific bond cleavage since no distinct bands could be identified. Increased heat treatment did not bring about the accumulation of protein in either the X₁- or X₂-bands, suggesting that it is unlikely that they consist largely of degradation products. It seems probable, therefore, that the X₁- and X₂-bands consist largely of γ -chains and consideration of electrophoretic mobility would suggest that the X₁-band contained γ_{112} -chain and the X₂-band, γ_{111} -chain.

Induction of cross-links in vitro. When a solution of collagen is gelled at neutral pH and a temperature of 37° it forms native-type fibrils (Jackson & Fessler, 1955; Gross, Highberger & Schmitt, 1955). If the gel is then cooled to 0–7° some of the collagen redissolves (Gross, 1958). Increasing the time for which the collagen is maintained in fibrillar form decreases the amount of collagen that will redissolve on cooling, the reason suggested being the formation of cross-links (Gross, 1958). In contrast with this behaviour collagen from lathyrus animals, which contains fewer β - and γ -chains than normal (Martin, Gross, Piez & Lewis, 1961), does not become progressively insoluble with time (Gross, 1963). The reason for this difference in behaviour is thought to involve the inhibition of a lysine-derived aldehyde group that is involved in the formation of the covalent cross-links (Rojkind & Juaréz, 1966). There is strong evidence that intramolecular cross-link formation involves the condensation of a lysyl residue in one α -chain with a lysine-derived aldehyde group in another (Piez,

1968), and that this is the major cross-linking mechanism *in vivo*. The results given in Table 3 demonstrate a rise in the content of X₁- and X₂-bands when collagen is gelled alone. This increase probably results from the above mechanism. However, a mechanism for cross-linking *in vivo* was suggested by Bensusan (1964) in which glucose attached to a lysyl residue underwent the Amadori rearrangement. The product (1-*N*-substituted 1-deoxyfructose) might then either form a Schiff base with other ϵ -amino groups or react with phenolic or histidyl residues by a Knoevenagel-type reaction. The experimental evidence produced was similar in nature to that found by Gross (1958), namely increased insolubility of the gel compared with that of a control incubated in the absence of glucose (Bensusan, McKnight & Naidu, 1966). Also compatible with this hypothesis is the finding that carbohydrate is a normal constituent of collagen (Oneson & Zacharias, 1960; Butler & Cunningham, 1966; François & Glimcher, 1966).

The present work (see Table 3), however, indicates no statistically significant differences between the percentage subunit composition of the control collagen sample and that incubated with glucose although the direction of the changes was towards a greater percentage of the cross-linked subunits.

The cross-linking of collagen in solution *in vitro* by using formaldehyde was demonstrated by Veis & Drake (1963), as shown by a conversion of α - into γ -chains. The results presented in Table 3 are consistent with this finding and demonstrate that the major increase is in the amount of X₁-band, which is believed to contain γ ₁₁₂-chain. It is not surprising that the main change should be in the number of intramolecular cross-links, but there was also some increase in the amount of X₂-band, indicating intermolecular cross-links. This is no doubt explained by the tendency of tropocollagen molecules to aggregate in solution (von Hippel, 1967).

Cross-linking with quinol was also attempted because it was shown that this reagent caused the gelation of soluble collagen to occur more rapidly, under defined conditions, than in its absence (Trnavská, Sit'aj, Grmelá & Malinsky, 1966). There was no induction of cross-links under the experimental conditions used above.

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