Thermal Denaturation of Soluble Calf-Skin Collagen

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1. Soluble calf-skin collagen has been denatured thermally between 37° and 60° and the component proteins have been separated on carboxymethylcellulose. 2. Four main fractions have been separated; α and β (in the nomenclature in common usage) and two other fractions. (The α and β components are complex owing to the presence of α_1 , α_2 , β_1 and β_2 parts). 3. Fractions 3 and 4 undergo rapid denaturation between 39° and 40° whereafter fraction 4 remains virtually unchanged even at 60°. 4. That portion of fraction 4 which remains at 60° is thought to be identical with the fraction designated γ by other workers, this fraction being composed of three α -chains in covalent linkage (such bonds are alkali-labile). 5. The equilibrium between α , β and fractions 3 and 4 is apparently reversible since acid-soluble collagen after denaturation at 45° or 60° followed by cooling to 0° for 30 min. was found to contain only fraction 4 when chromatographed at 37°.

Soluble collagens can be extracted from young tissues by neutral-salt solutions or by acidic solutions such as dilute acetic acid or citrate buffers. These soluble collagens are thought to be precursors of the insoluble fibrous protein (Gross, Highberger & Schmitt, 1955). Neutral-salt-soluble collagen appears to be the most recently formed collagen in young tissue, although it is probable that both salt-extracted and acid-extracted collagens are biologically heterogeneous (Jackson & Bentley, 1960).

Soluble collagens may be denatured in solution by heating or by means of chemical agents such as urea, and Engel (1962) has suggested that the denaturation of soluble collagen consists of two consecutive reactions: the first step being the breakdown of the helical structure of the molecule followed by a slower dissociation into smaller components. The neutral-salt-soluble material dissociates into three peptide chains, having similar molecular weights, the α components (Orekhovich, Shpikiter, Mazurov & Kounina, 1960). Acid-soluble collagen dissociates into at least two components, α and β , where β consists of two α -chains linked together by an alkali-labile bond (Orekhovich & Shpikiter, 1958; Nishihara & Doty, 1958). It has been reported that there are two different species of α and β in some denatured collagens (Piez, Lewis, Martin & Gross, 1961; Piez, Eigner & Lewis, 1963; Schleyer, 1962), and this is borne out in the present work. A third component, γ , has been found in small amounts and this appears to be three α -chains linked together by covalent bonds (Grassman, Hannig & Engel, 1961).

The separation of α and β components of heat-denatured acid-soluble collagen by means of column chromatography has been described by Piez, Weiss & Lewis (1960). A third component, designated Y, was obtained by Schleyer (1962) using a similar technique.

This paper deals with the separation by column chromatography of the products of the thermal denaturation of soluble calf-skin collagen, and suggestions are put forward for the relationship between them.

EXPERIMENTAL

Extraction of collagens. Neutral-salt-soluble collagens were extracted from the skin of an unborn calf by solutions of 0.2M- and 0.45M-sodium chloride as described by Gross *et al.* (1955). The collagen was, however, not freeze-dried, as there is considerable evidence that prolonged storage of freeze-dried collagen, or storage of a precipitate at -20°, leads to the formation of insoluble material.

Acid-soluble collagen was extracted from calf skin by the method of Steven & Tristram (1962).

The collagens were stored at 4° in 0.6% acetic acid. Stock solutions contained from 0.07 to 0.15% of salt-extracted collagen, and 0.2% of acid-extracted collagen.

Chromatography. The chromatography of denatured collagen was similar to that described by Piez *et al.* (1960). CM-cellulose was used as adsorbent and the columns were maintained at a constant temperature by circulating water through a jacket. Except where otherwise stated the column temperature was maintained at the temperature of denaturation. The stock solutions of collagen were dialysed against starting buffer for 24 hr. and then denatured by heating at various temperatures for 15 min.

The α and β components were separated by gradient

elution with sodium acetate buffer, pH 4.8, which was fed to the column from a nine-chambered Autograd. The third component was obtained by prolonged elution with the final buffer of the gradient (0.5M-acetate). A fourth component was finally eluted by a solution of 0.1M-sodium hydroxide in 0.1M-sodium acetate (added to minimize swelling of the cellulose). This component was occasionally separated as two poorly defined peaks.

The effluent fractions were analysed for protein colorimetrically by the Folin-Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951), analyses being carried out by means of an auto-analyser (Technicon Instruments Ltd., Chertsey, Surrey). For estimation of the concentrations of the four components a curve was plotted of extinction against concentration of collagen (obtained from hydroxyproline determinations). As reported by Lowry *et al.* (1951) it was found that the Beer-Lambert relationship is not obeyed, but that the concentration could be estimated fairly accurately from measurements of extinction by comparison with standard solutions of collagen.

RESULTS

The results of a typical separation on CM-cellulose is shown in Fig. 1. While the separation of the α and β components was similar to that reported by Piez *et al.* (1960, 1963), the further resolution of the α and β peaks into α_1 , β_2 , β_1 and α_2 fractions was dependent upon the type of CM-cellulose employed (cf. Piez *et al.* 1963); cf. Fig. 2. Satisfactory separations were obtained only with Cellex CM (California Biochemicals). The separation of the four fractions was less marked than was found by Piez *et al.* (1963) for the collagens of rat-tail tendon, rat skin, carp swim bladder and spiny-dogfish skin. The elution of component 3 and component 4 was independent of

Fig. 1. Effluent analysis from the chromatography on CM-cellulose of 45 mg. of denatured neutral-salt-extracted calf-skin collagen. The collagen was denatured by heating at 40° for 15 min. The 2.5 cm. x 30 cm. column was also maintained at 40° and was eluted with acetate buffer, pH 4.8. Each of the nine chambers of the Autograd contained 125 ml. of buffer, the concentrations of acetate being 0.025, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.30 and 0.50M. After approx. 800 ml. of the gradient had been fed to the column, the third component was eluted with 200 ml. of 0.50M buffer. The fourth component was finally eluted by a solution of 0.1M-sodium hydroxide and 0.1M-sodium acetate. The rate of elution was 150 ml./hr.

the type of CM-cellulose employed, both being quantitatively dependent only upon the temperature of denaturation.

Fig. 2. Elution pattern of 40 mg. of acetic acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation for 15 min. at 45°. Columns were eluted with acetate buffer, pH 4.8, a four-chambered Autograd (each chamber containing 200 ml. of buffer of concentration 0.06, 0.06, 0.16 and 0.16M) being used. CM-cellulose used: (a) Whatman (0.7 m-equiv./g.); (b) Serva (0.6 m-equiv./g.); (c) Cellex-CM (0.6 m-equiv./g.); (d) Cellex-CM (0.6 m-equiv./g.). With Cellex-CM (d) the buffers used were 200 ml. each of 0.08, 0.08, 0.12 and 0.16M.

Table 1. Composition of heat-denatured acid-extracted collagen

Temp.	No. of expts.	Components		
		α and β (%)	Component 3 (%)	Component 4* (%)
37°†	8			100
37.5	8	3, 6	13, 12	84, 82
38	8	9, 8	10, 13	81, 79
39	9	29, 30	7, 9	64, 61
40	10	59, 61	4, 4	37, 35
42	9	65	2	33
43	10	67, 70	2, 1	31, 30
60‡	6	67, 70	2, 1	31, 30

* Piez *et al.* (1963) have reported that excess of protein remaining on the column can be removed by elution with solutions of sodium hydroxide. This component was said by Piez to be absent when all the collagen was completely dissolved before chromatography. In our experience we have found that, after centrifuging at 20000g for 1 hr., when perfectly clear solutions were obtained, the amounts of component 4 were virtually unchanged.

† Collagen denatured at 43° for 15 min. and allowed to cool to 0° was found to contain the same proportions of all components as native collagen when chromatographed at 37°.

‡ Column temperature was maintained at 45° to prevent break-up of the packed column.

Table 2. *Composition of heat-denatured neutral-salt-extracted collagens*

	Temp.	No. of expts.	Components α and β (%)	Component 3 (%)	Component 4 (%)
(a) Collagen extracted by 0.2M-NaCl soln.	38°	4	21	30	49
(b) Collagen extracted by 0.45M-NaCl soln.	38	4	18	17	65
	40	4	71	9	20
	60*	4	84	0	16

* Column temperature was maintained at 45° to prevent break-up of the cellulose packing.

The amounts of the components eluted after denaturation at various temperatures are given in Table 1. Since α and β components were not completely separated a summed value is given. The recovery of protein, confirmed by the determination of total nitrogen on each bulked fraction, was usually approximately 100%.

Table 2 gives the composition of denatured salt-soluble collagens extracted (a) by 0.2M-sodium chloride and (b) by 0.45M-sodium chloride solution.

DISCUSSION

In their work on the separation of the components of calf-skin collagen, Piez *et al.* (1960) stated: 'It is not likely that the chromatographic separation of the α - and β -components is the result of different charge densities. . . . It is known, however, that the molecular weights of the two components differ approximately 2-fold. Under these conditions, the larger molecule would move more slowly than the smaller since its statistical chances of being attached to the adsorbent would be greater.'

Later work (Piez *et al.* 1963) reported the separation of α_1 , β_2 , β_1 and α_2 fractions, in that order of elution. This would suggest that the separation must be dependent upon charge density in view of the quoted molecular weights of α and β . The excess of cationic residues has been calculated from the analyses of Piez *et al.* (1960, 1963), assuming a pK CO_2H 4.0 (i.e. 86.3% of carboxyl groups are dissociated at pH4.8). The figures are given in Table 3.

In all cases (Piez *et al.* 1963) the order of resolution is predictable on the basis of the cationic charge at pH4.8 and the elution curves obtained reflect the range of charge distributions given in Table 3. The α and β components of calf-skin collagen are not well separated and this is borne out by the small variation in the charge densities.

The separations obtained in the present work cannot wholly be obtained on the basis of ionic characteristics. Since the amounts of components 3 and 4 are temperature-dependent (Fig. 3, Tables 1 and 2) it follows that the latter must be polymeric

Table 3. *Excess of cationic residues (per 1000 total residues) in soluble collagens at pH 4.8*

Calculated from the data of Piez *et al.* (1960, 1963).

	Original	α_1	β_2	β_1	α_2
Rat skin	24.1	18.3	20.4	25.2	32.1
Rat-tail tendon	22.0	14.7	12.3	20.8	27.9
Carp swim bladder	21.0	17.0	18.4	21.5	25.4
Spiny-dogfish skin	30.9	23.1		37.4	
Calf skin	28.0	24.4 (α)		26.3 (β)

Fig. 3. Elution patterns obtained from the chromatography on CM-cellulose of calf-skin collagen denatured (a) at 40° (solid line) and (b) at 37.5° (broken line).

forms, their separation being based upon molecular weight rather than ionic characteristics. This is supported by the finding that the system is reversible. If soluble collagen is denatured at 45° and thereafter cooled to 0° before chromatography at 37° the elution pattern is similar to that obtained for native acid-soluble collagen.

The form of polymerization of components 3 and 4 is not yet established but the rapid dissociation of component 3 between 37.5° and 40° coupled with a larger increase in the amount of α than in β (Fig. 4) suggests that this component (which is comparable with the Y component of Schleyer, 1962)

Fig. 4. Effect of temperature on the properties of collagen. ●, Proportion of α and β components; ○, collagen solubilized (cf. Comte *et al.* 1963); ■, component 4; □, optical rotation.

is a polymerized form of α held together by hydrogen bonding. This view is supported by the observation that neutral-salt-soluble collagen contains a far greater proportion of component 3 (Table 2) than does acid-soluble collagen (Table 1).

The fourth component which is eluted by sodium hydroxide is probably the same as the 'sodium hydroxide fraction' obtained by Kulonen, Virtanen, Viljanto & Seppälä (1962) and which is thought to resemble the parent collagen. Between 20 and 30% of the original collagen is eluted as component 4 after denaturation at 60°, and this remains intact unless heated at an alkaline pH, when it is presumably degraded into smaller units before elution. In this respect it resembles the γ -component reported by other workers (Grassmann *et al.* 1961; Altgelt, Hodge & Schmitt, 1961). At temperatures below 43° the additional material (up to at least 50% of the acid-extracted collagen) found in component 4 is probably composed of α and β chains linked together by weak bonds. This material is removed as α and β as the temperature is raised. It is thought that the true component 4 (i.e. that which remains at 60° and which is thought to comprise three covalently linked α chains) and the associated chains are polymeric and probably in helical configuration, which would account for the constituent chains not being eluted along with the third component.

Wood (1963) studied the fractionation of neutral-salt-soluble collagen and observed the presence of one fraction (fraction C) with a higher degree of cross-linking. This he assumed to be the 'nucleus forming collagen'. Component 4 reported in the

present work is probably the same type of collagen, in particular that which remains at 60°.

Comte, Balme & Villa (1963) have studied the effect of temperature on the extraction of collagen from comminuted calf skin. They showed that little or no collagen was extracted below 35° and their results suggest that the extraction is dependent upon the amount of α and β components present at a given temperature (cf. Fig. 4).

The high laevorotation of collagen is ascribed to the ordered helical configuration of the protein chains and the fall in rotation on thermal denaturation to the disruption of helical formation. The close similarity of the temperature curves for optical rotation and the amount of component 4 suggests that this component is the nucleus around which other components enter into helical configuration (cf. Wood, 1962).

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