Characterization of Pepsin-Solubilized Bovine Heart-Valve Collagen

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Collagens extracted from heart valves by using limited pepsin digestion were fractionated by differential salt precipitation. Collagen types were identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, amino acid analysis and cleavage with CNBr. Heart-valve collagen was heterogeneous in nature, consisting of a mixture of type-I and type-III collagens. The identity of type-III collagen was established on the basis of (a) insolubility in 1.7M-NaCl at neutral pH, (b) behaviour of this collagen fraction on gel electrophoresis under reducing and non-reducing conditions, (c) amino acid analysis showing a hydroxyproline/proline ratio greater than 1, and (d) profile of CNBr peptides on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showing a peak characteristic for type-III collagen containing peptides $\alpha 1(\text{III})\text{CB8}$ and $\alpha 1(\text{III})\text{CB3}$. In addition to types-I and -III collagen, a collagen polypeptide not previously described in heart valves was identified. This polypeptide represented approx. 30% of the collagen fraction precipitated at 4.0M-NaCl, it migrated between β - and $\alpha 1$ -collagen chains on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and its electrophoretic behaviour was not affected by disulphide-bond reduction. All collagen fractions from the heart valves contained increased amounts of hydroxylysine when compared with type-I and -III collagens from other tissues. The presence of β - and γ -chains and higher aggregates in pepsin-solubilized collagen indicated that these collagens were highly cross-linked and suggested that some of these cross-links involved the triple-helical regions of the molecule. It is likely that the higher hydroxylysine content of heart-valve collagen is responsible for the high degree of intermolecular cross-linking and may be the result of an adaptive mechanism for the specialized function of these tissues.

It has been known for a long time that collagen is a major component of heart valves and comprises approx. 50% of their dry weight (Bashey *et al.*, 1967). The biochemical characterization of heart-valve collagen has, however, been limited, probably because of its extreme insolubility (Bashey *et al.*, 1972). A precise knowledge of normal heart-valve collagen structure and composition could be helpful in establishing the nature of alterations that occur during loss of valvular functional capacity in certain human cardiac diseases. Previously we determined the amino acid composition of bovine heart-valve insoluble collagen and demonstrated for the first time the presence of increased amounts of hydroxylysine compared with skin collagen (Bashey *et al.*, 1972). Since those studies were performed, however, it has become apparent that there is genetic polymorphism in the molecular structure of collagen and at least four distinct collagen types have been described (for review see Miller, 1976). Type-I collagen is the most abundant species and is found mainly in tendon, bone and skin. It is composed of two distinct polypeptide chains, the $\alpha 1(\text{I})$ - and the $\alpha 2$ -chains, in a molar ratio

of 2:1. Type-II collagen is present in cartilage and is composed of three identical $\alpha 1(\text{II})$ -chains. Skin, vessels and foetal membranes contain variable proportions of type-III collagen, composed of three $\alpha 1(\text{III})$ -chains. Type-IV collagen has been found in basement membranes and is also composed of three identical chains, $\alpha 1(\text{IV})$.

Identification of the collagen types present in heart valves has yielded conflicting results. Mannschott *et al.* (1976) characterized the collagens extracted by limited pepsin digestion of pig heart valves, and showed the presence of both type-I and -III collagens in these extracts. In contrast, Collins *et al.* (1975) have suggested that type I is the exclusive type of collagen found in pig heart valves. In the present study, we have solubilized collagen from bovine heart valves by using limited pepsin digestion. The solubilized collagen has been purified by using differential salt fractionation, and characterized by NaDodSO₄/polyacrylamide-gel electrophoresis, cleavage with CNBr and amino acid analysis. Our studies show that both type-I and -III collagens are present in pepsin extracts from these tissues. We also found that these two collagens contain increased amounts of hydroxylysine compared with type-I and -III collagens from

Abbreviation used: NaDodSO₄, sodium dodecyl sulphate.

other tissues. During fractional NaCl precipitation, a collagen fraction with increased solubility at neutral pH was identified. This fraction comprised about 10% of the total collagen extracted and contained an additional collagenous polypeptide which migrated between $\alpha 1$ - and β -chains on NaDodSO₄/polyacrylamide-gel electrophoresis.

Experimental

Collagen extraction

Bovine hearts were obtained fresh from a local slaughterhouse and transported to the laboratory in ice. The valves were washed several times with 0.9% NaCl and adventitia and chordae tendinae were removed. The valves were then minced and homogenized in 1 M-NaCl/0.1 M-Tris/HCl/20 mM-EDTA, pH 7.4, at 4°C (20%, w/v) and extracted three times with 20–25 vol. of the same buffer over 7 days at 4°C. The remaining tissue was then similarly subjected to three extractions with 0.5 M-acetic acid at 4°C. The insoluble residue remaining after salt and acetic acid extractions was freeze-dried and used for subsequent pepsin digestion and collagen purification.

For pepsin digestion, about 0.5–1 g of the freeze-dried insoluble residues were suspended in 100–200 ml of 0.5 M-acetic acid containing 1 mg of pepsin (2× crystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml, and extracted at 15°C with shaking for 72 h. The pepsin-solubilized material was collected after removal of insoluble residue by centrifugation at 35000g for 40 min at 4°C and immediately neutralized by addition of 0.1 M-NaOH to inactivate the pepsin. Pepsin extraction of the residue was repeated under similar conditions. Samples from pepsin extracts were hydrolysed in 6 M-HCl and hydroxyproline was determined by the method of Woessner (1961). The pepsin extracts were combined and total collagen was precipitated by addition of solid NaCl to 20% (w/v). The mixture was left overnight at 4°C and precipitated collagen was then collected by centrifugation. The collagen was solubilized in 0.5 M-acetic acid and used for subsequent studies.

Collagen fractionation and purification

To separate the various collagen types present in the pepsin extracts, fractional salt precipitation was performed by methods described by Epstein (1974), Chung & Miller (1974) and Trelstad *et al.* (1976). After the collagen precipitated from the pepsin extracts was solubilized in 0.5 M-acetic acid, it was dialysed against 0.4 M-NaCl/0.1 M-Tris/HCl, pH 7.4, and solid NaCl added to give a final concentration of 1.7 M. The precipitated collagen was removed by centrifugation at 30000g for 30 min and it was further purified by solubilizing it in 1.0 M-NaCl/0.1 M-Tris/

HCl, pH 7.4, at 4°C and dialysing it for 3 days against several changes of 0.1 M-Tris/HCl buffer, pH 7.4 containing 2.5 M-NaCl. The collagen fraction that precipitated at 2.5 M-NaCl was separated out by centrifugation at 30000g for 30 min and the supernatant was then dialysed for 3 days against 4.0 M-NaCl in 0.1 M-Tris/HCl buffer, pH 7.4. The supernatant remaining after precipitation with 4 M-NaCl was subsequently dialysed for 3 days against 0.02 M-sodium phosphate, and no further precipitation occurred. Addition of solid (NH₄)₂SO₄ to 30% saturation also failed to produce visible precipitation, and this supernatant was therefore discarded.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed to examine the collagen polypeptide composition of the various fractions by using 5% gels in a modification of the method of Weber & Osborn (1969), as described previously (Jimenez & Rosenbloom, 1974). Samples were denatured with 1% NaDodSO₄ in the presence or absence of 1% mercaptoethanol (i.e. reducing or non-reducing conditions) by heating at 100°C for 10 min and the electrophoresis was carried out for 4 h with a constant current of 8 mA/tube. After electrophoresis the gels were stained with 0.25% (w/v) solution of Coomassie Brilliant Blue for 12 h and subsequently destained in acetic acid/methanol/water (2:3:35, by vol.). The destained gels were scanned at 560 nm in a Gilford spectrophotometer fitted with a linear transporter. Under these conditions, clear separation between $\alpha 1$ -, $\alpha 2$ -, β - and γ -collagen chains was obtained. The recorded tracings from the scans were used to calculate the area under the various peaks with a planimeter.

CNBr cleavage

A portion of the pepsin-solubilized collagen was freeze-dried and suspended in 25 ml of 70% (v/v) formic acid. The suspension was flushed with nitrogen and digested with twice the sample weight of CNBr for 4 h at 30°C (Bornstein & Piez, 1966). The digestion mixture was diluted 2-fold with water and freeze-dried. The freeze-dried material was redissolved in water and freeze-dried again to ensure complete removal of volatile products.

The CNBr-cleaved peptides were analysed by using NaDodSO₄/polyacrylamide disc gel electrophoresis as described by Scott & Veis (1976a) after denaturation in 1% NaDodSO₄ containing 1% mercaptoethanol at 100°C for 10 min. Electrophoresis was performed on 7.5% polyacrylamide gels by using 1 mA/tube for 20 min, then 3 mA/tube for 40 min, and 8 mA/tube for 4 h. The gels were stained, destained and scanned as described above.

Amino acid analysis

Samples were hydrolysed under nitrogen in 6M-HCl at 100°C for 20h and amino acids were separated on a Beckman 119 C amino acid analyser.

Results

Collagen extraction and solubilization

Extraction of heart valves with 1.0M-NaCl and 0.5M-acetic acid did not yield sufficient collagen for further characterization. To overcome this problem, we used prolonged and repeated pepsin digestion at 15°C. The results shown in Table 1 indicate that limited pepsin digestion solubilized 20–30% of the total heart-valve collagen. This amount may be slightly underestimated, as the collagen content of the extracts was compared with the total hydroxyproline content of the heart valves and the contribution of hydroxyproline from elastin was not taken into consideration.

Fractional salt precipitation

The collagen extracted by limited pepsin digestion was precipitated from 0.5M-acetic acid by addition of solid NaCl to 20% (final concentration) as described by Trelstad *et al.* (1974). This concentration of salt has been shown to precipitate all collagen types, including basement-membrane collagen. In the present study, we found that addition of solid NaCl to 20% (v/v) resulted in precipitation of over 75% of the collagen present in the extracts (Table 1).

The amounts of collagen precipitated at 1.7M-, 2.5M- and 4.0M-NaCl are shown in Table 2. Approximately equal amounts of collagen were precipitated at 1.7M- and 2.5M-NaCl, and about 90% of the total pepsin-solubilized collagen was precipitated at these two NaCl concentrations. A fraction of collagen which represented about 10% of the total pepsin-solubilized collagen was further precipitated with 4.0M-NaCl. No additional collagen was precipitated when the 4.0M-NaCl supernatant was dialysed against

0.02M-Na₂HPO₄ or when solid (NH₄)₂SO₄ to 30% saturation was added. There were no marked differences in the recoveries or in the fraction of collagen precipitated at various salt concentrations between aortic and mitral valves.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Further characterization of the collagens precipitated at the various salt concentrations was achieved by NaDodSO₄/polyacrylamide-gel electrophoresis of reduced and non-reduced denatured proteins. When the total collagen fraction precipitated with 20% NaCl was examined in the presence of reducing agent it was possible to identify clearly the α 1-, α 2-, β 11-, β 12- and γ -chains as shown in Fig. 1(a). Calculation of the areas under each peak by planimetry revealed that approx. 18% of the total collagen migrated in the region of γ -chains and 16% in the region of β -chains. Since the electrophoresis was performed after reduction of disulphide bonds, these results indicated that the β - and γ -chains were held together

Table 1. Amounts of hydroxyproline extracted by limited pepsin digestion of bovine heart valves

Values are total mg of hydroxyproline. For the first step, valves were freeze-dried after extraction with 1.0M-NaCl and 0.5M-acetic acid as described in the Experimental section. Pepsin extraction was performed at 15°C for 72h in 0.5M-acetic acid. Concentration of pepsin was 1 mg/ml.

	Aortic	Mitral
Freeze-dried heart valve (500mg)	52.6	35.79
First pepsin extract	11.45	4.37
Second pepsin extract	3.99	3.08
Total extracted	15.42	7.45
Percentage extracted	29	21
20% NaCl precipitate from pepsin extracts	11.90	6.89
Percentage precipitated	77	92

Table 2. Fractional NaCl precipitation of pepsin-solubilized collagen from bovine heart valves

Total pepsin-extracted collagen was precipitated with 20% NaCl. The precipitate was solubilized in 1M-NaCl/0.1M-Tris/HCl, pH7.4, at 4°C. Fractional NaCl precipitation was performed as described in the text.

	Hydroxyproline			
	Aortic		Mitral	
	mg	%	mg	%
Total collagen	11.90	100	7.45	100
1.7M-NaCl precipitate	8.08	48	2.58	42
2.5M-NaCl precipitate	5.12	48	2.76	44
4.0M-NaCl precipitate	0.46	4	0.78	14
Total collagen precipitated	11.66		6.12

Fig. 1. Spectrophotometric scans of sodium dodecyl sulphate/polyacrylamide gels of pepsin-solubilized collagen from mitral valve

Electrophoresis was performed on 5% polyacrylamide gels after heat denaturation of the sample in 1% sodium dodecyl sulphate under (a) reducing and (b) non-reducing conditions as described in the Experimental section. Arrows indicate the migration of $\alpha 1$ -, $\alpha 2$ -, $\beta 11$ -, $\beta 12$ - and γ -chains of standard type-I collagen which was run on a parallel gel under identical conditions. Direction of migration was from left (cathode) to right (anode).

by non-disulphide cross-links. The molar ratio of $\alpha 1$ - to $\alpha 2$ -polypeptides was 3.36:1, which indicated that there was an excess of $\alpha 1$ -chains. When the total collagen fraction was electrophoresed without reduc-

tion of disulphide bonds, a marked decrease in the proportion of $\alpha 1$ -chains and a concomitant increase in the collagen migrating in the region of γ -chains were demonstrated (Fig. 1b). These results indicated

that the excess $\alpha 1$ -polypeptides were present as a disulphide-bonded trimer and suggested therefore that type-III collagen was present (Chung & Miller, 1974). This was further confirmed when the collagen fraction precipitated at 1.7M-NaCl was electrophoresed under similar conditions. When electrophoresis was performed after disulphide-bond reduction, a great excess of $\alpha 1$ -chains was demonstrated and very few $\alpha 2$ -chains were present (Fig. 2a). The molar ratio of $\alpha 1$ - to $\alpha 2$ -chains in this fraction was 5.82:1. When the electrophoresis was performed without reducing agent, however, this ratio was 1.9:1 (Fig. 2b). Changes in the relative proportion of collagen migrating in the position of γ -chains were also detected, since before disulphide-bond reduction the γ -region contained 38% of the total scanned protein and only 11% after reduction. The small amounts of $\alpha 2$ -chains and the failure to detect collagen migrating in the position of $\beta 12$ -chains also supports the notion that type-III collagen was present in this fraction. The electrophoretic patterns of the collagen precipitated at 2.5M-NaCl showed $\alpha 1$ - and $\alpha 2$ -polypeptides as well as β - and γ -chains (Fig. 3). These patterns were not appreciably different if they were obtained in the presence or absence of reducing agents (results not shown). These findings indicated that type-I collagen was the main component of this fraction. When the collagen fractions precipitated at 4.0M-NaCl were similarly examined, it was found that in addition to the usual collagen polypeptides migrating in the position of $\alpha 1$ - and $\alpha 2$ -chains, another band migrating between β -chains and $\alpha 1$ -chains was detected (Figs. 4 and 5). This band was found in all preparations examined and constituted about 30% of the total collagen in this fraction. A mol.wt. of approx. 119000 was calculated from comparison of the migration of these polypeptides with the migration of $\alpha 1$ - and β -chain standards (Sykes & Bailey, 1971). The electrophoretic patterns did not show any significant differences when electrophoresis was performed in the presence or in the absence of reducing agent (results not shown).

CNBr cleavage

The electrophoretic patterns in NaDodSO₄/polyacrylamide gels of CNBr peptides from heart-valve collagen were compared with the profiles of CNBr peptides from rat skin and chick tendon collagens extracted with acetic acid. They were also compared with similarly obtained data reported by Eyre & Muir (1975) and Scott & Veis (1976a,b). A representative scan of heart-valve CNBr peptides is shown in Fig. 6. A characteristic peak exclusive to type-III collagen which contains peptides $\alpha 1$ (III)CB8 and $\alpha 1$ (III)CB3 co-migrating in the electrophoretogram was seen towards the end of the gel profile (shaded area). This peak, which is well separated from that

of peptide $\alpha 1$ (I)CB3, was only seen in heart-valve samples and was not detected in skin or tendon collagens (results not shown). Besides this characteristic peak indicating the presence of type-III collagen, the profile of heart-valve CNBr peptides also showed characteristic peaks derived from type-I collagen. These were identified by the method of Scott & Veis (1976a) as follows. The first major peak represents the uncleaved peptide $\alpha 2$ CB3-5; the next two peaks, which were not completely resolved in our electrophoretograms, represent the incompletely cleaved peptides $\alpha 1$ CB2, 4, 5, 8, 3 and $\alpha 1$ CB5, 8, 3. The next major peak is a mixture of peptides $\alpha 1$ CB4, $\alpha 1$ CB8 and $\alpha 1$ CB7. Following this peak, two smaller peaks corresponding to peptides $\alpha 1$ CB6 and $\alpha 1$ CB3 of type-I collagen are seen. The last peak in the profile represents peptides $\alpha 1$ (III)CB8 and $\alpha 1$ (III)CB3, which, owing to their similar molecular weights, co-migrate in the electrophoretogram. In addition, there were other minor peaks not yet identified in the heart-valve profile. This electrophoretic pattern therefore showed that type-I collagen is the predominant collagen in the heart valve, but the presence of type-III collagen was also clearly demonstrated.

Amino acid analysis

Amino acid analysis of the total collagen solubilized by pepsin and of the collagens precipitated at 1.7M, 2.5M- and 4.0M-NaCl are presented in Table 3. The analyses from all the fractions clearly showed the characteristic features of collagen, such as a very high content of glycine, alanine and the imino acids proline and hydroxyproline. The analyses also confirm our previous finding of high content of hydroxylysine in the valvular collagens, since it was observed that all collagen fractions contained 2-3 times as many hydroxylysine residues as did skin collagen. The amino acid composition of the collagen obtained by precipitation at 1.7M-NaCl showed a high content of 4-hydroxyproline, a hydroxyproline/proline ratio greater than 1 and a greater number of glycine residues compared with the total and with collagens precipitated with 2.4M- and 4.0M-NaCl. These are distinctive features of type-III collagen. Low values for valine and high values for histidine, which are characteristic of type-III collagen, are also noted in the collagen fraction precipitated with 1.7M-NaCl. The collagen precipitated with 2.5M-NaCl had an amino acid composition consistent with type-I collagen, except that more hydroxylysine residues were present in this fraction. The hydroxylysine/lysine ratio was 0.52, which is about 3 times that found in type-I collagen from skin. The amino acid analysis of the fraction precipitated with 4.0M-NaCl also showed characteristic features of collagen, but one striking finding was the marked increase in lysine hydroxylation. The ratio of hydroxylysine/

Fig. 2. Spectrophotometric scans of sodium dodecyl sulphate/polyacrylamide gels of collagen precipitated with 1.7M-NaCl from mitral valve under (a) reducing and (b) non-reducing conditions. Electrophoresis was performed as described in Fig. 1 legend.

lysine was approx. 0.81, which is almost twice the ratio in collagens precipitated at 1.7M- and 2.5M-NaCl and almost 5 times the ratio in skin collagen. The finding of 349 residues of glycine and 104 residues of 4-hydroxyproline/1000 amino acid residues in this fraction indicated that it contained essentially pure collagen.

Discussion

The extreme insolubility of bovine heart-valve collagen was again documented in this study and limited pepsin digestion was necessary to extract sufficient collagen for characterization.

Mannschott *et al.* (1976) were able to extract around 70% of total pig heart-valve collagen with

Fig. 3. Spectrophotometric scans of sodium dodecyl sulphate/polyacrylamide gels of collagen precipitated with 2.5M-NaCl from mitral valve under reducing conditions. Electrophoresis was performed as described in Fig. 1 legend.

Fig. 4. Spectrophotometric scans of sodium dodecyl sulphate/polyacrylamide gels of collagen precipitated with 4M-NaCl from mitral valve under non-reducing conditions. Electrophoresis was performed as described in Fig. 1 legend.

pepsin; this amount is much higher than the fraction extracted from bovine tissues reported here. This difference is probably due to the age of the experimental animals used, since older animals were used

in the present study. The conditions of pepsin extraction may also contribute to the differences, since our extraction was carried out at 15°C, whereas it was performed at 20°C by Mannschott *et al.* (1976).

Fig. 5. Photograph of Coomassie Blue-stained gels of collagens from mitral valve

All gels were obtained under non-reducing conditions, except for gel 3, which was obtained in the presence of 0.1% mercaptoethanol. 1, Total pepsin-solubilized collagens; 2, 1.7M-NaCl-precipitated collagen; 3, 1.7M-NaCl-precipitated collagen under reducing conditions. 4, 2.5M-NaCl-precipitated collagen; 5, 4.0M-NaCl-precipitated collagen. Electrophoresis was performed as described in Fig. 1 legend.

This extracted collagen has been further characterized by using salt fractionation, NaDodSO₄/polyacrylamide-gel electrophoresis, CNBr cleavage and amino acid analysis. One of the remarkable observations in the pepsin extracted bovine heart-valve collagen was the presence of β , γ and higher-molecular-weight aggregates. Since pepsin cleaves only the non-helical collagen telopeptides, which contain most of the cross-links, the presence of β - and γ -components in pepsin digests suggests that either some non-helical portion resisted pepsin digestion or that intermolecular cross-linking within the helical regions of the molecules was present. The finding that the electrophoretic migration of a significant fraction of β - and γ -chains remained unaffected after disulphide-bond reduction indicated that these chains were not held together by disulphide bonds and it seems likely that lysine- and hydroxylysine-derived cross-links may be involved.

The finding of excess $\alpha 1$ -polypeptides, with an $\alpha 1/\alpha 2$ ratio of 3.36 in the total solubilized collagen,

suggested heterogeneity of collagen molecules in valvular tissues and indicated that in addition to type-I collagen another collagen type or types comprising entirely $\alpha 1$ -chains were present. Comparison of electrophoretic patterns of the total solubilized collagen and of collagen precipitated at 1.7M-NaCl under reducing and non-reducing conditions suggested the presence of type-III collagen. The patterns showed a marked increase in the proportion of $\alpha 1$ -chains and a concomitant decrease in the collagen migrating in the position of γ -chains after disulphide-bond reduction. Further support for this notion was obtained from amino acid analysis of the 1.7M-NaCl fraction, since only this fraction showed the high content of 4-hydroxyproline and a hydroxyproline/proline ratio greater than 1 characteristic of type-III collagen. In addition, in the electrophoretic pattern of the CNBr digest from heart-valve collagen, a single peak containing the two peptides $\alpha 1(\text{III})\text{CB3}$ and $\alpha 1(\text{III})\text{CB8}$ was found. This peak is specially characteristic of type-III collagen (Fig. 6). The finding of $\alpha 1$ - and $\alpha 2$ -polypeptides in a ratio close to 2:1 and of $\beta 12$ -chains in the 2.5M-NaCl fraction, as well as the amino acid analysis shown in Table 3, demonstrated that type-I collagen was the main component of this fraction. Furthermore, the profile of CNBr peptides indicated that the majority of heart-valve collagen was probably type-I, since prominent peaks corresponding to peptides $\alpha 2\text{CB3-CB5}$ and $\alpha 1\text{CB4}$, $\alpha 1\text{CB8}$ and $\alpha 1\text{CB7}$, were obtained.

There has been significant controversy in the literature about the types of collagen present in the heart valves. For example, type-III collagen in heart valve was previously shown by Mannschott *et al.* (1976). Collins *et al.* (1975), on the other hand, suggested the exclusive presence of type-I collagen in this tissue, and Morris & McClain (1972) showed similarities in some CNBr peptides between valvular collagen and collagen from cartilage. Our studies are in agreement with those of Mannschott *et al.* (1976), since type-I and -III collagens were clearly shown. An interesting finding in our studies was the identification of a new collagen polypeptide in the fraction precipitated with 4.0M-NaCl from the heart-valve collagen. This polypeptide had peculiar electrophoretic behaviour, since it migrated in a position intermediary between that of β - and $\alpha 1$ -chains. Although its physiological significance is not clear at present, it has some similarities with the αA -chain described by Burgeson *et al.* (1976) in foetal human membranes and with the X-chain demonstrated by Benya *et al.* (1977) in cultured chondrocytes. Although we have isolated collagenous peptides with similar characteristics in pepsin extracts of embryonic-chick tendon and calvaria (S. A. Jimenez, R. Yankowski & R. I. Bashey, unpublished work), to our knowledge the present work is the first demonstration of such a polypeptide in adult tissue. Similarities between these

Fig. 6. Spectrophotometric scans of gels containing the CNBr peptides from pepsin-solubilized collagen of mitral valve. Electrophoresis was performed on 7.5% acrylamide gels after heat denaturation of the sample in 0.1% sodium dodecyl sulphate containing 0.1% mercaptoethanol. The nomenclature of the peptides is similar to that used by Scott & Veis (1976a,b) described in the text. Shaded area shows a peak characteristic for type-III collagen which contains peptides $\alpha 1(\text{III})\text{CB3}$ and $\alpha 1(\text{III})\text{CB8}$ co-migrating in the electrophoretogram due to their similar molecular size.

Table 3. Amino acid composition of collagens from bovine heart valve

Values given are residues per 1000 total amino acid residues. For total collagen, collagen was extracted from mitral valves by limited pepsin digestion at 15°C. Pepsin-solubilized collagen was precipitated by addition of 20% NaCl. This precipitated collagen was dissolved in 1 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4, and used for amino acid analysis after hydrolysis. This collagen was then used for differential NaCl precipitation, giving fractions obtained at 1.7 M-, 2.5 M- and 4.0 M-NaCl. Each collagen was redissolved in 1.0 M-NaCl/0.1 M-Tris buffer, pH 7.4, and used for amino acid analysis after hydrolysis.

	Total collagen	Collagen fractions		
		1.7 M-NaCl	2.5 M-NaCl	4 M-NaCl
4-Hydroxyproline	101	121	100	104
Aspartic acid	48	49	42	44
Threonine	15	16	17	20
Serine	24	41	33	31
Glutamic acid	65	68	69	70
Proline	115	99	112	115
Glycine	342	353	348	349
Alanine	110	91	111	91
Valine	26	16	22	22
Methionine	N.D.	8	5	6
Isoleucine	18	15	13	14
Leucine	26	19	24	27
Tyrosine	1	3	2	2
Phenylalanine	11	10	12	12
Hydroxylysine	17	12	12	17
Lysine	25	24	23	21
Histidine	7	8	5	6
Arginine	50	47	51	50
4-Hydroxyproline				
Proline	0.88	1.22	0.89	0.90
Hydroxylysine				
Lysine	0.68	0.50	0.52	0.81

chains included their apparently higher molecular weight and their increased solubility at neutral pH. Although we failed to demonstrate clearly the presence of chains similar to the α B-chains of foetal membrane collagens or to the Y-chain of collagen from cultured chondrocytes, it is possible that the analogous polypeptide of heart-valve collagen co-migrated with α 1-chains and it was not resolved under the electrophoretic conditions used.

The increased hydroxylation of lysine residues has been again documented and it is noteworthy that both the type-I and type-III collagens from heart valves have a higher hydroxylysine content than do similar collagens from skin or other vascular tissues. Since it has been suggested that hydroxylysine-derived cross-links are more stable than lysine-derived cross-links, it seems likely that large amounts of these cross-links may be responsible for the marked insolubility of heart-valve collagen. We also speculate that increased number and strength of cross-links may represent an evolutionary adaptation of the heart-valve collagen to become capable of sustaining the constant mechanical stress of the circulation. Although the mechanisms responsible for increased hydroxylation of lysine residues are not known, it is possible that conditions resulting in decreased hydroxylysine content of heart valves may alter the mechanical properties of these tissues and be responsible for pathological alterations.

Note Added in Proof (Received 20 June 1978)

Since the original manuscript of the present paper was completed, a paper on collagen of heart valve has been published by Collins *et al.* (1977).

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