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The Chemical Composition of Bovine Vitreous-Humour Collagen Fibres

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The insoluble protein fraction was prepared from the central and posterior peripheral fraction of bovine vitreous humour. The collagen present in this fraction was solubilized by pepsin and fractionated by gel chromatography. Analysis of the solubilized collagen fractions showed that the α-chain component had an amino acid composition and yielded a series of CNBr-cleavage peptides that showed it was very similar to type II collagen obtained from articular cartilage. Bovine vitreous-humour collagen α-chains differed, however, from those of cartilage collagen in that they had a lower alanine content and differed in their susceptibility to cleavage by CNBr. Satisfactory cleavage was obtained after two CNBr treatments involving reduction and alkylation. In addition, significant quantities of other peptide constituents were present in the vitreous-humour collagen fractions, and the galactose and glucose content of the α-chain fraction was more than double that of the same fraction obtained from articular cartilage. Although the origin of the additional peptide constituents in the vitreous-humour collagen preparations is not known, the results obtained indicate that they are probably not derived from a distinct type of α-chain component but may be terminal peptides covalently linked to the α_1 type-II helical portions of the collagen. The differences in the chemical composition of the vitreous-humour collagen indicate that vitreous-humour fibres are composed of a special type-II collagen.

The major fibrous component in the bovine vitreous humour has a diameter of 8–23 nm (Matoltsy *et al.*, 1951; Matoltsy, 1952; Olsen, 1965), and early studies established that the major component in the fibre was collagen (Gross *et al.*, 1955). In a later study it was shown that after treatment with 4 M-guanidinium chloride to extract associated proteins and glycoproteins the amino acid composition was similar to that of cartilage collagen. These findings, together with the absence of α_2-chains after thermal denaturation, indicated that vitreous-humour fibres are composed of type-II collagen (Swann *et al.*, 1972). Subsequently it was shown (Swann *et al.*, 1976) that about three-quarters of the vitreous-humour collagen was solubilized by pepsin treatment and that the soluble fraction contained significant amounts of a high-molecular-weight α-chain component. The amino acid composition of the fractions obtained by pepsin treatment, however, differed somewhat from those of similar fractions derived from type II collagen. The vitreous-humour collagen had a higher content of aspartic acid, serine, valine and leucine and a lower content of alanine. The distribution of CNBr-cleavage peptides obtained from pepsin-solubilized vitreous-humour collagen from bovine, sheep, rabbit and dog also differed from those obtained from cartilage collagen (Swann *et al.*, 1977; Swann & Sotman, 1978) in that in addition to the cartilage-like CNBr-cleavage peptides the vitreous-humour samples contained high-molecular-weight peptides. These results indicated that type-II collagen components were present in vitreous-humour fibres, but that the vitreous-humour collagen either differed in its susceptibility to CNBr cleavage or that it contained additional peptide constituents and possibly an as yet unidentified α-chain component. The apparent homogeneity of the preparations of adult bovine vitreous-humour fibres used in these studies (Swann *et al.*, 1974) indicated that the chemical differences between the solubilized vitreous-humour and cartilage collagen fractions were not due to the occurrence of significant quantities of a second type of fibre in the vitreous humour. It was found previously, however, that α-

Abbreviation used: SDS, sodium dodecyl sulphate.

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chains prepared from lathyritic chick vitreous humour by CM-cellulose chromatography (Trelstad & Kang, 1974) had a different amino acid composition from that of other types of collagen. Evidence was also obtained (Newsome *et al.*, 1976) that vitreous-humour collagen was synthesized by different cell types at different stages of development. At early stages the neural retina apparently synthesized most of the collagen, whereas in later stages of development cells derived from the vitreous humour were more active. It was also shown that chick-embryo retina cells in culture synthesized a collagenous product that yielded CNBr-cleavage peptides with the same mobility on SDS/polyacrylamide gels as those derived from cartilage collagen (Smith *et al.*, 1976).

During development different types of extracellular macromolecules are synthesized at different stages, and these may differ from the extracellular products that determine the structure of the mature functional tissues (Toole *et al.*, 1972). In the case of the bovine vitreous humour, a large part of the growth of the eye and the accumulation of collagen and hyaluronic acid in the vitreous humour occur at a time when the vitreous humour is avascular and contains few cells (Balazs *et al.*, 1959; Berman & Voaden, 1970; Swann & Constable, 1972). It is therefore not clear to what extent the collagens synthesized at early stages of development contribute to the structure and function of the adult tissue, particularly since morphological studies have shown that the vitreous-humour collagen fibres are continuous with the basement membrane of the ciliary epithelium and Müller cells (Spira & Hollenberg, 1973). These tissues therefore may contribute to the synthesis of the secondary vitreous humour that represents the predominant part of the tissue in the adult. To provide more information about the types of collagen present in the adult vitreous humour and ultimately about the cells responsible for their synthesis, the present studies were performed with samples of adult bovine vitreous humour. Preliminary results of these experiments were reported previously (Swann *et al.*, 1977; Swann & Sotman, 1978).

Materials and Methods

Intact enucleated adult cow eyes were collected at the slaughterhouse and transported on ice to the laboratory. After equatorial section (Swann & Constable, 1972), samples of the gel portion of the posterior–peripheral and central vitreous humour were removed by forceps, washed in saline (0.9% NaCl), pooled and separated by centrifugation (90000*g* for 30min at 4°C) into an insoluble residue and a supernatant (Swann *et al.*, 1972). The insoluble residues were combined, resuspended in water, dialysed against water and freeze-dried to yield fraction VR–I. The average volume of vitreous humour collected per eye was 11ml and the yield of fraction VR–I was 103μg/ml of pooled vitreous humour.

Pepsin treatment

Method 1. Suspensions of fraction VR–I in 0.5M-acetic acid (1mg of sample/ml) were treated with pepsin (2× crystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.) (10mg of enzyme/100mg of sample) at 4°C for 8 days. After the enzyme treatment the reaction mixture was centrifuged (90000*g* for 30min at 4°C) to yield an insoluble fraction and a supernatant. The insoluble fraction was resuspended in 0.1M-acetic acid and then re-centrifuged. This procedure was repeated a second time, and the insoluble fraction was then freeze-dried to give fraction VR–IA. The supernatant was adjusted to pH7.0 by the addition of 0.1M-NaOH, and the collagen was precipitated by the addition of solid NaCl to a concentration of 4.4M. The precipitated collagen was recovered by centrifugation (90000*g* for 30min of 4°C), dissolved in 0.5M-acetic acid, re-precipitated with NaCl, recovered by centrifugation, dissolved in 0.5M-acetic acid, dialysed against 0.1M-acetic acid and freeze-dried to yield fraction VR–IB.

Method 2. This procedure involved a digestion step performed as described above but at a temperature of 16°C for 16h. The reaction mixture was then reduced with dithiothreitol (Calbiochem, San Diego, CA, U.S.A.) (200mM; at 16°C for 16h), alkylated with excess of iodoacetic acid (Eastman Kodak Co., Rochester, NY, U.S.A.) (at 16°C for 2h) and then re-treated with pepsin at 16°C for a further 16h. The reaction mixture was then handled as described for method 1 to yield a non-pepsin-solubilizable fraction (VR–IIA) and a pepsin-solubilized fraction (VR–IIB).

Preparation of bovine articular-cartilage collagen fractions

After extraction of proteoglycans, proteins and glycoproteins from bovine metacarpal–phalangeal articular-cartilage samples with 4M-guanidinium chloride (Swann *et al.*, 1979), the tissue residue was dialysed successively against water, 1M-NaCl and water and then freeze-dried. The freeze-dried cartilage slices were then ground to a powder in a Wiley Mill (Thomas–Wiley Intermediate Mill; Arthur H. Thomas, Philadelphia, PA, U.S.A.). The ground cartilage was suspended in 0.5M-acetic acid and digested with pepsin at 4°C for 8 days as described above for pepsin method 1. After the pepsin treatment the reaction mixture was centrifuged, and the collagen in the supernatant was precipitated with NaCl, dissolved, dialysed and freeze-dried as described for the vitreous-humour samples.

The non-pepsin-solubilizable fraction was labelled AC–IA and the pepsin-solubilized fraction AC–IB.

Acid-soluble calf skin collagen, fraction CLCS, was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.).

Fractionation of pepsin-solubilized collagens

(*a*) *Gel-permeation chromatography.* Fractions VR–IB, VR–IIB and AC–IB dissolved in 1M-$CaCl_2$/50mM-Tris/HCl buffer, pH 7.6 (5mg of sample/ml of solvent), were denatured by heating at 42°C for 15min. The samples were then fractionated by chromatography on a descending-flow column (200cm × 2.5cm) packed with Bio-Gel A-1.5m (100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.), as described by Miller *et al.* (1969).

(*b*) *CM-cellulose chromatography.* The α-chain fraction isolated from fraction VR–IB by gel-permeation chromatography was re-fractionated on a column (10cm × 1cm) packed with CM-cellulose (Whatman, Clifton, NJ, U.S.A.) and equilibrated with a starting buffer composed of 1M-urea/50mM-sodium acetate buffer, pH 4.8 (Miller, 1971*a*). Samples were dialysed successively against water, 1M-NaCl, water and then the column starting buffer. After application of the sample the column was eluted with starting buffer until a stable base-line was achieved. The column was then eluted with a linear gradient composed of 250ml of starting buffer and 250ml of limiting buffer (1M-urea/0.15M-NaCl/50mM-sodium acetate buffer, pH 5.8). The column was operated at 42°C and the effluent was monitored at 230nm by using a flow-through recording spectrophotometer (LKB Instruments, Rockwell, MD, U.S.A.).

CNBr treatment

The initial studies were performed by using the procedure described by Miller *et al.* (1971) (method 1). Samples were dissolved or suspended in 70% (v/v) formic acid to give a concentration of 3mg/ml. An amount of CNBr equal to that of the collagen sample was added, the reaction tube was flushed with N_2 and the mixture was incubated at 37°C for 4h. The reaction mixture was then diluted with water and freeze-dried. In later studies the products obtained by an initial CNBr treatment were reduced and then treated a second time with CNBr (Hudson & Spiro, 1972) (method 2). After the first CNBr treatment the freeze-dried product was dissolved in 0.1M-acetic acid (10ml/20mg of sample) and reduced with β-mercaptoethanol (Eastman Kodak Co.) (20%, v/v) at 37°C for 16h. The reaction mixture was then fractionated on a Bio-Gel P-2 column (100cm × 1cm) equilibrated with 0.1M-acetic acid. The column effluent was monitored at 230nm. The peptides eluted in the void-volume peak were freeze-dried, re-treated with CNBr as described above and then freeze-dried.

The CNBr-cleavage peptides obtained by treating fractions VR–IB and AC–IB were fractionated on a CM-cellulose column by using the method described by Miller (1971*b*).

Analytical procedures

Amino acids were determined after hydrolysis with 6M-HCl at 105°C for 24h in a laboratory-constructed semi-automated micro-modification of the two-column three-buffer system described by Moore *et al.* (1958). The acidic and the neutral amino acids were eluted from a column (21cm × 0.3cm) packed with Durrum DC-4A resin (Durrum Chemical Corp., Sunnyvale, CA, U.S.A.) with the pH 3.25 and the pH 4.25 buffers. Basic amino acids and hexosamines were eluted from a column (13cm × 0.35cm) packed with the same Durrum DC-4A resin with the pH 5.28 buffer. The analyser was constructed with parts obtained from the Durrum Chemical Corp., and employed a ninhydrin reagent to detect and measure the presence and quantities of amino acids. Tryptophan was not determined. Glycosamine analyses were performed with the amino acid analyser after hydrolysis with 6M-HCl at 105°C for 3h (Swann & Balazs, 1966). The mannose, glucose and galactose contents of samples were determined by g.l.c. after methanolysis and preparation of the pertrimethylsilyl derivatives (Reinhold, 1972). The types of peptide constituents were determined by SDS/polyacrylamide-gel electrophoresis. Samples were dissolved in 0.1M-sodium phosphate buffer, pH 7.2, containing 4M-urea and 0.5% (w/v) SDS. For reduction of samples β-mercaptoethanol (10%, v/v) was added, followed by incubation at 37°C for 16h. Polyacrylamide gels containing 5% and 7.5% (w/v) acrylamide were run in the presence of 0.1% SDS by using a continuous 0.1M-sodium phosphate buffer system, pH 7.2 (Furthmayr & Timpl, 1971), at 6mA/gel for 6h. Samples were also analysed by using the LKB Multiphor slab gel system.

Results

The recoveries of collagen fractions after pepsin treatment and fractionation of the pepsin-solubilized material on Bio-gel A-1.5m column (Fig. 1) are given in Table 1. The values shown for the non-pepsin-solubilizable and pepsin-solubilized fractions are the ranges of freeze-dried weights obtained with five different preparations expressed as percentages (w/w) of the initial VR–I fraction. The values for the gel-chromatography fractions are average recoveries of the individual column fraction expressed as percentages of the total column fractions after fractionation of the pepsin-solubilized collagen fractions (Fig. 1).

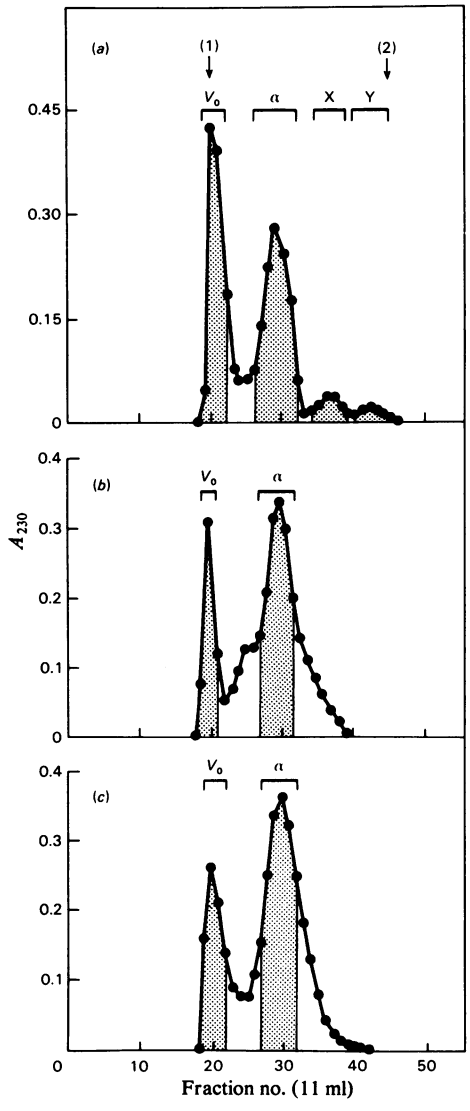

Fig. 1. *Fractionation of pepsin-solubilized collagen fractions VR–IB (a), VR–IIB (b) and AC–IB (c) on a Bio-Gel A-1.5m column (200cm × 2.5cm) eluted with 1M-$CaCl_2$/50mM-Tris/HCl buffer, pH 7.2*

Experimental details are given in the text. The column effluent was analysed by measuring the absorbance at 230nm. The elution positions of Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and ^3H_2O are indicated by the arrows at (1) and (2) respectively. The effluent fractions were pooled to yield the fractions indicated. The effluent fractions between the V_0 and α-chain fractions were pooled to yield fraction B_1, and those between the α-chain and X fractions were pooled to yield fraction B_2.

When pepsin method 1 was used (4°C for 8 days) 15–25% of the vitreous-humour insoluble protein fraction (VR-I) was recovered as a non-pepsin-solubilizable residue (fraction VR–IA) and 50–65% was solubilized (fraction VR–IB). Similar quantities of soluble collagen were obtained by pepsin method 2, but smaller quantities were recovered in the non-pepsin-solubilizable fraction VR–IIA. When VR–IB sample was fractionated on the Bio-Gel A-1.5m column four peaks were obtained (Fig. 1*a*). The effluent volumes in between the void-volume (V_0) and α fractions and the α and X fractions were also collected to yield the B_1 and B_2 fractions respectively. An average of 50% of the total recovered column-fraction constituents was present in the α-chain fraction after treatment with pepsin method 1. The void-volume fraction accounted for 22% of the total fractions. As shown in Fig. 1, the X and Y fractions after pepsin method 1 were obtained as defined fractions well separated from the α-chain fraction. After treatment with pepsin method 2 distinct X and Y fractions were not obtained (Fig. 1*b*). There was a lower recovery of constituents in the void-volume fraction and slightly higher recovery of α-chain constituents (Table 1).

The types of peptide constituents present in the pepsin-solubilized fractions and the Bio-Gel V_0, α, X and Y fractions (Fig. 1) determined by using 5% SDS/polyacrylamide tube gels are shown in Fig. 2. As shown previously (Swann *et al.*, 1976), the pepsin-solubilized fraction (gel 2) contained two high-molecular-weight α-chain components, when compared with calf skin type I collagen (gel 1), and no α_2-chains were detected. In addition, two low-molecular-weight constituents were detected (gel 2), which had mobilities similar to those of the constituents present in the Bio-Gel X and Y fractions. The V_0 fraction (gel 4) was largely composed of constituents that did not enter the gel. The major component in the X fraction (gel 6) had a mobility slightly less than that of bovine serum albumin (gel 8), and the Y fraction was composed of low-molecular-weight components (gel 7). The same profile of peptide components was obtained in all preparations examined, the only difference observed being the resolution obtained between column fractions, for example the X fractions sometimes contained small amounts of the α-chain components, and the high-mobility peptide usually present in the X fraction (gel 6) was sometimes present in the Y fraction.

When the pepsin method 2 involving reduction, alkylation and further pepsin treatment was used, the higher-molecular-weight α-chain component was either absent or present in small amounts (Fig. 2, gel 3), and the mobility of the remaining α-chain component was increased. Significantly greater amounts of peptides were also obtained in the B_2–X fraction region of the chromatogram (Fig. 1 and Table 1). These peptides had mobilities between those of the α-chain and X-fraction component, and no well-defined X- and Y-fraction components were ob-

Table 1. *Recovery of fractions after pepsin digestion of the vitreous-humour residue fraction (VR–I)*
Experimental details are given in the text

Source of collagen	Pepsin treatment	Recovery (%)* Pepsin-insolubilizable	Pepsin-solubilized	Recovery from gel chromatography (Fig. 1)† V_0	B_1	α-Chain	B_2	X	Y
Vitreous humour									
	Method 1	VR–IA	VR–I B			VR–IB			
		15–25	50–65	22	17	50	8	2.5	0.5
	Method 2	VR–IIA	VR–IIB			VR–IIB			
		7–14	54–67	11	12	57		20	0
Articular cartilage		AC–IA	AC–IB			AC–IB			
	Method 1	18–24	52–64	8	10	66		16	0

* The range of freeze-dried samples recovered expressed as a percentage of the initial weight of fraction VR–I for five different preparations.

† The average recoveries of individual column fractions expressed as a percentage of the total column fractions for three different preparations of fractions VR–IB, VR–IIB and AC–IB.

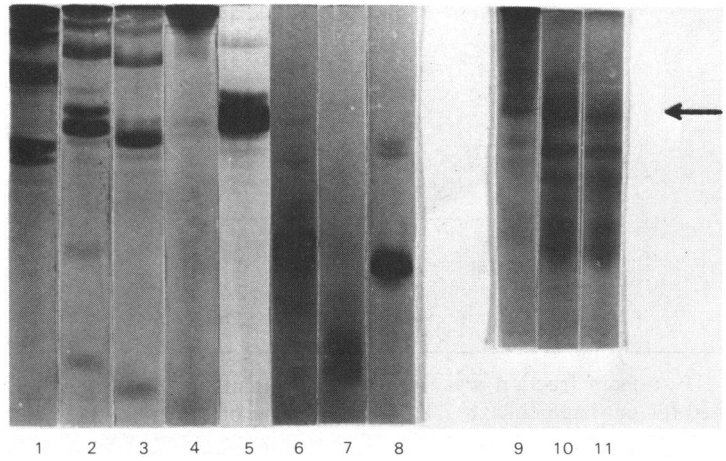

Fig. 2. *Electrophoresis of reduced collagen fractions in the presence of SDS*
Experimental details are given in the text. Gel 1 was acid-soluble calf skin collagen. Gels 2 and 3 were fractions VR–IB and VR–IIB respectively (Table 1). Gels 4, 5, 6 and 7 were VR–IB V_0, α-chain, X and Y fractions respectively (Table 1 and Fig. 1*a*). Gel 8 is bovine serum albumin. Gel 9 was fraction VR–I after a single CNBr treatment (method 1). Gel 10 is fraction VR–I after cleavage by CNBr by method 2. Gel 11 is the VR–IB V_0 fraction after cleavage with CNBr by method 2. Gels 1–8 and 9–11 contained 5% and 7.5% acrylamide respectively. The arrow indicates the mobility of bovine serum albumin run on a 7.5% gel at the same time as gels 9–11. The gels were stained with Coomassie Blue.

served. Analysis of the VR–IB α-chain and VR–IIB α-chain fractions, acid-soluble calf skin soluble collagen and the AC–IB α-chain fraction on 5% SDS/polyacrylamide tube gels consistently showed that the mobilities of the α-chains had the following relationship: α_1(I)-chain > AC–IB α-chain ≏ VR–IIB α-chain > VR–IB α-chain.

The amino acid analyses of the various collagen fraction are shown in Table 2. The vitreous-humour insoluble protein (fraction VR–I) and the non-pepsin-solubilizable fraction (VR–IA) had compositions similar to those reported previously (Swann *et al.*, 1976). Compared with the composition of the pepsin-solubilized articular-cartilage collagen α-chain fraction (Table 2, column 10), all of the vitreous-humour collagen fractions had a lower content of alanine, although the content was increased by the procedure involving two pepsin treatments. Fraction VR–IIB α-chain had an alanine content of 92 residues/1000 residues compared with a value of 102 residues/1000 residues for the AC–IB α-chain fraction. Both the VR–IB α-chain and VR–IIB α-chain fractions had lower leucine contents than did the other vitreous-humour fractions, and the values obtained were very similar to the leucine content of the AC–IB α-chain fraction. The amino acid com-

Table 2. *Chemical composition of collagen fractions*
Experimental details are given in the text.

Fraction ...	VR–I	VR–IA	VR–IIA	VR–IB				VR–IIB		AC–IB
				V_0	α-Chain	X	Y	V_0	α-Chain	α-Chain
Amino acid	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	Content (residues/1000 residues)									
Aspartic acid	52	50	52	51	48	62	53	63	47	48
Threonine	54	23	30	24	23	26	24	36	24	22
Serine	30	30	35	31	27	42	32	52	27	28
Glutamic acid	93	98	92	95	91	90	78	92	94	93
Proline	102	108	82	106	114	99	125	103	105	111
Glycine	298	314	308	317	333	287	313	265	334	335
Alanine	80	79	58	78	85	81	58	77	92	102
Half-cystine	5	2	4	2	—	2	2	2	—	—
Valine	25	22	23	23	21	28	29	35	20	17
Methionine	10	11	6	11	9	10	1	10	11	11
Isoleucine	18	12	19	15	14	22	28	27	12	10
Leucine	38	35	40	34	27	44	47	49	26	25
Tyrosine	5	7	9	4	2	7	2	15	3	2
Phenylalanine	15	20	24	14	12	11	7	22	13	11
Lysine	18	17	26	18	16	28	22	11	18	17
Histidine	10	8	6	6	4	11	4	5	5	4
Arginine	46	47	55	46	45	44	38	41	50	48
4-Hydroxyproline	108	95	107	100	104	91	121	82	102	101
Hydroxylysine	23	22	24	25	25	15	16	13	17	15
Carbohydrate	Content (%, w/w)									
Glucosamine	0.6	0.6		0.1	0			0.1	0	0
Mannose	0.08	0.08		Trace	0			Trace	0	0
Galactose	2.1				2.7				3.2	1.2
Glucose	2.0				2.6				2.7	1.1

position of the VR–IIB α-chain fraction was very similar to that reported for cartilage collagen (Trelstad *et al.*, 1970; Strawich & Nimni, 1971; Miller, 1971*a*). Carbohydrate analyses were also performed on some of the collagen samples, and the results are given in Table 2. The galactose and glucose content of the VR–I fraction was the same as those reported previously (Swann *et al.*, 1976), but the VR–IB α-chain and VR–IIB α-chain fractions had higher contents of these two sugars than was observed previously in the whole pepsin-solubilized fraction. The galactose and glucose content of the VR–IB α-chain and VR–IIB α-chain fractions was more than double that present in the articular-cartilage α-chain fraction. Significant quantities of glucosamine and mannose were present in the VR–I and VR–IA fractions, and small amounts were also present in the VR–IB V_0 and VR–IIB V_0 fractions.

Chromatography of the pepsin method-1 Bio-Gel α-chain fraction (VR–IB α-chain) on CM-cellulose (Fig. 3) gave a single broad peak eluted at relatively low ionic strength compared with acid-soluble calf skin collagen. SDS/polyacrylamide-gel analysis showed that the major α-chain component together with smaller amounts of the high-molecular-weight α-chain component (Fig. 2, gel 2) were present in all regions of the peak, and that the amino acid composition was the same as that given for the Bio-Gel α-chain fraction (Table 2, column 5) and did not vary with elution position.

The profiles of peptide constituents in various fractions are shown in Fig. 4. The α-chain component in fraction AC–IB (gels 2 and 7) had a slightly slower mobility than that of the α_1-chains in acid-soluble calf skin collagen (gels 1 and 8). Two different preparations of fraction VR–IB were analysed on gels 3 and 4. For gel 3 the pepsin treatment was carried out for 12 days at 4°C, compared with the standard 8 days at 4°C for the sample on gel 4. Gels 5 and 6 were analyses of VR–IB fractions derived from sheep and dog vitreous humour respectively. Although consistent small differences were observed in the mobilities of the different α-chain components when analysed by the slab gel system (Fig. 4), these differences were smaller than those obtained when tube gels were employed (Fig. 2, gels 2 and 3).

CNBr treatment

The elution profiles of the CNBr-cleavage peptides derived from VR–IB and AC–IB fractions

after CM-cellulose chromatography are shown in Figs. 5(*a*) and 5(*b*). Although the profiles were somewhat different in that three fractions were obtained

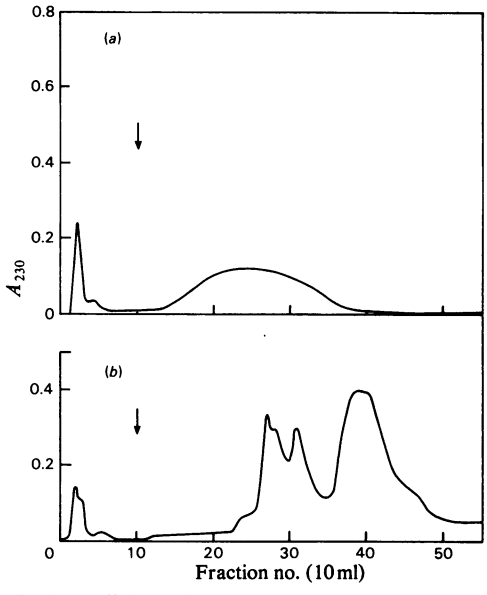

Fig. 3. *CM-cellulose chromatography of* (*a*) *the VR–IB α-chain fraction* (*Fig.* 1*a*) *and* (*b*) *acid-soluble calf skin collagen*

Experimental details are given in the text. The elution of peptide components was monitored by measuring the absorbance at 230nm. The arrows indicate the beginning of the linear NaCl gradient (see the text).

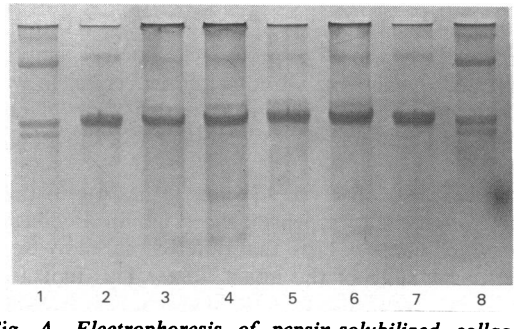

Fig. 4. *Electrophoresis of pepsin-solubilized collagen fractions*

Experimental details are given in the text. Gels 1 and 8 were acid-soluble calf skin collagen. Gels 2 and 7 were fraction AC–IB. Gels 3 and 4 were two different VR–IB fractions. The sample on gel 3 was prepared after pepsin treatment for 12 days at 4°C, compared with 8 days at 4°C for the sample on gel 4. Gels 5 and 6 were VR–IB fractions obtained from sheep and dog vitreous humour respectively. Electrophoresis was carried out with a slab gel containing 5% acrylamide. The peptide constituents were detected by staining with Coomassie Blue.

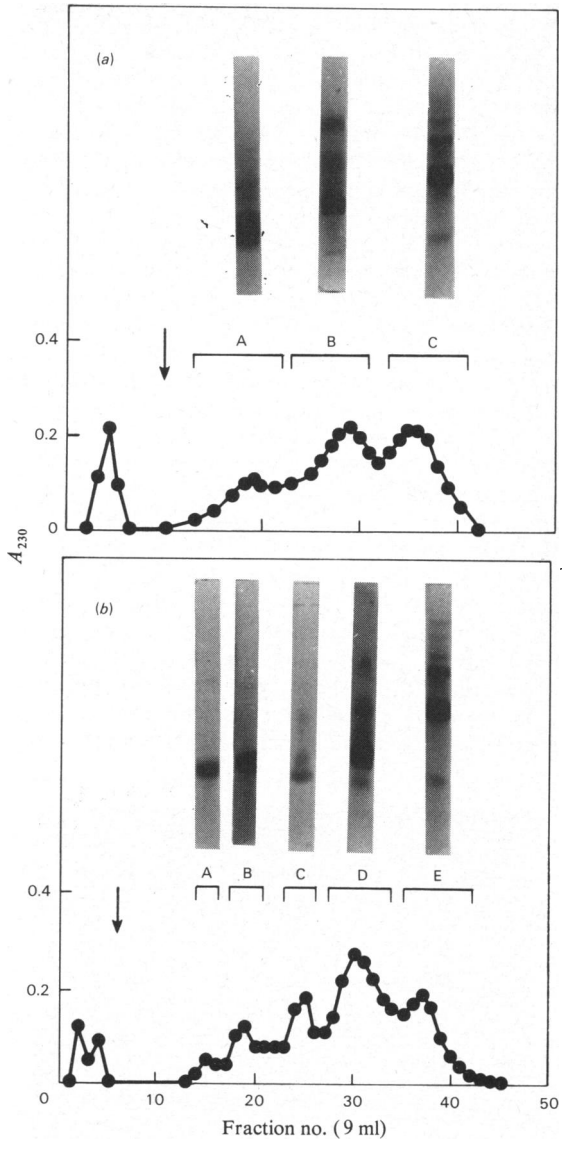

Fig. 5. *CM-cellulose chromatography of the VR–IB α-chain fraction* (*a*) *and the AC–IB α-chain fraction* (*b*) *after cleavage by CNBr* (*method* 2)

Experimental details are given in the text. After application of the samples the column was eluted with a linear NaCl gradient (beginning at the point indicated by the arrows). The column effluents were analysed by measuring the absorbance at 230nm, and the fractions indicated were pooled to yield subfractions A, B and C (*a*) and A–E (*b*). The fractions were then concentrated, desalted by chromatography on Bio-Gel P-2 and then analysed by electrophoresis in tube gels containing 7.5% acrylamide in the presence of SDS. The types of CNBr-cleavage peptides by gel analysis are shown above the indicated effluents pooled to yield the respective column fractions.

from the vitreous-humour sample and five from the cartilage sample, SDS/polyacrylamide-gel analysis of these fractions (Fig. 5) revealed a very similar distribution of peptide constituents. By comparison with reported analysis for the types of CNBr-cleavage in cartilage collagens (Miller, 1971*b*; Eyre & Muir, 1975), the following assignments can be made for the constituents present in the vitreous-humour fractions (Fig. 5*a*): peak A appears to contain α_1(II) peptide CB8, and peaks B and C contain α_1(II) CB10 and α_1(II) CB11 peptides as the major components respectively. The types of peptides present after CNBr treatment of other vitreous-humour collagen fractions are shown in Fig. 2. With a single CNBr treatment (method 1) high-molecular-weight CNBr-cleavage peptides were obtained from fraction VR–I (Fig. 2, gel 9). After reduction and alkylation and re-treatment with CNBr (method 2) the distribution of CNBr-cleavage peptides (Fig. 2, gel 10) was similar to that shown in Fig. 4. Also shown in gel 11 (Fig. 2) are the types of CNBr-cleavage peptides obtained by treating the VR–IB V_0 fraction by CNBr method 2. Even when method 2 was employed, however, small quantities of the high-molecular-weight CNBr-cleavage peptides were still observed in many vitreous-humour samples. Essentially the same distribution of CNBr-cleavage peptides was obtained with VR–I, VR–IA, VR–IB V_0, VR–IB α-chain, VR–IIB V_0 and VR–IIB α-chain fractions. The types of CNBr-cleavage peptides obtained from several of the vitreous-humour collagen fractions were compared with those obtained from the AC–IB α-chain fraction by using slab gel electrophoresis (Fig. 6). The vitreous-humour and cartilage samples contained peptides with the same electrophoretic mobilities.

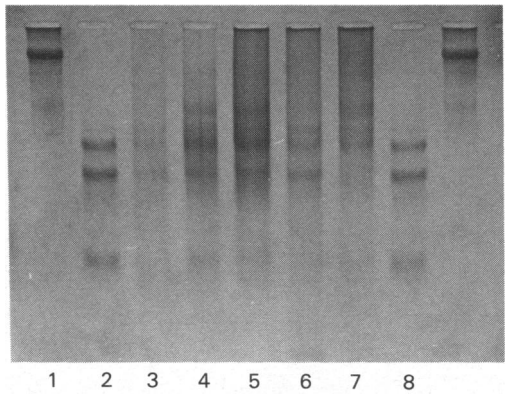

Fig. 6. *Electrophoresis of CNBr-cleavage peptides derived from vitreous-humour collagen fractions (gels 3–7) compared with those derived from the fraction AC–IB α-chain (gels 2 and 8)*

Experimental details are given in the text. Samples of untreated fraction AC–IB α-chain were run in gels 1 and 9. Gel 3 was fraction VR–IB V_0; gel 4 was fraction VR–IA; gel 5 was fraction VR–IIB V_0; gel 6 was fraction VR–IIB α-chain; gel 7 was fraction VR–I. The gel-7 sample was treated by CNBr method 1. CNBr method 2 was used for the remainder of the treated samples. Electrophoresis was performed by using a slab gel containing 7.5% acrylamide in the presence of SDS. The peptide constituents were detected by staining with Coomassie Blue.

Discussion

The chemical structure of vitreous-collagen is of interest because this collagen occurs in a distinctive fibrous form. In addition to the possibility that a knowledge of its chemical structure will shed some light on the events that regulate collagen fibrillogenesis, vitreous-humour collagen is also important because it is the macromolecular constituent that is largely responsible for the gel state of the vitreous humour in most species (Duke-Elder, 1930).

Although more than one type of fibre has been observed in bovine vitreous humour (Matoltsy *et al.*, 1951), the samples used in the present study had a very uniform appearance and diameter (Swann *et al.*, 1974), and only rarely was a second type of fibre observed. The earlier studies concerned with the structure of bovine vitreous-humour fibres established that they were composed of collagen, and, although amino acid analyses were performed (Young & Williams, 1954), they did not indicate that it was a distinct type of collagen that differed in composition from the then accepted form of collagen (type I). Later, however, after the discovery (Miller & Matukas, 1969; Trelstad *et al.*, 1970) that cartilage contained a distinct type of collagen (type II), amino acid analysis and SDS/polyacrylamide-gel analysis indicated that bovine vitreous-humour collagen was also a type-II collagen and that this form of collagen was not unique to cartilage (Swann *et al.*, 1972).

It was also observed (Swann *et al.*, 1976) that, after treatment with pepsin, the solubilized collagen re-formed fibres *in vitro* that differed markedly from the morphology of the intact fibres. This indicated that the terminal cross-linking peptide regions cleaved by pepsin are important in fibre formation. The present study confirmed that vitreous-humour collagen did not contain an α_2-chain component, and showed that after pepsin treatment it did contain a significant amount of a high-molecular-weight α-chain component. These findings indicated that the differences in the amino acid compositions of pepsin-solubilized vitreous-humour and cartilage collagens maybe due to differences in the structure of the terminal peptide regions, but they did not rule out the possibility that vitreous-humour collagen contained either a unique type of α-chain or a small

amount of an α-chain with a different composition from that of most of the chains. The present data do not rule out the possibility that vitreous-humour collagen fibres contain either a unique type of α-chain or a small amount of an α-chain with a different composition from that of most of the chains. By analogy with studies carried out with basement-membrane collagens (Trelstad & Lawley, 1977), it seems clear that the vitreous-humour fibres contain peptide constituents that were incompletely cleaved by the pepsin treatment at 4°C (method 1), but that were digested when two pepsin treatments at 16°C involving reduction and alkylation were used. Mayne *et al.* (1977) have shown that guinea-pig aorta smooth-muscle cells in culture synthesize a collagenous peptide with an apparent mol.wt. of 45000. The X and Y constituents have certain features in common with fractions isolated from basement membranes (Trelstad & Lawley, 1977; Chung *et al.*, 1976), and may have been derived from a small amount of a distinct type of collagen that was partially cleaved by pepsin treatment at 4°C.

The recoveries and chemical composition of the vitreous-humour collagen fractions, however, appear to favour the view that the X and Y constituents are derived, not from either the occurrence of more than one type of fibre or a unique type of α-chain component, but from the peptides covalently linked to the α_1(II)-chains present in most of the vitreous-humour fibres. The differences between the cartilage and vitreous-humour collagens were not due to the prior extraction of the cartilage sample with 4M-guanidinium chloride (used to remove proteoglycans and glycoproteins), because when the VR–I sample was also extracted with 4M-guanidinium chloride essentially the same results were obtained when the collagenous residue was treated with pepsin. The X and Y constituents were also not released by reduction and alkylation of fraction VR–I, and were not observed unless cleavage with pepsin was employed. It was also observed that, despite significant differences in the amino compositions of the V_0 and α-chain fractions obtained by fractionation of the VR–IB and VR–IIB samples (Table 2), these V_0 and α-chain fractions contained a similar distribution of CNBr-cleavage peptides as the major helical regions of the collagens (Figs. 2, 5 and 6). The differences in composition between the V_0 and α-chain fractions therefore are thought to be due to differences in the composition of the terminal peptide constituents and the extent to which these are cleaved by pepsin. Both of the V_0 fractions and the X and Y fractions had low alanine and high leucine contents compared with the VR–IIBα and AC–IB α-chain fractions. The occurrence of glucosamine and mannose in the VR–I and VR–IA fractions and their absence from the α-chain fractions also indicate that the collagens in these fractions contain glycopeptide constituents, which are thought to reside in the terminal regions of the molecule (Clark & Kefalides, 1976; Clark *et al.*, 1978).

In addition to the possible presence of terminal peptide constituents, the vitreous-humour and cartilage collagens differ also in their susceptibilities to cleavage by a single CNBr treatment. Hudson & Spiro (1972) observed that a single CNBr treatment caused only a limited cleavage of basement-membrane collagen, and they indicated that this was due to the occurrence of oxidized methionine residues. Whether the vitreous-humour collagen contained oxidized methionine residues *in vivo* or underwent oxidation during preparation and handling is not known. Even though the distributions of methionine residues along the α-chain appear to be the same in vitreous-humour and cartilage collagens, there may be minor differences in sequence that result in the resistance of the methionine bond to CNBr cleavage in vitreous-humour collagen. It has been observed that the methionine–cystine bond was only partially cleaved by CNBr (Doyen & Lapresle, 1979). Sequence heterogeneities have also been detected in bovine α_1(II)-chains (Butler *et al.*, 1977). In addition, the different susceptibility to CNBr cleavage may also be related to the higher carbohydrate content of the vitreous-humour collagen (Table 2). The galactose and glucose content of the VR–IIB α-chain fraction indicates that most if not all the hydroxylysine residues are linked to carbohydrate side chains.

In conclusion, it is thought that the overall similarity of the amino acid composition to that of cartilage collagen and the types of CNBr-cleavage peptides clearly indicate that the fibres in the central and posterior peripheral regions of adult bovine vitreous humour contain a collagen that should be classified as a type-II collagen. There are, however, distinct differences in the chemical composition of vitreous-humour and cartilage collagens. The present data indicate that these differences may be due to the presence of terminal peptide constituents in the vitreous-humour collagen.

Although there are differences in composition of the vitreous humour in different species (Berman & Voaden, 1970), analyses of pepsin-solubilized collagen fractions from dog, sheep (Fig. 3) and also rabbit and human (Swann *et al.*, 1977) have shown that the major fibrous component in these species has a similar structure to that present in bovine vitreous humour.

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