Biochemical and Physicochemical Characterization of Pepsin-Solubilized Type-II Collagen from Bovine Articular Cartilage

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Solubilization of collagen from bovine articular cartilage with pepsin requires the preliminary extraction of proteoglycans from the ground substance. Biochemical and physicochemical properties of this pepsin-solubilized collagen are independent of the pretreatment (extraction with 1.5 M-CaCl₂, 5 M-guanidinium chloride or 0.2 M-NaOH) and of the age range (2–4-year-old and 2-month-old animals). Characterization of the denatured components, of the CNBr peptides and of the amino acid and cross-link composition shows that the collagen of the hyaline cartilage is all type II. Electrical birefringence measurements showed the presence of tropocollagen molecules (length 280 nm) and molecules whose length is slightly less than twice that of the tropocollagen molecules. This latter molecule may be a dimer composed of two monomers linked by intermolecular head-to-tail bonds and whose theoretical length (530 nm), according to the quarter-stagger theory, is in good agreement with our measured values (510–530 nm). We have verified that the β -components of this collagen are formed of two α -chains linked by the stable intermolecular bond, dehydrodihydroxylysinylnorleucine. These dimeric molecules are absent from solutions of skin collagen whose β -components possess only aldol-type intramolecular cross-links. Although reconstituted fibres from solutions of skin and cartilage collagen are similar, the segment-long spacing crystallites formed with pepsin-solubilized cartilage collagen present a symmetrical and dimeric form corresponding to the lateral aggregation of two monomers with an overlap (90 nm) of the C-terminal ends.

So far, four different types of collagen have been found in connective tissues. Type-II collagen, composed of three identical $\alpha_1(\text{II})$ chains and denoted as [$\alpha_1(\text{II})$]₃, was shown for the first time to exist in chicken sternal cartilage by Miller & Matukas (1969). Numerous other studies revealed that this type-II collagen was characteristic of cartilaginous tissues. Since it is very insoluble, its solubilization necessitates either the use of lathyrin animals for which the formation of collagen cross-links is inhibited, or the selective elimination of the N- and C-terminal regions by enzymic digestion with papain or pepsin, with or without preliminary extraction of the proteoglycans of the ground-substance. Miller (1972) showed that pepsin treatment dissolved 60–70% of the sternal-cartilage collagen from chickens.

In the case of bovine articular cartilage, Strawich & Nimmi (1971) solubilized 20% of collagen by incubation of the tissue with papain. We have shown in preliminary studies that, providing the proteoglycans are removed by a suitable treatment, the major part of collagen from this tissue can be solubilized with pepsin (Herbage & Buffevant, 1974; Herbage, 1975). These studies, as well as those of Miller & Lunde (1973), Deshmukh & Nimmi (1973) and Eyre & Muir (1975a,b), using the action of CNBr

for the characterization of the insoluble or soluble collagens, demonstrated that the hyaline cartilage consists entirely of type-II collagen.

However, Seyer *et al.* (1975a,b) showed the presence of type-I collagen in articular cartilage of post-natal chickens and in embryonic bovine epiphyseal cartilage. From these results and from the amino acid composition of adult bovine cartilage (from 2-year-old steers) Lipshitz *et al.* (1975) postulated that the collagen of this cartilage contained a collagen with a low content of hydroxylysine, presumably type-I, in addition to type-II collagen.

We have therefore undertaken a systematic study of these cartilaginous tissues to determine the exact nature of the collagen. We present here the biochemical and physicochemical analysis of the pepsin-solubilized collagen from bovine articular cartilage for two age ranges (2–4 years and 2 months). This soluble fraction of collagen represents, according to the pretreatment used for extraction of proteoglycans, up to 95% of the total collagen.

Materials and Methods

Articular cartilages from 2-month-old calves and 2–4-year-old steers were obtained from articular

surfaces of the femora. Finely sliced articular cartilage was homogenized in liquid N₂ with a grinder (10s, IKA; Janke und Kunkel, Staufen, Germany). Acid-soluble collagen from calf skin was prepared by the method of Piez *et al.* (1961).

Cartilage pretreatment

The proteoglycans were extracted with either (a) guanidinium chloride solution (4M in 0.05M-sodium acetate, pH 5.8, 24h at 4°C), (b) CaCl₂ solutions (1.5M at pH 5.5, 24h at 20°C), (c) NaOH solution (0.2M, 24h at 4°C) or (d) by the method of Steven & Thomas (1973) (H₂O₂ followed by trypsin and EDTA treatments). The insoluble residues were washed in absolute ethanol then vacuum-dried. The hexosamine content of the residue was determined by the Elson & Morgan (1933) method by using the modification of Cessi & Piliego (1960). The percentage of collagen extracted was determined by measuring the hydroxyproline content of the extracts and of the insoluble residues by the method of Stegemann (1967) as adapted for the Technicon autoanalyser (Grant, 1964).

Pepsin treatment of insoluble collagen

Articular cartilage with or without pretreatment was digested by pepsin (twice-crystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.) as described by Miller (1972). Cartilage powder was added to the pepsin solution in 0.5M-acetic acid to give a collagen/pepsin ratio of 10:1 (w/w). After constant stirring for 18h at 20°C, the insoluble residue was separated by ultracentrifugation (1h at 20000g). Soluble collagen was precipitated by the addition of NaCl to a concentration of 0.9M. Pepsin was inactivated by dissolution of the collagen in 1.0M-NaCl/0.05M-Tris/HCl, pH 7.5. After 3 days the solution was dialysed against 0.02M-Na₂HPO₄, pH 9.4. The precipitate was collected by centrifugation for 1h at 30000g, redissolved in 0.01M-acetic acid and freeze-dried.

Biochemical characterization of pepsin-solubilized collagen

Collagen samples were hydrolysed overnight with 6M-HCl in sealed tubes under N₂ at 108°C. The hydrolysates were dried and analysed by using a Jeol JLC 5 AH amino acid analyser. Total hexoses were measured by the orcinol method (Brown, 1946) with a standard glucose/galactose mixture (2:1, v/v). Glucose content was evaluated with glucose oxidase (God-Perid method; Boehringer, Mannheim, Germany). The percentage of O-glycosidically bound hydroxylysine was determined by the method of Aronson *et al.* (1967). The amide groups of asparagine and glutamine residues were determined by the method of Eastoe *et al.* (1961).

Subunit composition of pepsin-solubilized collagen

The subunits of heat-denatured skin and cartilage collagen were separated by ion-exchange and molecular-sieve chromatography and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Chromatography was performed as follows. (a) A column (2.5cm × 12cm) of Whatman CM-52 CM-cellulose was equilibrated at 40°C with 0.05M-sodium acetate buffer, pH 4.8, containing 1.0M-urea, and was eluted with a linear gradient of 0.05–0.18M-NaCl at a flow rate of 115 ml/h. (b) A column (2.5cm × 100cm) of Bio-Gel A-15m (200–400 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) was equilibrated and eluted with 1M-CaCl₂ in Tris/HCl buffer (0.05M, pH 7.5). Fractions (3ml) were collected at a flow rate of 25 ml/h, and those corresponding to the β-components were pooled, dialysed against 0.01M-acetic acid and freeze-dried.

Electrophoretograms were prepared by the flat-bed technique of Sykes & Bailey (1971). A 6.75% (w/v) acrylamide gel containing 0.2% (w/v) sodium dodecyl sulphate was run for 2h at 350V, then stained with Coomassie Blue.

Reduction of collagen, and identification of the cross-links

β-Components (recovered from Bio-Gel chromatography) from pepsin-solubilized cartilage collagen and acid-soluble skin collagen (100mg of freeze-dried samples) were suspended in 0.9% NaCl (pH 7.4) and reduced with KB³H₄ diluted with non-radioactive KBH₄ to 10mCi/mmol (collagen/KBH₄ 30:1, w/w). After hydrolysis in boiling 6M-HCl for 24h, the cross-links were analysed by ion-exchange chromatography (Bailey *et al.*, 1970). Confirmation of the identity of the reduced cross-links was achieved by comparison with authentic standards (supplied by Dr. A. Bailey) by using the extended basic column of the Beckman amino acid analyser (Bailey *et al.*, 1970).

Collagen cleavage with CNBr

Pepsin-solubilized cartilage collagen and acid-soluble skin collagen were treated with CNBr in 70% (v/v) formic acid. A weight of CNBr equal to a 150-fold molar excess relative to the methionine residues (ten residues/1000; Miller, 1972) of the dissolved collagen was added. The reaction mixture, flushed with N₂, was incubated at 30°C for 5h, diluted tenfold with water and freeze-dried. The peptides were redissolved in 0.1M-acetic acid and passed through a column (2.5cm × 10cm) of Bio-Gel P₂ (100–200 mesh; Bio-Rad Laboratories). The fraction of the Bio-Gel P₂-column eluate containing the peptides was freeze-dried.

Electrophoresis of the CNBr peptides was performed as described above for the subunits of collagen. Control of the exact nature of the CNBr peptides was achieved by ion-exchange and molecular-sieve

chromatography. A column (1 cm × 24 cm) of CM-cellulose (Whatman CM-52) was equilibrated at 40°C with 0.02M-sodium citrate adjusted to pH 3.8 with citric acid. Peptides were eluted at a flow rate of 50 ml/h by a linear gradient of 0–0.1M-NaCl in 500 ml (total volume) of starting buffer.

A column (2.5 cm × 150 cm) of Bio-Gel A 1.5m (200–400 mesh; Bio-Rad Laboratories) was equilibrated and eluted with 1M-CaCl₂ in Tris buffer (0.05M, pH 7.5, adjusted with 0.1M-HCl), at a flow rate of 25 ml/h. The *A*₂₃₀ of the effluents was continuously monitored.

Physicochemical characterization

The different collagens were all studied in 0.1M-acetic acid solutions.

Viscosity. This was determined at 20°C with a solvent flow-time of 150 s, by using an Ubbelohde-type viscometer as modified by Vallet (1952).

Sedimentation coefficient. This was calculated from the sedimentation observed in an MSE analytical ultracentrifuge. All measurements were made at a speed of 60000 rev./min (*r*_{av} = 6.49 cm), with a rotor temperature of 15°C.

Thermal stability. Two methods were used to characterize the thermal stability of the collagens; differential thermal analysis and polarimetry. (a) Collagen samples (2 mg) swollen in 0.1M-acetic acid (0.1 ml) were studied in a sealed cell (Dupont 990 thermal analyser). The thermogram is obtained with a heating rate of 5°C/min and a starting temperature of 10°C. The extrapolated onset temperature is the best estimate of transition temperature as found by McClain & Wiley (1972).

(b) A Jouan micropolarimeter equipped with a 2 cm-path-length jacket cell was used. Optical rotation was measured at 540 nm. Denaturation was accom-

plished by increasing the temperature of the solutions at a rate of 5°C/min. The denaturation temperature (*T*_D) was defined as the midpoint of the phase transition from collagen to gelatin.

Molecular length. Length of molecules present in our solutions was measured by electrical birefringence measurement. The instrument used was entirely built in our laboratory (Bernengo *et al.*, 1976). The birefringence decay curve is obtained for calf skin and cartilage collagen in 0.1M-acetic acid at 20°C (at a concentration of 50 mg/litre).

Electron microscopy. Segment-long-spacing crystallites are precipitated by dialysis of collagen solutions against 0.4% ATP solution (in 0.1M-acetic acid).

Reconstituted native-type fibrils were obtained by dialysis of collagen solutions against 0.02M-Na₂HPO₄, pH 9.4.

Segment-long-spacing crystallites and fibrils were stained with phosphotungstic acid (0.4%, pH 3.5) and with unbuffered uranyl acetate solution (0.1%) as described by Stark & Kühn (1968). The electron-microscopic studies were carried out on the Philips EM 300 instrument from the Centre de Microscopie Electronique Appliquée à la Biologie de l'Université Claude Bernard, Lyon, France.

Results

Collagen solubilization

The direct action of pepsin on bovine articular cartilage does not solubilize collagen; therefore pretreatment to remove (totally or partially) the proteoglycans is necessary for extraction of large quantities of collagen (Table 1). Similar results are obtained for the two age groups. Increasing amounts of proteoglycans are extracted with CaCl₂ and guanidinium

Table 1. *Collagen solubilization from bovine articular cartilage*
For experimental details, see the Materials and Methods section.

Articular cartilage	Nature of pretreatment	Collagen extracted after pretreatment (% of total)	Proteoglycan extracted after pretreatment (% of total)	Collagen extracted after pepsin treatment (% of total)
Adult bovine (2–4-year-old)	None	—	—	Traces
	CaCl ₂ (1.5M)	1	70	60–70
	Guanidinium chloride (4M)	1.5	80	60–80
	NaOH (0.2M)	1.5	96	80–95
	H ₂ O ₂ + trypsin (Steven & Thomas, 1973)	—	>99	1st treatment, 20–30 2nd treatment, 70
Calf (2-month-old)	None	—	—	Traces
	CaCl ₂ (1.5M)	1.5	65	70–80
	Guanidinium chloride (4M)	2	70–80	80–90
	NaOH (0.2M)	2	90	90–95

chloride. The concentrations selected are those that permit maximum extraction of proteoglycans [the 'optimum concentrations' of Sajdera & Hascall (1969)], but preserve the helical structure of the native collagen (Herbage *et al.*, 1974). For total extraction, the proteoglycans had to be degraded, for example, by the action of 0.2M-NaOH (Herbage & Buffevant, 1974) or by a H_2O_2 treatment followed by trypsin digestion (Steven & Thomas, 1973).

The quantity of collagen extractable by pepsin increases with proteoglycan concentration, except in the case of cartilage pretreated by the method of Steven & Thomas (1973). In spite of total removal of proteoglycans, only 20–30% of collagen could be solubilized. We have, however, observed that a second pepsin treatment permitted extraction of the remaining insoluble collagen (Table 1).

After a NaOH pretreatment it is possible to extract almost all the collagen with pepsin. It should be noted, however, that alkaline treatment alone extracts only 1.5–2% of the total collagen.

Biochemical characterization

The biochemical and physicochemical properties of collagen extracted with pepsin after pretreatment

Fig. 1. CM-cellulose chromatography of skin and cartilage collagen

(a) Acid-soluble collagen from calf skin, and pepsin-solubilized collagens from (b) 2–4-year-old and (c) 2-month-old bovine articular cartilage. The column (2.5 cm $\times$ 12 cm) was equilibrated at 40°C with acetate buffer (0.05 M, pH 4.8) containing 1.0 M-urea and eluted with a linear 0.05–0.18 M-NaCl gradient at a flow rate of 115 ml/h (total volume 750 ml).

Fig. 2. Molecular-sieve chromatography of skin and cartilage collagens

(a) Acid-soluble collagen from calf skin, and pepsin-solubilized collagens from (b) 2–4-year-old and (c) 2-month-old bovine articular cartilage. The column (2.5 cm $\times$ 100 cm) of Bio-Gel A 15 m (200–400 mesh), was equilibrated and eluted with 1 M- CaCl_2 in Tris buffer (0.05 M, pH 7.5). Fractions (3 ml) were collected at a flow rate of 25 ml/h. The β -components in the pooled fractions marked by the bars were recovered for cross-links analysis (see Fig. 3).

with CaCl_2 or guanidinium chloride are identical. Therefore we describe only the results obtained after pretreatment with guanidinium chloride, noting the differences observed after pretreatment with 0.2 M-NaOH.

Subunit composition. The CM-cellulose chromatography (Fig. 1) and the electrophoresis in sodium dodecyl sulphate/polyacrylamide gel (Plate 1) show the total absence of the α_2 -chains in the denaturation components of our collagen and therefore the absence of type-I collagen. As already observed (Herbage & Buffevant, 1974), the peak corresponding to the $\alpha_1(\text{II})$ -chains has two components (Figs. 1b and 1c). This effect, certainly caused by pepsin action, is more marked for the older tissue. The presence of β - and γ -components is confirmed by molecular-sieve chromatography (Fig. 2). The relative proportions of the β - and γ -components with the α -chains are higher in the adult bovine cartilage (Fig. 2b).

The nature of the cross-links giving rise to the β -components has been determined by the method of Bailey *et al.* (1970) after reduction with KB^3H_4 and chromatography of the β -components recovered from molecular-sieve chromatography (Fig. 2). For the

acid-soluble collagen from calf skin, the β -components arise from two α -chains joined by intramolecular bonds of the 'aldol' type (Fig. 3a). For cartilage collagen the α -chains are joined by the intermolecular bond dehydrodihydroxylysinonorleucine. In the case of the collagen from adult bovine cartilage (2–4 years old) large quantities of hexosyl-lysine derivatives (peaks A₁, A₂, pA) have been found (Fig. 3b). The absence of 'aldol' type cross-links, and by consequence the absence of intramolecular bonds in the β -components of cartilage collagen, has been shown by using the extended basic column of a Beckman amino acid analyser (Bailey *et al.*, 1970).

Characterization of CNBr peptides. To verify the

absence of type-I collagen, we compared the CNBr-derived peptides from acid-soluble collagen from skin and cartilage. The peptides were identified by polyacrylamide-gel electrophoresis using the flat-bed technique of Sykes & Bailey (1971) (Plates 1e and 1f). In contrast with the disc-electrophoresis method of Furthmayr & Timpl (1971) a semi-logarithmic plot of migration distance against the molecular size of the peptides does not give a straight line, showing that charge on the peptides contributes to the migration, in spite of the presence of sodium dodecyl sulphate. However, by this method it is possible to separate the peptides $\alpha_1(I)CB7$ and $\alpha_1(I)CB8$ and also more clearly the four characteristic peptides of type-II

Fig. 3. Elution chromatogram showing the radioactive components from an acid hydrolysate of collagen β -components. The components (recovered from molecular-sieve chromatography, Fig. 2) were reduced with KB^3H_4 and separated as described by Bailey *et al.* (1970). (a) β -Components from acid-soluble skin collagen; (b) and (c) β -components from pepsin-solubilized collagen from 2–4-year-old (b) and 2-month-old articular cartilage (c). [O^6Nle = hydroxynorleucine, $Cl(Nle)$ = chloronorleucine, $O^{5,6}Nle$ = dihydroxynorleucine, $O^5Lys(O^5\omega Nle)$ = dihydroxylysinonorleucine.]

Fig. 4. CM-cellulose (a, b) and Bio-Gel (c, d) chromatography of CNBr peptides of collagen from skin and articular cartilage (a) and (b) A column (1 cm $\times$ 24 cm) of Whatman CM-52 CM-cellulose was equilibrated at 40°C with 0.02 M-sodium citrate adjusted to pH 3.8 with citric acid. Peptides were eluted at a flow rate of 50 ml/h by a linear gradient of 0–0.1 M-NaCl in a total volume of 500 ml of starting buffer. (a) 40 mg of CNBr digest of acid-soluble skin collagen. (b) 40 mg of CNBr digest of pepsin-solubilized collagen from bovine articular cartilage. (c) and (d) a column (2.5 cm $\times$ 150 cm) of Bio-Gel A 1.5 m (200–400 mesh) was equilibrated with 1 M-CaCl₂ in Tris buffer (0.05 M, pH 7.5). Peptides were eluted at a flow rate of 25 ml/h. (c) 8 mg of CNBr digest of acid-soluble skin collagen; (d) 8 mg of CNBr digest of pepsin-solubilized cartilage collagen. α_2 CB3.5 or α_1 CB9.7 represent uncleaved peptides and α_1 CB4 + 5 or α_1 (II)CB11 + 12 represent a mixture of two peptides.

collagen, namely α_1 (II)-CB10, -CB11, -CB8, -CB9.7. Moreover, the use of the flat-bed technique allows a better comparison of band position (Plate 1). Exact characterization of peptides was verified by CM-

cellulose and Bio-Gel chromatography (Fig. 4), which gave similar chromatograms to those of the literature for type-I and type-II collagens (Eyre & Muir, 1975; Deshmukh & Nimni, 1973; Miller, 1972).

EXPLANATION OF PLATE I

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels of collagens and CNBr peptides of collagens from calf skin and bovine articular cartilage

The flat-bed technique of Sykes & Bailey (1971) was used. The 6.75% (w/v) acrylamide gel contained 0.2% sodium dodecyl sulphate; both gel and electrode buffers were Tris/borate buffers, pH 8.5. Samples were run for 3 h (a, b, c and d) or 2 h (e and f) at 350 V (2.5 V/cm). The gel was sliced to remove the top and bottom faces and the centre section stained with Coomassie Blue and destained in 5% (v/v) acetic acid containing 40% (v/v) methanol. (a) Acid-soluble collagen from calf skin; (b) and (c) pepsin-solubilized cartilage collagen (2-4-year-old) pretreated with guanidinium chloride (b) or 0.2 M-NaOH (c); (d) pepsin-solubilized collagen from calf cartilage (2-month-old) pretreated with guanidinium chloride (mobility of α_2 = 5 cm). (e) and (f) CNBr peptides of acid-soluble skin collagen (e) and of pepsin-solubilized collagen from bovine cartilage (f). (Mobility of $\alpha_1\text{CB12}$ = 8 cm.) $\alpha_1(\text{I})\text{CB3,8}$ and $\alpha_1(\text{II})\text{CB9,7}$ represent uncleaved peptides.

EXPLANATION OF PLATE 2

Reconstituted fibres of skin and cartilage collagen

Reconstituted collagen fibres were obtained by dialysis of collagen in 0.1M-acetic acid against 0.02M- Na_2HPO_4 . (a) Acid-soluble collagen from calf skin; (b) and (c) pepsin-solubilized collagen from cartilage pretreated with guanidinium chloride (b) or 0.2M-NaOH (c). Fibres were stained with 0.4% phosphotungstic acid, pH 3.5, followed by 1% uranyl acetate. For (a), (b) and (c), D = 64 nm and represents the well-known periodicity of collagen fibres.

EXPLANATION OF PLATE 3

Segment-long-spacing crystallites of skin and cartilage collagen

The segment-long-spacing crystallites were prepared from (a), acid-soluble skin collagen and (b), pepsin-solubilized cartilage collagen by dialysis of 0.1% collagen solution in 0.1 M-acetic acid against 0.4% ATP at pH 3.0 and stained by phosphotungstic acid and uranyl acetate. Arrows indicate direction of alignment of the molecules with N- and C-terminal ends in register (a, i) and overlapped in (b, ii). In (a, i) and (b, i) the bar represents 300 nm.

The only difference observed with pepsin-solubilized collagen after a NaOH pretreatment was a lower electrophoretic migration of the $\alpha_1(\text{II})$ -chains, in good agreement with the elution of these chains at a lower ionic strength from the CM-cellulose column (Herbage & Buffevant, 1974). This difference is due to a partial de-amidation of peptide-bound asparagine and glutamine residues (0.41 mol/g instead of 0.45 mol/g of amide groups) (Table 2).

Table 2. *Amino acid composition of acid-soluble skin collagen and of pepsin-solubilized cartilage collagen*
Each value is given as the average of a minimum of five analyses. For details, see the Materials and Methods section.

	Content (residues/1000 amino acid residues)		
	Acid-soluble calf skin collagen	Pepsin-solubilized collagen from bovine articular cartilage (pretreated with 4M-guanidinium chloride or with 0.2M-NaOH)	
		2-4 year-old animal	2-month-old animal
Hyp*	94	97	96
Asp	45	48	48
Thr	18	23	23
Ser	33	28	26
Glu	75	92	93
Pro	121	115	121
Gly	330	330	326
Ala	119	98	103
Val	21	18	18
Met	6	9	8
Ile	11	10	9
Leu	23	26	26
Tyr	3	1	1
Phe	13	14	13
Hyl†	7	20	21
Lys	26	18	17
His	5	2	2
Arg	50	51	51
Percentage of Hyl residues glycosylated	15	55	
Total hexoses (% dry wt. of tissue)	0.7	4	
Glucose (% dry wt. of tissue)	0.2	1.4	
Amide groups (mol/g)	0.44	{ 0.45 (guanidinium pretreatment) 0.41 (NaOH pretreatment)	

* Hyp, hydroxyproline

† Hyl, hydroxyllysine

Amino acid composition. The composition of the amino acids of pepsin-solubilized collagen from bovine cartilage collagen is shown in Table 2. It becomes apparent that this composition is independent both of cartilage pretreatment (except for the quantity of the amide groups) and of the age of the animal.

The hydroxylysine (20-21 residues/1000) and the lysine (17-18 residues/1000) content agree well with the values of Eyre & Muir (1975) and Miller & Lunde (1973), but differ from those of Strawich & Nimni (1971). Approx. 55% of hydroxylysine residues are glycosylated with a galactose/glucose ratio of approx. 2:1.

Electron microscopy. The fibres reconstituted from the different solutions of collagen by dialysis against 0.02M-sodium phosphate, pH9.4, have either the periodicity of 64nm characteristic of the native structure of collagen fibres (Plate 2) or indistinct cross-striation bands (Plate 2b) as already observed by Stark *et al.* (1972). Some fibrils (Plate 2b) show oblique bands that could correspond to fibre twisting. In contrast with the results of Kühn *et al.* (1968) obtained with a desamido collagen, the partial de-amidation (10%) induced by the NaOH pretreatment is not sufficient to prevent the re-formation of native-type fibres (Plate 2c).

The segment-long-spacing crystallites formed from cartilage collagen are very different from those obtained from calf skin collagen (Plate 3) and show a dimeric structure. These dimers correspond to the parallel alignment of two monomers with an overlap of the C-terminal ends. By using the calculation method of Bruns & Gross (1973), these dimers are about 470nm in length, with an overlap length of about 90nm (Table 3). These dimers are different from those of skin collagen, which correspond to an end-to-end aggregation.

Physicochemical characterizations

Electrical and hydrodynamic properties. Molecules that have asymmetric charge distributions are orientated by an external field. Partial orientation of these macromolecules modifies the optical properties of the solution, which becomes birefringent. Our apparatus displays directly the logarithm of birefringence decay on a storage oscilloscope (Bernengo *et al.*, 1974). For acid-soluble collagen from calf skin in 0.1M-acetic acid, a plot of the logarithm of the birefringence against time gives a straight line (Fig. 5). From the slope of this straight line, by assuming a cylindrical rod model and from Burgers' (1938) formula, we obtain a molecular length of 275nm. With the two cartilage collagens the data (Fig. 5b) can be fitted well to a two-relaxation-step process. Essentially two species are present: some 90% of the solution birefringence is due to the monomer (280nm), and 7-9% of the solution birefringence is

Table 3. Physicochemical characterization of acid-soluble collagen from calf skin and of pepsin-solubilized collagen from bovine articular cartilage
For details, see the materials and methods section.

	Specific optical rotation $[\alpha]_{D}^{20}$	Intrinsic viscosity (η) (dl/g)	Sedimentation coefficient s_{20} (S)	Denaturation temperature T_D (°C)		Electrical birefringence		Segment-long-spacing crystallite	
				Differential thermal analysis	Polarimetry	% total birefringence	Molecular length	% of total	Molecular length
Acid-soluble collagen from calf skin	-400	14.5	2.7	39	36	>98	274 nm	(80% → 290 nm 20% → 580 nm)	
Pepsin-solubilized collagen from bovine articular cartilage	-350	14	2.6	38	36	90 7-9	280 nm 510-530 nm	>95% → 480 nm	
2-4-year-old animal	-350	13		41	38			>90% → 480 nm	
2-month-old animal									

Fig. 5. Plot of the logarithm of normalized birefringence against time for collagen solutions (concentration, 50 mg/litre in 0.1 M-acetic acid)
(a) Acid-soluble collagen from calf skin (applied field = 750 V/cm); (b) pepsin-solubilized collagen from bovine articular cartilage (applied field = 250 V/cm). This curve is fitted by the theoretical curve of equation:
$$\Delta n = -a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$$

with a relative precision better than 2%. The following values have been found: $a_1 = 0.91$; $\tau_1 = 150 \mu s$; $a_2 = (1 - a_1) = 0.09$; $\tau_2 = 1.3 \text{ ms}$.

due to a rigid molecule whose length is approx. 1.8-1.9 times the length of the monomer (510-530 nm).
Because of its low concentration, this dimeric molecule has not been observed by analytical ultracentrifugation. The sedimentation coefficient of the monomer (2.6S) is close to that found for skin collagen (2.7S). The intrinsic viscosities of cartilage and skin collagens are nearly identical (Table 3).
Helical-structure properties. The thermal stability of our collagens has been measured by differential thermal analysis and by polarimetry. It has been found that the denaturation temperature of the 2-month-old-calf cartilage collagen is slightly higher than that from skin and adult bovine cartilage. On the other hand, irrespective of the age, the specific optical

rotation of cartilage collagen is lower than that of calf skin collagen (-350° instead of -400°).

Discussion

Collagen from bovine articular cartilage (from less-than-4-year-old animals) can be solubilized with pepsin after prior removal of the proteoglycans of the ground-substance. In particular, the major part of the collagen is soluble with pepsin after a pretreatment of the cartilage with 0.2M-NaOH.

The proteoglycans seem to make an important contribution to the insolubility of this cartilage collagen. This does not rule out the possibility that pretreatment acts directly on the collagen cross-links. Indeed, in spite of total degradation of proteoglycans by the method of Steven & Thomas (1973), a prolonged pepsin action is necessary to solubilize the same quantity of collagen as after a NaOH pretreatment. Further, pepsin digestion of adult human articular cartilage, even after complete elimination of proteoglycans by these same pretreatments, fails to solubilize the collagen (Herbage *et al.*, 1975), which has stable and non-reducible cross-links (Herbert *et al.*, 1973).

The biochemical analysis of our pepsin-solubilized cartilage collagens, the chromatographic analyses of their denaturation components and of their CNBr peptides show that they are essentially all type II. Their amino acid composition is very close to that found by Miller & Lunde (1973). However, the hydroxylysine and lysine contents (20–21 and 17–18 residues/1000 residues respectively) are different from those given by Strawich & Nimni (1971) for the papain-solubilized fraction of bovine cartilage collagen (28 and 15 residues/1000 residues). This point is important, since these last values have been used as references for numerous authors, particularly Lipshitz *et al.* (1975) who used these values to suggest the existence of type-I collagen in bovine articular cartilage. This hypothesis, based on the hydroxylysine and lysine contents of the total tissue, should be reconsidered if the hydroxylysine content of purified type-II collagen is about 21 instead of 28 residues/1000 residues. We have equally verified that the non-pepsin-solubilized fractions of this collagen [fraction extracted during pretreatment (1–2% of total collagen) and fraction remaining in the insoluble residue after pepsin treatment (5–30% of total collagen)] was equally type-II collagen (D. Herbage & C. Michel, unpublished results).

The physicochemical properties of pepsin-solubilized collagens from bovine cartilage are independent of the pretreatment and of the age range (except for the denaturation temperature of the calf cartilage collagen, which is higher than that of the adult bovine cartilage collagen). On the other hand,

these results are similar to those obtained on chicken cartilage collagen (Igarashi *et al.*, 1973).

However, with the electrical-birefringence technique (Kerr effect), we have shown differences between the nature of the molecules present in the solutions (0.1M-acetic acid) of acid-soluble skin collagen and of pepsin-solubilized cartilage collagen. Although solutions of acid-soluble skin collagen contain a single type of molecule, corresponding to the well-known rigid rod whose calculated length is 275 nm (Bernengo *et al.*, 1974), the solutions of cartilage collagens are composed of a mixture of two types of molecules, namely the rigid rod (calculated length 280 nm) and molecules (7–8% of the solution birefringence) whose length is slightly smaller than twice the length of the rigid rod (510–530 nm). This latter molecule is different from the aggregates (of electrostatic nature) appearing in acid solutions of acid-soluble skin collagen when the pH is increased (J. C. Bernengo & D. Herbage, unpublished results) and may be a dimer composed of two monomers linked by intermolecular 'head-to-tail' bonds. The theoretical length of 530 nm according to the 'quarter stagger' theory (Schmitt, 1956) is in good agreement with our measured value.

We have confirmed this hypothesis by showing that the β -components isolated from the denaturation products of cartilage collagen, are composed of two α -chains linked by the intermolecular cross-link, dehydrodihydroxylysinonorleucine. The presence of this intermolecular cross-link and the absence of 'aldol'-type intramolecular cross-link is consistent with the nature and location of the cartilage collagen cross-links (Miller, 1971; Bailey & Shimokomaki, 1971; Balian & Bailey, 1975).

The presence of this dimeric molecule in 0.1M-acetic acid does not, however, explain the particular structure of the segment-long-spacing crystallites re-formed from these solutions. Indeed nearly all is in a dimeric form that corresponds to a lateral aggregation of the molecules with overlapping of the C-terminal regions (overlap length 90 nm). They are similar to those obtained by Olsen (1965) and by Swann *et al.* (1976) with vitrosin and by Ludowieg *et al.* (1973) with Pronase-solubilized collagen from whale foetal nucleus pulposus. A relationship between the presence in solution of dimeric molecules (observed by electrical birefringence) and the form of the segment-long-spacing crystallites precipitated from the same solution does not seem to exist, since the solutions of pepsin-solubilized collagen from osteoarthritic articular cartilage (Herbage, 1975) possess dimeric molecules, whereas the segment-long-spacing crystallites are monomers (D. Herbage, unpublished results).

The fibrils reconstituted from pepsin-solubilized cartilage collagen generally show a structure identical with those obtained from acid-soluble skin collagen,

even after pretreatment with NaOH. The structure described by Bruns *et al.* (1973) and Bruns (1976) presenting a regular staggered array of parallel subfibrils has not been found. Thus as for the pepsin-solubilized collagen from bovine vitreous humour (Swann *et al.*, 1976), the reconstituted fibres from solutions of pepsin-solubilized cartilage collagen differ from the native fibres and are similar to those obtained with calf skin collagen. The peptides cleaved by pepsin may be one of the fractions that determines the particular fibrous form of collagen, another one being the interactions with the ground substance.

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