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Further Biochemical and Physicochemical Characterization of Minor Disulfide-Bonded (Type IX) Collagen, Extracted From Foetal Calf Cartilage

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Minor disulfide-bonded collagen (previously termed X_1–X_7 and now called type IX collagen) was isolated from foetal calf cartilage after pepsin treatment. At least three native fractions, containing, respectively, the $X_1X_2X_3$, X_4, and $X_5X_6X_7$ chains, were separated; and from further biochemical and physicochemical experiments (differential scanning calorimetry, electrical birefringence, rotary shadowing), we propose a tentative model for their organization within a parent molecule. X_1 and X_2 are molecules composed of three chains of apparent M_r 62,000 and 50,000 linked by interchain disulfide bonds and containing pepsin-sensitive regions. The cleavage of at least three of these sites, present within X_2, gives rise to the X_3 and $X_5X_6X_7$ fractions composed of molecules 80–100 nm and 40–55 nm in length, respectively. The $X_5X_6X_7$ fraction is not digested by pepsin at 30°C owing to its high thermal stability (certainly explained by its high hydroxyproline + proline content). This organization is in good accordance with that proposed for chicken cartilage type IX collagen; differences could only exist in the number and (or) the location of the pepsin-sensitive sites.

Key words: type IX collagen, foetal calf cartilage, pericellular matrix

Type II collagen is the major collagen of cartilage. Other minor collagens have been recently isolated from pepsin digests of different cartilaginous tissues. These are the collagen chains $1\alpha 2\alpha 3\alpha$ [1] and several collagenous disulfide-bonded fragments called M, CF_2, CF_1 [2,3], CPS_1, CPS_2 [4,5], HMW, LMW [6,7], M_1, M_2 [8], and X_1–X_7 in our laboratory [9]. These different fragments, corresponding to type IX collagen as proposed in [10], are probably derived from the same (pro)collagen. Indeed, a disulfide-bonded collagen of apparent M_r 300,000, called p-MHW [11] and pM-collagen [12], has been isolated without proteolytic treatment from organ cultures of embryonic chick cartilage. These collagens are primarily located in the pericellular environment of the chondrocytes, as shown by light [9,13,14] and electron microscopy [15].

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Recently a cDNA coding for a polypeptide, containing three collagenous domains connected by short noncollagenous peptides, has been characterized in chick cartilage. It was proposed that it represents one of the three subunits of the parent molecule of HMW and LMW [16].

In this report we describe further biochemical and physicochemical analysis of the X_1–X_7 components, isolated from foetal calf epiphyseal cartilage, and the molecular model deduced from these experiments. This structure is compared to the model of type IX collagen [10].

MATERIALS AND METHODS

Collagen Preparation

Epiphyseal cartilage was obtained from foetal calves (7–9 months). The tissue was sliced, homogenized under liquid nitrogen with a grinder (IKA A 10 S, Janke und Kunkel), and extracted with 4 M guanidinium chloride in 0.05 M sodium acetate, pH 5.8, for 24 hr at 4°C to remove the proteoglycans. After extensive washing with deionized water, the insoluble residue was suspended in 0.5 M acetic acid containing 0.2 M NaCl and subjected to pepsin (Sigma, P 7012 twice crystallized) treatment for 24 hr at 8°C, as described in [1]. The pepsin digest was neutralized to pH 8.6 with 4 M NaOH and centrifuged for 40 min at 30,000g to remove undigested material. The supernatant was dialyzed against deionized water to precipitate collagens, and the resulting precipitate was then dialyzed against 0.1 M acetic acid. The different collagen types were then salt-fractionated at 0.7 M, 1.2 M, 2.0 M, and 3.0 M NaCl as in [9].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Lyophilized samples were analyzed on 6.75% or 10% polyacrylamide slab gels as in [17]. The gel was stained overnight with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 5% acetic acid.

Carboxymethyl (CM)-Cellulose Chromatography

CM-cellulose chromatography of the 2.0 M NaCl precipitate was carried out as in [3].

Cyanogen Bromide Cleavage

About 5 mg of collagen was dissolved in 5 ml of 70% formic acid. Cleavage was performed for 4 hr at room temperature with 20 mg of cyanogen bromide (CNBr). The samples were then diluted with deionized water, freeze-dried, and analyzed by polyacrylamide gel electrophoresis.

Staphylococcus - aureus V_8 Protease Cleavage

Two-dimensional gel electrophoresis was performed as described in [18]. For the first dimension the different collagenous components were separated on a linear gradient of 5–7.5% polyacrylamide. After digestion by V_8 protease during the second dimension, the peptides obtained were separated on a 15% polyacrylamide gel. The gel was then stained by silver stain as in [19].

Second Pepsin Treatment of the 2.0 M NaCl Precipitate

The 2.0 M NaCl precipitate was dissolved in 0.5M acetic acid containing 0.2 M NaCl and pepsin at 1 mg/ml. Pepsin treatment was performed for 16 hr, either at 8°C or at 30°C, with constant stirring. Pepsin was inactivated by the addition of 4 M NaOH to adjust the pH to 8.6. The mixture was then dialyzed against 0.1 M acetic acid and precipitated by 2.0 M NaCl. The resultant precipitate was collected by centrifugation and the supernatant was precipitated by 3.0 M NaCl. The different fractions obtained were dialyzed against dilute acetic acid and freeze-dried.

Differential Scanning Calorimetry (DSC)

Lyophilized samples ($\simeq$ 5 mg) were swollen in 0.5 M acetic acid and immediately sealed in the cell of a programmed differential calorimeter (Setaram, Lyon). The thermograms were obtained with a heating rate of 1.1°C/min from a starting temperature of 4°C.

Electrical Birefringence Measurements

The length of the molecules of the 2.0 M NaCl precipitate in solution was measured by electrical birefringence. The apparatus that we have used was entirely built by Bernengo et al [20]. The birefringence decay curves were obtained at 20°C in 0.1 M acetic acid at a collagen concentration of 0.2 g/liter and analyzed as previously described in [21].

Rotary Shadowing Experiments

The rotary shadowing technique was adapted from [22]. The proteins were dissolved in 1 M ammonium acetate at a concentration of 30 μg/ml. After addition to 70% glycerol (v/v) the mixture was sprayed over freshly cleaved mica. Shadowing was made in an Edwards evaporator after evacuation of the chamber to 10^{-6} torr. The sample was shadowed with tungsten platinum (0.5-mm-diameter tungsten rods with 4 cm of 0.02-mm-diameter platinum wire at 10 V, 2 mA) at an angle of 9° for complete platinum evaporation, and followed by carbon coating at 90° for 10 sec. The replicas were floated in distilled water and picked up on 200-mesh uncoated grids. They were observed on a Philips EM 300 or Jeol 1200 Ex electron microscope from the Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie de l'Université Claude Bernard, Lyon I. Calibration of magnification was made with grafting replicas.

RESULTS

We have separated from each other three different fractions containing triple helical molecules characterized by their denaturation components on polyacrylamide gel electrophoresis: $X_1X_2X_3$, X_4, and $X_5X_6X_7$. The 3.0 M NaCl precipitate contained the $X_5X_6X_7$ fraction. The 2.0 M NaCl precipitate, composed mostly of X_1–X_4, was separated by CM-cellulose chromatography at 10°C in two native fractions (Fig. 1), which were analyzed on SDS-polyacrylamide gel electrophoresis (data not shown). The first eluted fraction contained $X_1X_2X_3$ only, and the second fraction a mixture of $X_1X_2X_3$, and X_4.

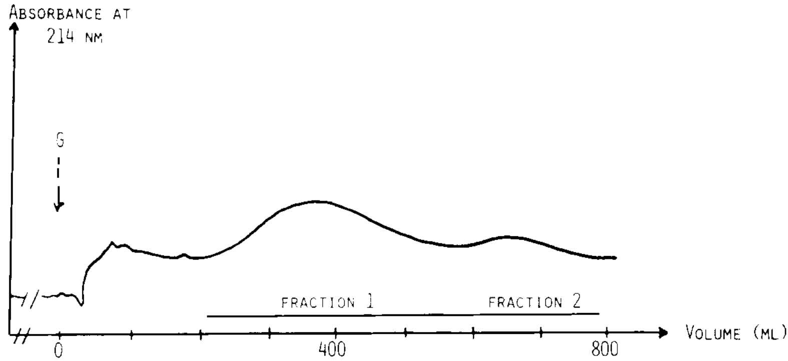


Fig. 1. Carboxymethyl-cellulose chromatography of the 2.0 M NaCl precipitate. The column was equilibrated with 0.04 M sodium acetate, pH 4.8, at 10°C. The different fractions were eluted with a linear gradient from 0.03–0.3 M NaCl in a total volume of 800 ml.

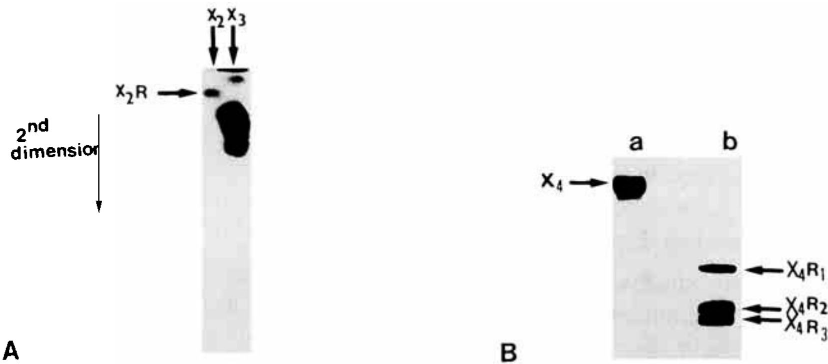


Fig. 2. A) Two-dimensional electrophoresis of the $X_1X_2X_3$ fraction. The first dimension was performed on a 6.75% polyacrylamide gel and the second one on a 10% polyacrylamide gel in presence of dithiothreitol (DTT). B) SDS-polyacrylamide gel (10%): a, X_4; b, X_4 + DTT.

As already described [9], X_5,X_6, and X_7 were not affected by reduction with dithiothreitol, whereas $X_1X_2X_3$ and X_4 were disulfide-bonded. As shown by two-dimensional electrophoresis (Fig. 2A), X_2 gave rise to only one peptide, called X_2R, of apparent M_r 50,000. X_3 was reduced into three major components—X_3R_1, X_3R_2, and X_3R_3—of apparent M_r 40,000, 37,000, and 31,000, respectively; and X_4, into three peptides—X_4R_1, X_4R_2, and X_4R_3—(Fig. 2B) of apparent M_r 19,500, 12,000, and 10,000.

When the mixture containing X_1–X_3 was subjected to CNBr cleavage, we observed at least seven peptides on polyacrylamide gel electrophoresis (Fig. 3, track b). X_4 was cleaved by CNBr into two peptides (Fig. 3, track d), which were disulfide-bonded, for they gave rise by reduction to three other peptides of lower molecular weight (Fig. 3, track e).

The peptides resulting from cleavage of X_2 and X_3 by V_8 protease of S aureus were very similar but, nevertheless, showed some differences (Fig. 4). Several prominent peptides were present in the digests of both X_2 and X_3. Some peptides

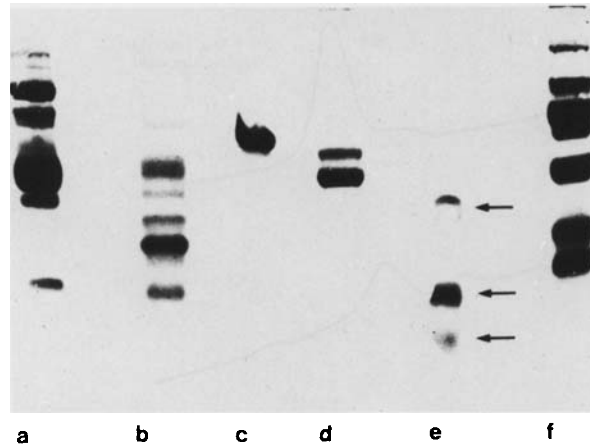


Fig. 3. SDS-polyacrylamide gel (10%) electrophoresis of (c) X_4 chain and of the CNBr peptides from (a) type I collagen (b) $X_1X_2X_3$ fraction, (d) X_4, (e) X_4 + DTT and (f) type II collagen.

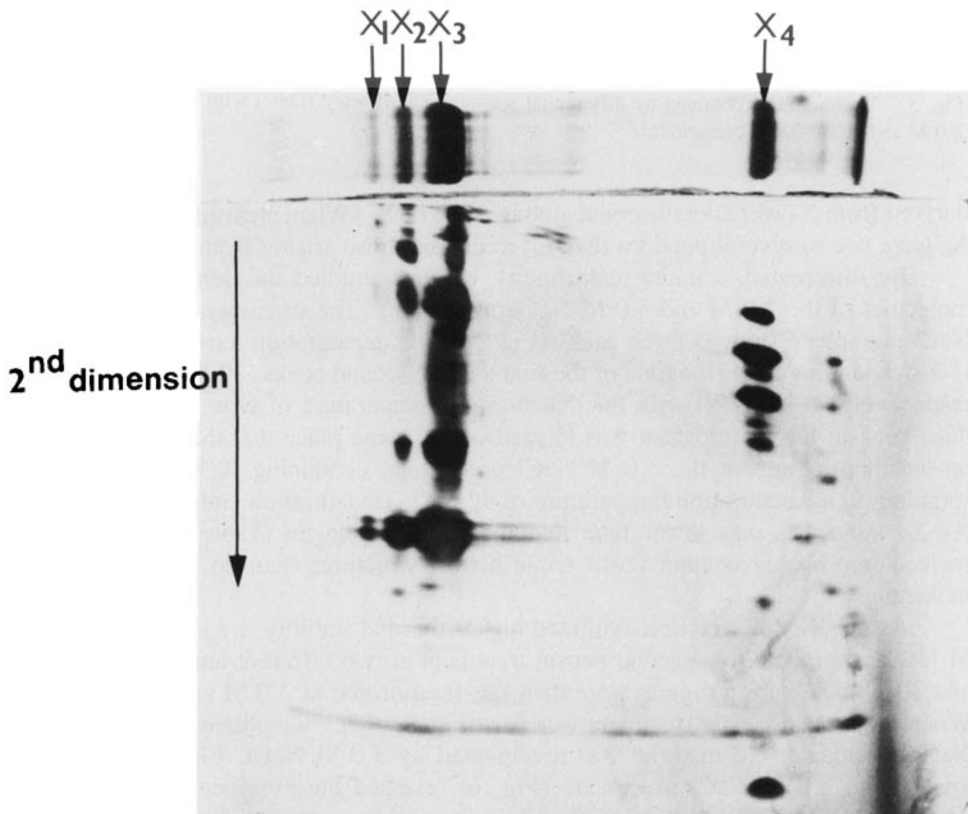


Fig. 4. Two-dimensional electrophoresis of the 2.0 M NaCl precipitate (silver staining). The first dimension was performed on a slab gel formed by a linear gradient of 5–7.5% polyacrylamide. A gel lane containing 100 μg of the 2.0 M NaCl precipitate was transferred to 15% polyacrylamide gel and overlayed with 10 μg of S aureus V_8 protease in sample buffer in the second dimension. The gel was run in the second dimension as described in Materials and Methods.

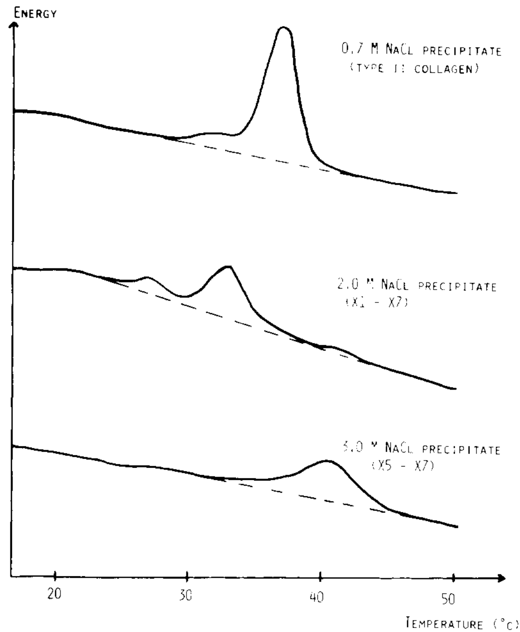


Fig. 5. Thermograms obtained by differential scanning calorimetry (DSC) with type II collagen and 2.0 M and 3.0 M NaCl precipitates.

derived from X_3 were not observed among those of X_2. When cleaved by V_8 protease, X_4 gave rise to several peptides that differed from those arising from X_2 and X_3.

By differential scanning calorimetry we have studied the denaturation of the molecules of the 2.0 M and 3.0 M NaCl precipitates. The thermogram of the 2.0 M NaCl precipitate showed three peaks (Fig. 5). The denaturation temperatures (Table I) corresponding to the maxima of the first and the second peaks (29.8°C and 35.5°C, respectively) were lower than the denaturation temperature of type II collagen. The third peak of the thermogram was located on the same place that the peak observed on the thermogram of the 3.0 M NaCl precipitate, containing $X_5X_6X_7$ and corresponding to a denaturation temperature of 42.8°C. Denaturation enthalpy change for X_1–X_7 molecules was lower than that for type II collagen (Table I). The X_1–X_7 molecules probably contain fewer triple helical structures than do type II collagen molecules.

As the $X_5X_6X_7$ fraction exhibited higher thermal stability, we subjected the 2.0 M NaCl precipitate to a second pepsin treatment at two different temperatures: 8°C and 30°C. The pepsin digests were then salt-fractionated at 2.0 M and 3.0 M NaCl. When the second pepsin treatment was performed at 8°C, we obtained only a 2.0 M NaCl precipitate. No material was precipitated by 3.0 M NaCl. Electrophoresis of this second 2.0 M NaCl precipitate (Fig. 6) revealed the presence of X_3 and X_4, which were not degraded by pepsin at 8°C. X_2 was at least partially digested by the enzyme at this temperature, for in the presence of dithiothreitol (DTT), the second 2.0 M NaCl precipitate lacked for X_2R (Fig. 6) arising from the reduction of X_2. Furthermore, in the absence of DTT, electrophoresis of the 2.0 M NaCl precipitate showed a broadening of the bands corresponding to X_2 and X_3. When the second

TABLE I. Analysis by Differential Scanning Calorimetry (DSC) and Electrical Birefringence of Type II Collagen, 2.0 M and 3.0 M NaCl Precipitates Dissolved in 0.1 M Acetic Acid

	DSC		Electrical birefringence	
	T_d[a] (°C)	ΔH[a] (J/mg collagen)	τ[a] (μsec)	Length (nm)
Type II collagen	39.5	6.15×10^{-2}	173	276
2.0 M NaCl precipitate	1st peak 29.8	0.58×10^{-2}	2	62
	2nd peak 35.5	2.6×10^{-2}	7	95
3.0 M NaCl precipitate	42.8	2.8×10^{-2}		

[a]T_d = denaturation temperature; ΔH = enthalpy change; τ = relaxation time.

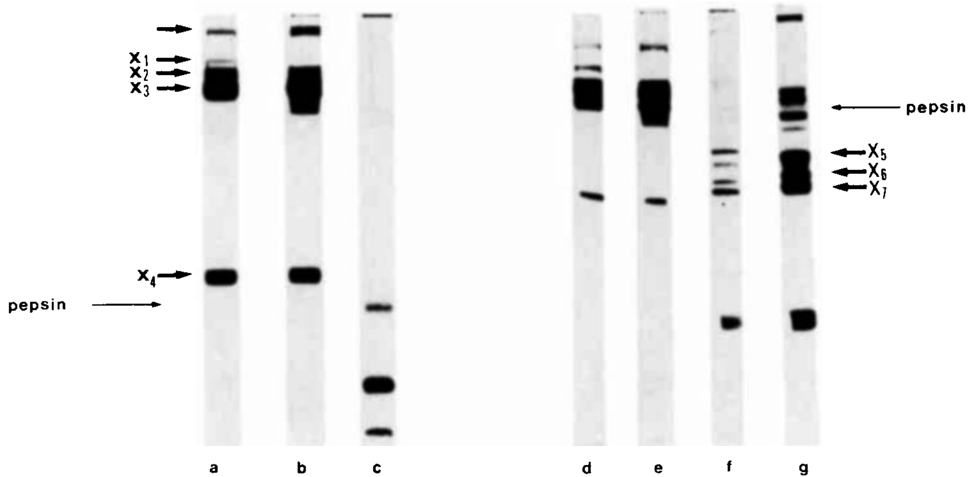
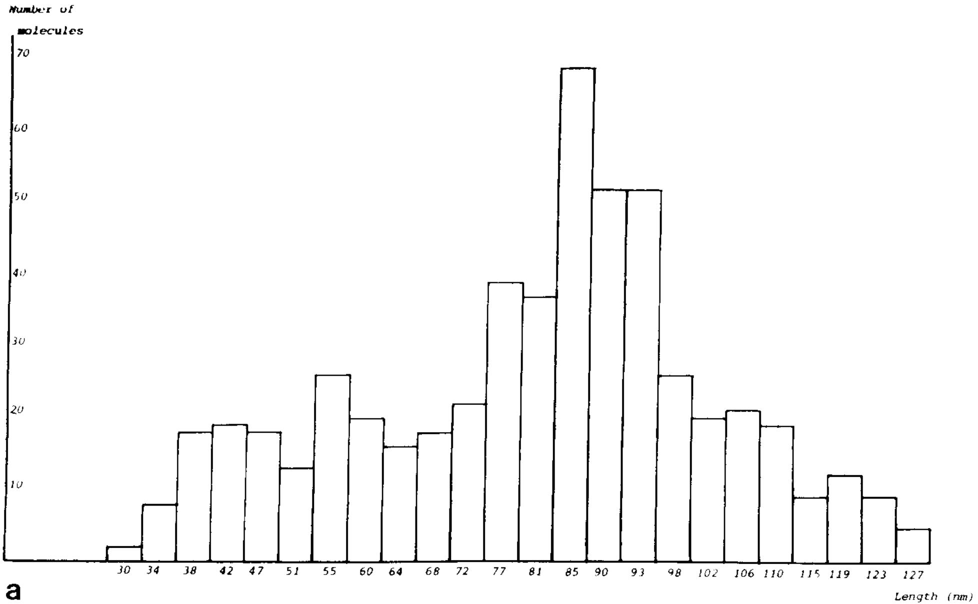


Fig. 6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (6.75% gel: a, b, c; 10% gel: d, e, f, g) of the 2.0 M NaCl precipitate (a) before the second pepsin treatment, (b) after the second pepsin treatment at 8°C, and (c) after the second pepsin treatment at 30°C. d. As in lane a + DTT. e. As in lane b + DTT. f. As in lane c + DTT. g. SDS-PAGE of the 3.0 M NaCl precipitate after the second treatment at 30°C.

pepsin treatment was performed at 30°C, the prominent precipitate was the 3.0 M NaCl, and only a barely visible precipitate was obtained at 2.0 M NaCl. These two precipitates contained $X_5X_6X_7$ and also several peptides probably arising from the digestion of X_1–X_4 by the enzyme. It should be noted that after the second pepsin treatment at 30°C, the $X_5X_6X_7$ fraction was not digested and was present in higher amounts than in the initial 2.0 M NaCl precipitate.

A microscopic approach, using a rotary shadowing technique, allowed the visualization of the form, length, and association of collagen molecules. Figure 7a shows a histogram of the length of the molecules present in the 2.0 M NaCl precipitate. The lengths varied from 30 to 140 nm, with at least two predominant groups: 40–60 nm and 80–100 nm. With respect to the molecular form, we observed at one end of the molecules some small hook (Fig. 8a). A kink was only observed on



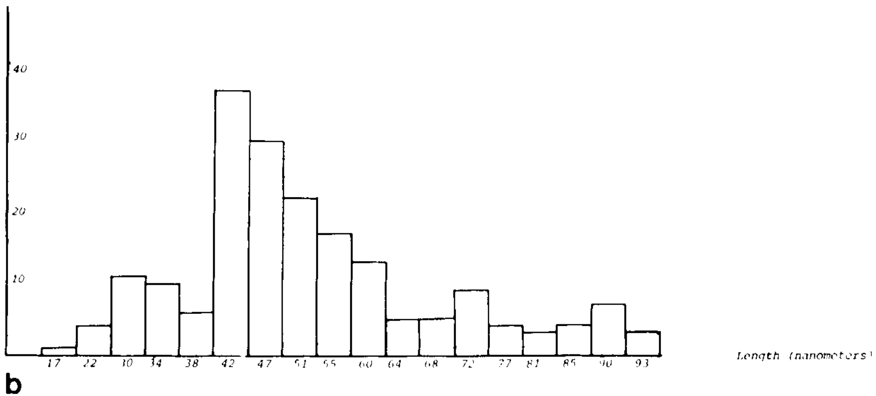


Fig. 7. Histograms of the molecular lengths measured by the rotary shadowing technique (a) in the 2.0 M NaCl precipitate and (b) in the 3.0 M NaCl precipitate.

some molecules whose length was superior to 100 nm. The histogram of the lengths of the 3.0 M NaCl precipitate is shown in Figure 7b. They were dispersed in a single class of 40 to 55 nm. Only stiff rod forms with any inflexion point can be seen in Figure 8b.

Molecular lengths were also measured by means of the electrical birefringence technique. The variation of birefringence against time is shown in Figure 9. For type II collagen molecules, a plot of the logarithm of the birefringence against time gave a straight line. From the slope of the straight line, assuming a cylindrical rod model and according to Burgers's formula, we obtained a molecular length of 276 nm (Table I). With the collagen molecules of the 2.0 M NaCl precipitate, birefringence decay was a two-relaxation-step process. Essentially, two lengths were measured, confirming the existence of two groups whose lengths were shown by the rotary shadowing

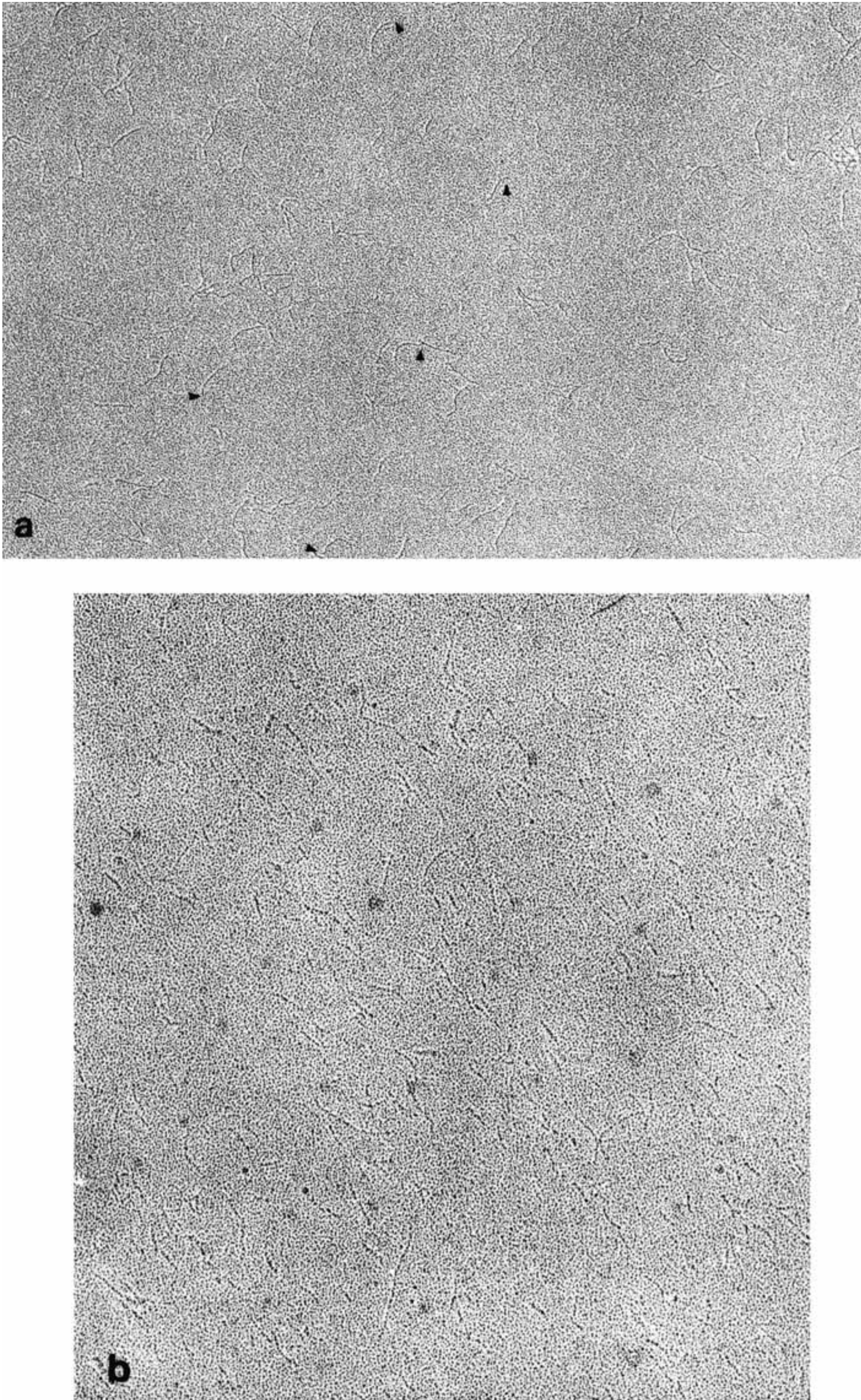


Fig. 8. Rotary shadowing images (a) of the 2.0 M NaCl precipitate (arrowheads show a small hook at one end of the molecule) and (b) of the 3.0 M NaCl precipitate. a,b: ×90,000.

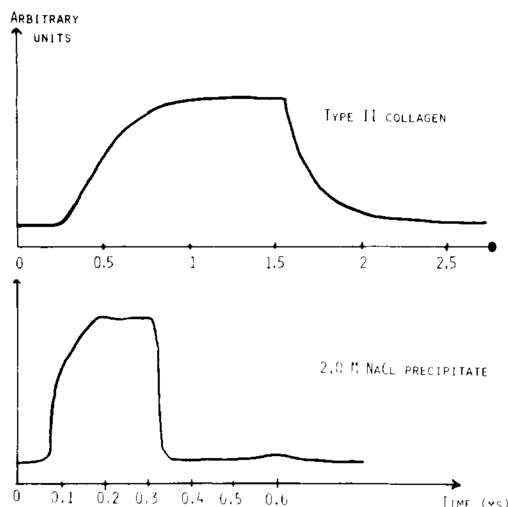


Fig. 9. Electrical birefringence pattern of type II collagen and 2.0 M NaCl precipitate dissolved in 0.1 M acetic acid. Experimental conditions as in [20, 21].

technique: about 52% of the solution birefringence arose from molecules 95 nm long, and the remainder came from molecules 62 nm long.

DISCUSSION

From the biochemical similarities observed between the minor disulfide-collagens extracted from different cartilaginous tissues from different animal species and called M [2,3], CPS [4,5], HMW, LMW [6,7], M_1, M_2 [8], or X_1–X_7 [9], it is tempting to assemble all these collagens under a single name: type IX collagen, as proposed by Mayne et al [10]. The biochemical and physicochemical results presented in this paper are in accordance with this hypothesis. We propose for the X_1–X_7 collagen extracted from foetal calf cartilage a parent molecular form, containing the different collagenous fragments (X_1–X_7) present in the pepsin digest, which is very similar to the model proposed for type IX collagen from chicken cartilage and deduced from the results of Ninomiya and Olsen [16] and Reese and Mayne [6].

X_2 gives rise, by reduction, to a single component, X_2R, of apparent M_r 50,000. Thus, the X_2 fragment is probably composed of three X_2R, chains of molecular weight 50,000, linked by interchain disulfide bonds to form a triple helix.

X_3 is the major component of the 2.0 M NaCl precipitate with a molecular weight of 125,000 and a length varying in the 80–100-nm range. By reduction, X_3 gives rise to three major peptides—X_3R_1, X_3R_2, and X_3R_3—of apparent M_r 40,000, 37,000, and 31,000, respectively.

As shown by two-dimensional electrophoresis after V_8 protease cleavage, X_2 and X_3 give rise to several identical peptides. So X_2 and X_3 contain a portion of common sequence and are related molecules. Furthermore, in some preparations of the 2.0 M NaCl precipitate X_1 and X_2 are present only in small amounts or are altogether absent, whereas X_3 is always present. The existence of X_1 and X_2 seems to be dependent on the conditions of pepsin treatment. Thus X_1 and X_2 are probably higher M_r forms of X_3, containing further pepsin-sensitive sites with regard to X_3.

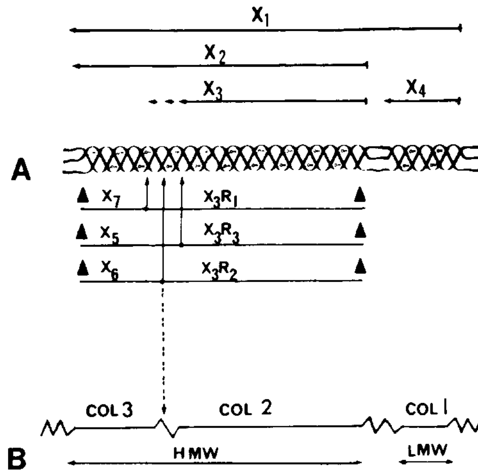


Fig. 10. A) Tentative model of X_1–X_7 collagen deduced from our experiments. B) Model proposed by Ninomiya and Olsen [16].

$X_5X_6X_7$ are involved in triple helical structure (as shown by salt-fractionation) with a higher thermal stability compared to the other fragments and with a length of 40–55 nm.

When the second pepsin treatment was performed at 30°C, X_1–X_3 were completely digested by the enzyme, whereas we observed an increase in the amounts of $X_5X_6X_7$.

This result suggests that X_5–X_7 are pepsin-resistant portions of the whole structure.

According to the M_r of X_5–X_7 and X_3R_1, X_3R_2, X_3R_3, we observe that $X_5 + X_3R_3 = 50{,}500$; $X_6 + X_3R_2 = 52{,}000$; and $X_7 + X_3R_1 = 53{,}000$, which is similar to the molecular weight of X_2R (50,000).

From these results we propose the following model (Fig. 10A):

The $X_5X_6X_7$ fraction and the X_3 fraction were separated by regions containing pepsin-sensitive sites as shown for type IV collagen. These sites were located at different levels of the molecule and should correspond to the hooks observed using the rotary shadowing technique. To explain our results we have to admit that these regions are not entirely cleaved during the first pepsin treatment.

These results can be compared with the recent findings of Ninomiya and Olsen [16]:

•X_1 should correspond to HMW + LMW (containing Col 3 + Col 2 + Col 1) and X_2 to HMW (containing Col 3 + Col 2).

•X_3 (analogous to M [2] and CPS_1 [4]) and the $X_5X_6X_7$ fraction (analogous to CF_1 [3]) may be compared to the triple helical structure containing, respectively, Col 2 and Col 3. Indeed, $X_5X_6X_7$ exhibits a high thermal stability, similar to that found for the short arm of HMW [11], and a high amino acid content (325 residues/1,000) [9] close to the 290 residues/1,000 found in Col 3 [16]. However, contrary to the results found in chicken cartilage, the X_3 and $X_5X_6X_7$ fractions are composed of helical fragments of different lengths, and (Fig. 10B) the similarity of the M_r of X_6 and X_3R_2 (15,000 and 37,000, respectively) to the M_r of the subunits of HMW

(14,000 and 36,400) is worth noting. The other subunits present in X_3 (X_3R_1 and X_3R_3), just as X_5 and X_7, could come from the cleavage of the parent molecule (or molecules) in different pepsin-sensitive regions.

•The sum of the molecular weights of X_4 and X_2 (31,000 + 150,000) corresponds approximately to the molecular weight of X_1 as determined by polyacrylamide gel electrophoresis (180,000) [9]. As we have shown that X_4 is not related to X_2 and X_3, by analogy with the results of Ninomiya and Olsen [16], we can tentatively propose that X_4 may be separated from X_2 by noncollagenous domains, which are always destroyed by the pepsin treatment performed during extraction.

In conclusion it seems that type IX collagen present in calf cartilage is similar to type IX collagen extracted from chick cartilage with some differences in the pepsin-sensitive sites present in the molecule. The exact nature of type IX collagen remains unclear: the presence of one or several molecules containing one or several genetically different chains has yet to be determined.

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