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An Electron Microscopical Study of the Influence of Different Glycosaminoglycans on the Fibrillogenesis of Collagen Type I and II in vitro

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Summary. Proteoglycans (PG) and glycosaminoglycans (GAG) bind to collagen, and thus influence fibril formation. Polysaccharides interfere with the aggregation of collagen molecules and affect pattern formation.

The morphological structure of type I and type II collagen was studied after adding different GAG to collagen solutions in test tubes in vitro. Electron microscopical investigations suggest that sulfated GAG change the aggregation behaviour of collagen molecules. Thus, the cross-striation pattern is changed. This effect seems to be based on the degree of sulfatation and not on the molecular weight of the GAG. Furthermore, GAG appear to have a stabilizing influence on the in vitro fibril formation.

Key words: Electron microscopy – Effect of various GAG on fibril formation in vitro – Collagen structure

Introduction

Proteoglycans (PG) and glycosaminoglycans (GAG) have been shown to possess numerous morphogenic effects. Directly surrounding the cells, they influence differentiation, proliferation and migration processes in many cell types (Toole 1973; Meier and Hay 1974; Pratt et al. 1975; Slavkin and Greulich 1975; Merker et al. 1978). Another important biological function of PG and GAG is based on their ability to bind to collagen. This has been demonstrated in different connective tissues such as skin, cartilage, synovium, cornea, and aorta (Serafini-Fracassini and Smith 1966; Smith et al. 1967; Highton et al. 1968; Smith and Frame 1969; Kajikawa et al. 1970; Myers et al. 1969, 1973; Ruggeri et al. 1975; Myers 1976). The binding of PG and GAG to collagen has also be shown in vitro. It has been suggested, for example, that with the exception of keratan sulfate and hyaluronate, chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin all bind to collagen (Öbrink 1972). Other studies reported by Gelman and Blackwell (1974) indicate that chondroitin-4-sulfate, dermatan sulfate, hyaluronate, and keratan sulfate become attached to collagen in various degrees.

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GAG affect the aggregation of collagen molecules during fibrillogenesis (Wood 1960) and therefore may also influence the aggregation pattern. Thus, aggregation patterns of collagen other than native fibrils can develop, such as segment-long-spacing (SLS) collagen or fibril-long-spacing (FLS) collagen. Electron microscopical studies have demonstrated various cross-striation patterns of SLS and FLS (Kühn and Zimmer 1961; Kühn 1962; Chapman and Armitage 1972; Merker et al. 1978).

The aim of the present study was to examine the effects of different GAG on the collagen structure during fibril formation with special attention to the morphological changes, by means of electron microscopy. Since GAG are weakly permeable substances, this work was done using in vitro methods.

Materials and Methods

Collagen. Collagen extraction and purification were carried out at 4° C. Collagen type I was prepared from bovine aorta (media). After salt extraction (Chung and Miller 1974) and subsequent acetic acid extraction the insoluble collagen was suspended in 0.5 M acetic acid and incubated with pepsin (Serva, Heidelberg, Germany) for 48 h. The pepsin solubilized collagen was precipitated with 5% NaCl, redissolved in 0.5 M acetic acid and neutralized. The collagen was washed twice by precipitation with 20% NaCl, 0.03 M Tris-HCl (pH 7.5) and finally dissolved in 0.2 M NaCl, 0.03 M Tris-HCl (pH 7.5). The solubilized collagen was subjected to a fractionated precipitation with 1.2 M NaCl, 1.5 M NaCl, 1.7 M NaCl and 2.5 M NaCl (Trelstad et al. 1976a). Type I was precipitated with 2.5 M NaCl, dissolved in 0.5 M acetic acid, dialyzed against 0.15 M acetic acid and lyophilized. The purified collagen type I was characterized by 5% SDS-polyacrylamide gel electrophoresis (Fig. 1).

Collagen type II was prepared from rat chondrosarcoma and salt-extracted according to the method of Smith and Martin (1975). From the time the tumors became visible the rats were given 0.3% β-aminopropionitrile fumarate (βAPN) (3 g/kg food) until the tumors were removed. Collagen type II was characterized by 5% SDS-gel electrophoresis (Fig. 1).

Type II collagen from chicken cartilage was extracted according to von der Mark et al. (1976). This material was a gift from Dr. Klaus von der Mark (Max-Planck-Institut für Biochemie, Martinsried, München, Germany).

Glycosaminoglycans. The following GAG were used: non-sulfated hyaluronate (Miles Research Products Division), naturally occurring, normally sulfated chondroitin-4-sulfate (whale cartilage; Sigma, München, Germany), chondroitin-6-sulfate (shark cartilage; Sigma, München, Germany), chondroitin-4-6-sulfate (bovine trachea extracted according to the method of Stuhlsatz and Greiling 1976b), keratan sulfate (bovine cornea; Greiling and Stuhlsatz 1966), and dermatan sulfate (pig skin; Stuhlsatz and Greiling 1976a). Chondroitin-4-6-sulfate, keratan sulfate and dermatan sulfate were kindly provided by Prof. H. Greiling, Aachen, Germany. Besides the normally sulfated GAG, synthetic, highly sulfated GAG were used: SP54 (pentosan-polysulfoester, molecular weight 2,000 daltons, 3,8 SO_4/disaccharide; Benechemie, München, Germany), Arteparon (mucopolysaccharide polysulfoester, molecular weight 12,000 daltons; Luitpold-Werke, München, Germany) and SMC (mucopolysaccharide polysulfoester; Dr. Rentschler Arzneimittel, Laupheim, Germany).

Formation of Segment-Long-Spacing Crystallites (SLS) with ATP in vitro (Stark and Kühn 1968; Modified). 0.6 mg/ml collagen was dissolved in 1% acetic acid at +4° C and dialysed against 3% acetic acid for 8 h. The solutions were dialysed (+4° C) against 0.4% adenosine triphosphate (ATP) (Sigma, München, Germany) in 1% acetic acid (pH 2.8) for 16 h.

In vitro Formation of Collagen Fibrils and SLS After the Addition of GAG. The lyophilized collagen preparations were dissolved at a concentration of 1.2 mg/ml in 0.5 M acetic acid (pH 2.8), centrifuged at 165,000 × *g* for 1 h (starting material) and dialysed against 0.1 M citrate buffer (pH 4.3) for 20 h. All steps were carried out at +4° C. The following concentrations of GAG in citrate buffer (pH 4.3) were used: 1.2 mg/ml, 0.6 mg/ml, 0.06 mg/ml and 0.012 mg/ml. In most experiments, howev-

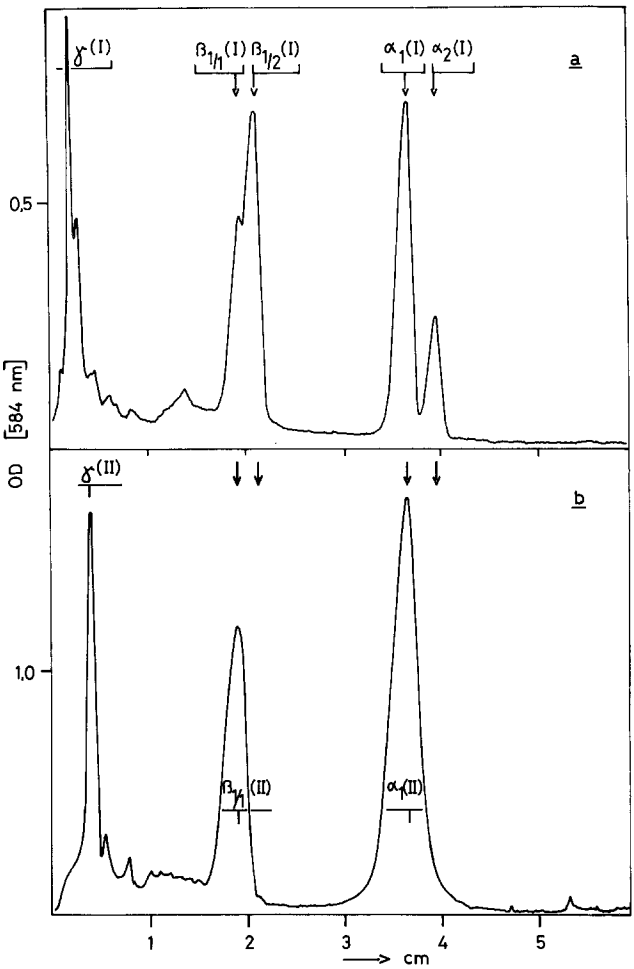


Fig. 1 a and b. Electrophoresis of collagen fractions on 5% SDS-polyacrylamide flat gels (by Maurer 1971; Davies gel system 1). **a** Type I collagen isolated from mouse skin; **b** type II collagen prepared from rat chondrosarcoma. After the run the gels were stained with coomassie brilliant blue and cut in strips. Subsequently the protein bands were measured densitometrically with a Gilford Spectrophotometer Type 240 at 584 nm

er, the GAG were used at concentrations of 0.06 mg/ml and 0.012 mg/ml. The collagen and GAG solutions were mixed at a ratio of 1+1. After mixing collagen and GAG and slowly stirring the solutions for 3 h (+4° C) the in vitro fibril formation was induced by dialysis against a large volume of 0.02 M $Na_2HPO_4 \cdot 2H_2O/KH_2PO_4$ (pH 7.0) for 48 h at +4° C. Electron microscopical observations were made from the starting material, after slowly stirring the collagen solutions with GAG for 3 h, and after dialysis against Na-/Ka-buffer. GAG was not added to the control experiments.

Electron Microscopy. Formvar-coated copper grids were used. For visualization in the electron microscope, suspensions of long-spacing collagen and native collagen fibrils were negatively stained with a solution of 2% phosphotungstic acid, pH 7.0, for 10 min. For positive contrast, the preparations of long-spacing collagen were stained by application of a solution of 0.5% phosphotungstic

acid, pH 3.5, for 10 min and 1% uranyl acetate, pH 4.0 for 15 min. Native-type fibres were positively stained like the long-spacing collagen except that the phosphotungstic acid had a concentration of 2%. All specimens were examined with a Siemens Elmiskop 101.

Results

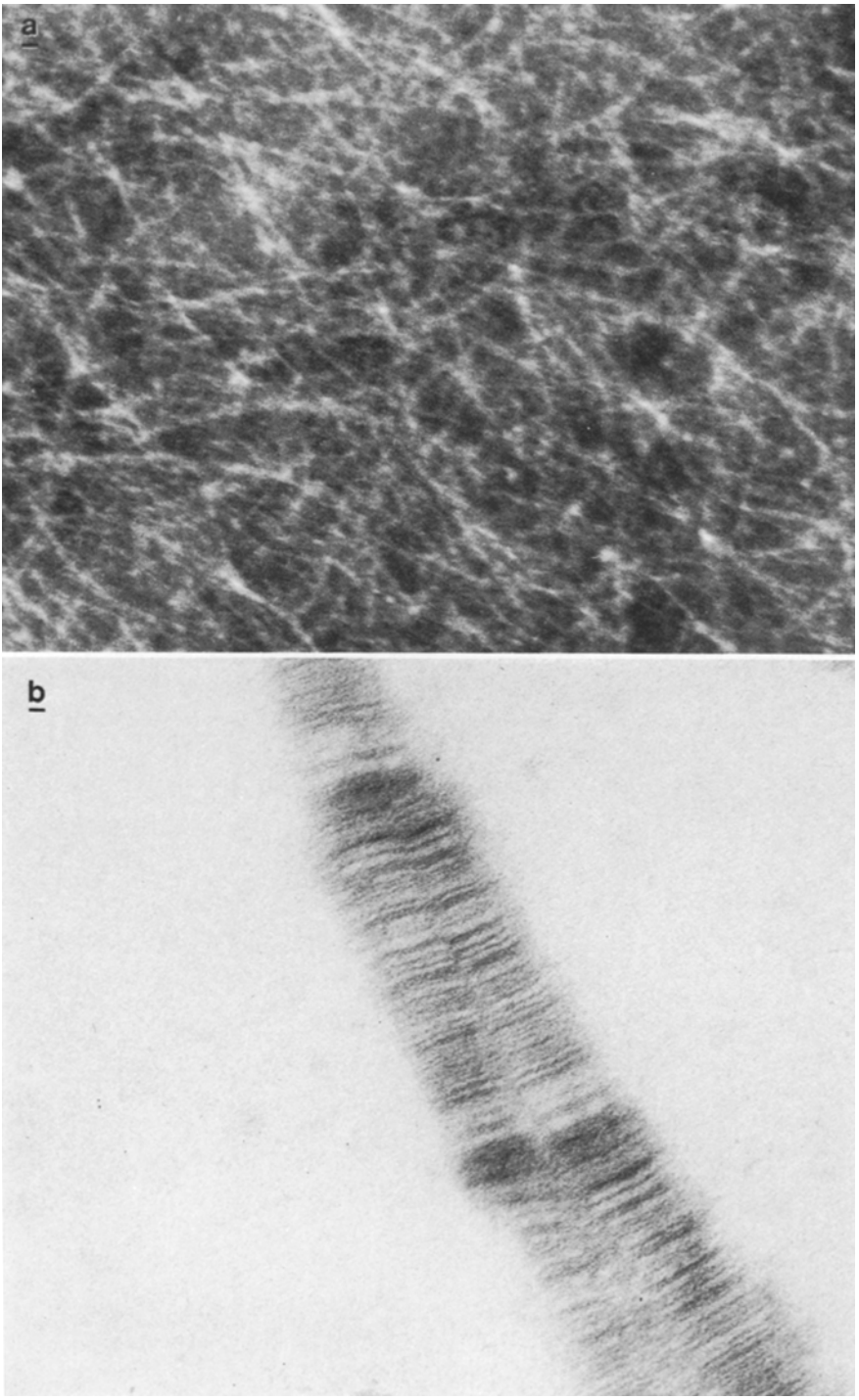
(1) Appearance of Starting Material. Electron microscopical visualization of the collagen solutions in acetic acid after centrifugation at 165,000 × *g* at +4° C for 1 h (starting material) revealed thin filaments without cross-striation (Fig. 2a). These filaments were seen in type I and type II collagen solutions. Diameters between 40 Å and 180 Å were measured.

(2) The Effect of Normally Sulfated GAG After 3 h Incubation (+4° C). Addition of naturally occurring, normally sulfated GAG and the non-sulfated hyaluronate, independent of the concentrations, resulted in thin filaments with diameters of 40 Å–180 Å and no cross-striation. No difference between type I and II collagen could be seen. Control experiments gave identical results.

(3) The Effect of Highly Sulfated GAG After 3 h Incubation (+4° C). If the synthetic, highly sulfated GAG were used in place of the normally sulfated GAG, segment-long-spacing collagen (SLS) was produced. Differences between typ I and II collagens and between the two type II collagen preparations were observed. Type I collagen formed SLS fibrils with a diameter of 300–450 Å. These fibrils showed a tail-to-head (C–N) connection (Fig. 2b) with the ends of the segments overlapping with 300 Å. Dialysis against ATP results in similar fibrils (Fig. 3). Type II collagen from rat chondrosarcoma formed SLS aggregates (500–800 Å diameter) which continue tail-to-tail (C–C) and head-to-head (N–N). The ends of the single segments overlap with 850–900 Å (C–C) and 1,150–1,200 Å (N–N) (Fig. 4 and 5a). Type II collagen from chicken cartilage formed a different aggregation pattern. SLS fibrils (C–C, N–N), about 1,200 Å thick, overlap at the C-terminals with 900 Å, at the N-terminals with 100 Å (Fig. 5b and 6).

Besides the SLS collagen we could detect thin filaments without cross-striation. The ratio of SLS and thin filaments was dependent upon the type and dose of GAG used in the preparation. With lower concentrations of the highly sulfated GAG more thin filaments were formed. As compared to SP54 and Arteparon, the addition of SMC resulted in more filaments and less SLS.

Fig. 2. a Thin filaments from type I collagen after centrifugation at 165,000 × *g* (+4° C) for 1 h (starting material). The filaments show no cross-striation. Negative staining with 2% phosphotungstic acid (pH 7.0). × 140,000. **b** Type I collagen SLS fibrils with tail-to-head connections (*C-N*) and an overlapping length of 300 Å. These fibrils have formed after addition of SMC in high doses (0.3 mg/ml) and dialysis (+4° C) against Na-/Ka-buffer; they are identical to fibrils formed after 3 hours incubation of SMC with type I collagen. Positive staining with 0.5% phosphotungstic acid (pH 3.5) and uranyl acetate (pH 4.0). × 220,500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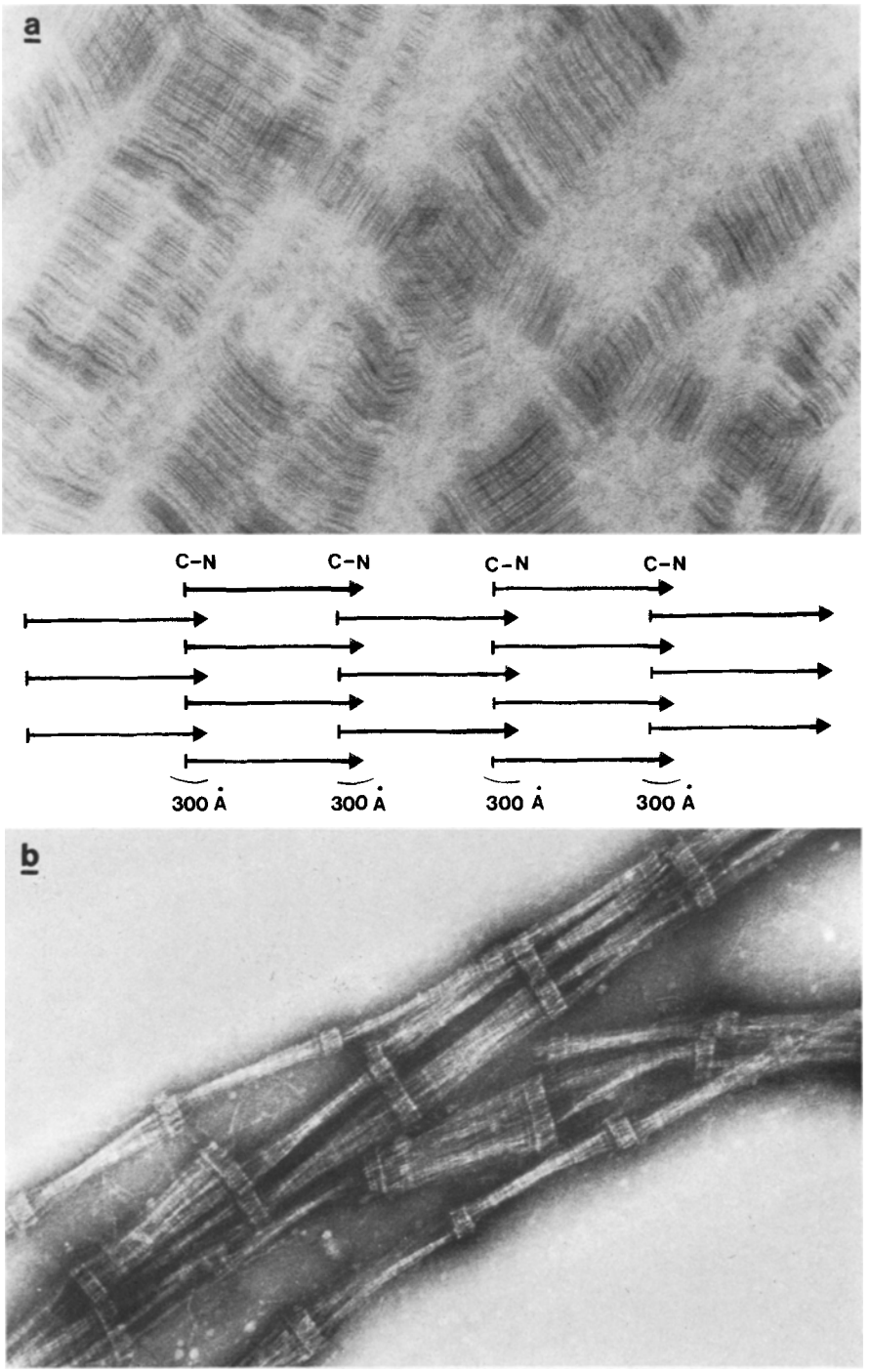
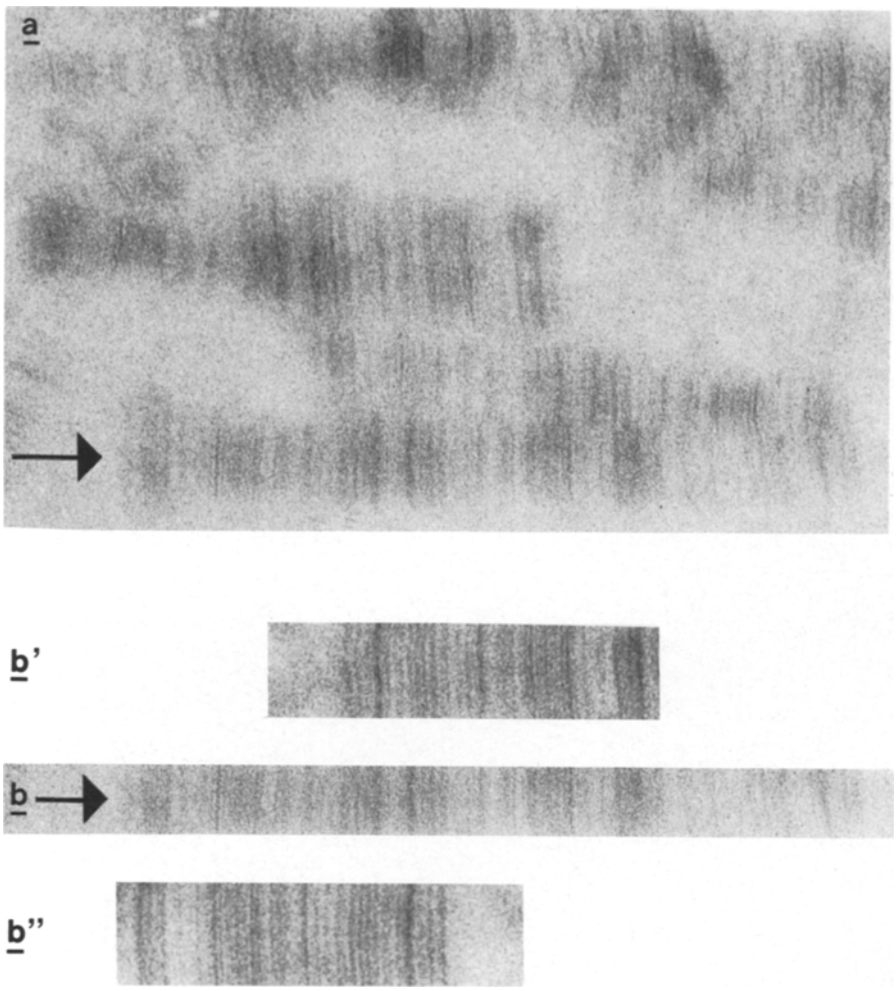


Fig. 3. a Tail-to-head (*C–N*) connected SLS fibrils (type I collagen) formed after dialysis against 0.4% ATP. Positively stained with 0.5% phosphotungstic acid (pH 3.5) and 1% uranyl acetate (pH 4.0). ×90,000. **b** Negative staining (2% phosphotungstic acid, pH 7.0) reveals an overlapping length of 300 Å. ×90,000. Between **a** and **b** a schematic presentation of the arrangement of the collagen molecules



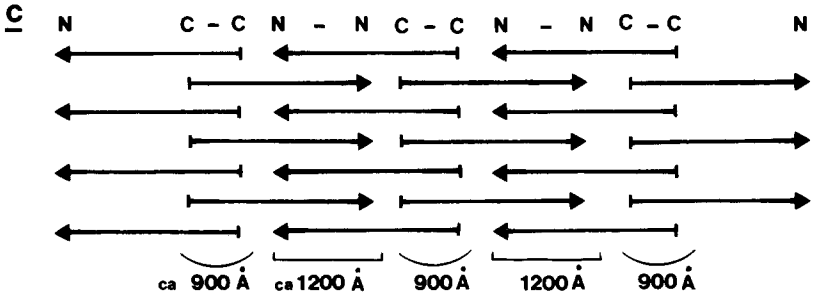


Fig. 4. **a** SLS aggregates formed by type II collagen from rat chondrosarcoma after 3 h incubation with Arteparon in high doses (0.03 mg/ml). ×160,000. **b** Section of a) →. The aggregate shows a *C–C* and *N–N* joining. The terminal contact area of the *C–C* connection overlaps with 850–900 Å, the *N–N* connection with 1,150–1,200 Å. **b′**, **b″** (photo montage). The cross-striation pattern of a single SLS crystallite from type II collagen (rat chondrosarcoma) indicates the overlapping area of the C-terminal (850–900 Å). The ends of a single SLS crystallite were turned by 180° and compared with the cross-striation of the aggregate (**b**). **a**, **b**, **b′**, **b″**, positively stained with 0.5% phosphotungstic acid (pH 3.5) and 1% uranyl acetate (pH 4.0). **c** Schematic presentation of the arrangement of the collagen molecules

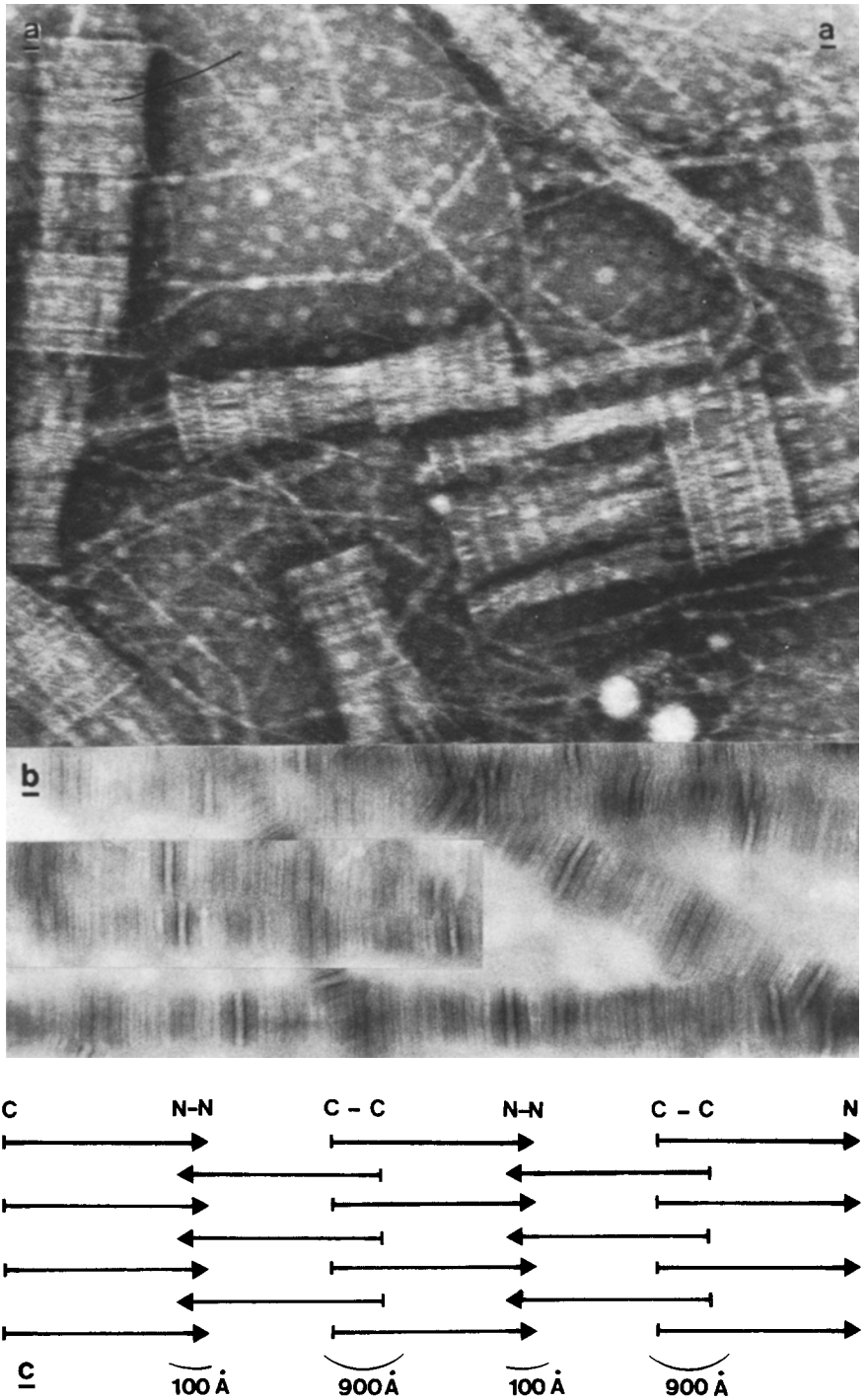


Fig. 5. a Negative staining (2% phosphotungstic acid, pH 7.0) of SLS aggregates from type II collagen (rat chondrosarcoma). These structures have formed after 3 h influence of Arteparon in high doses (0.03 mg/ml). The overlapping lengths were determined to be 850–900 Å (*C–C*) and 1,150–1,200 Å (*N–N*) (compare with Fig. 4). ×168,000. **b** SLS fibrils of type II collagen from

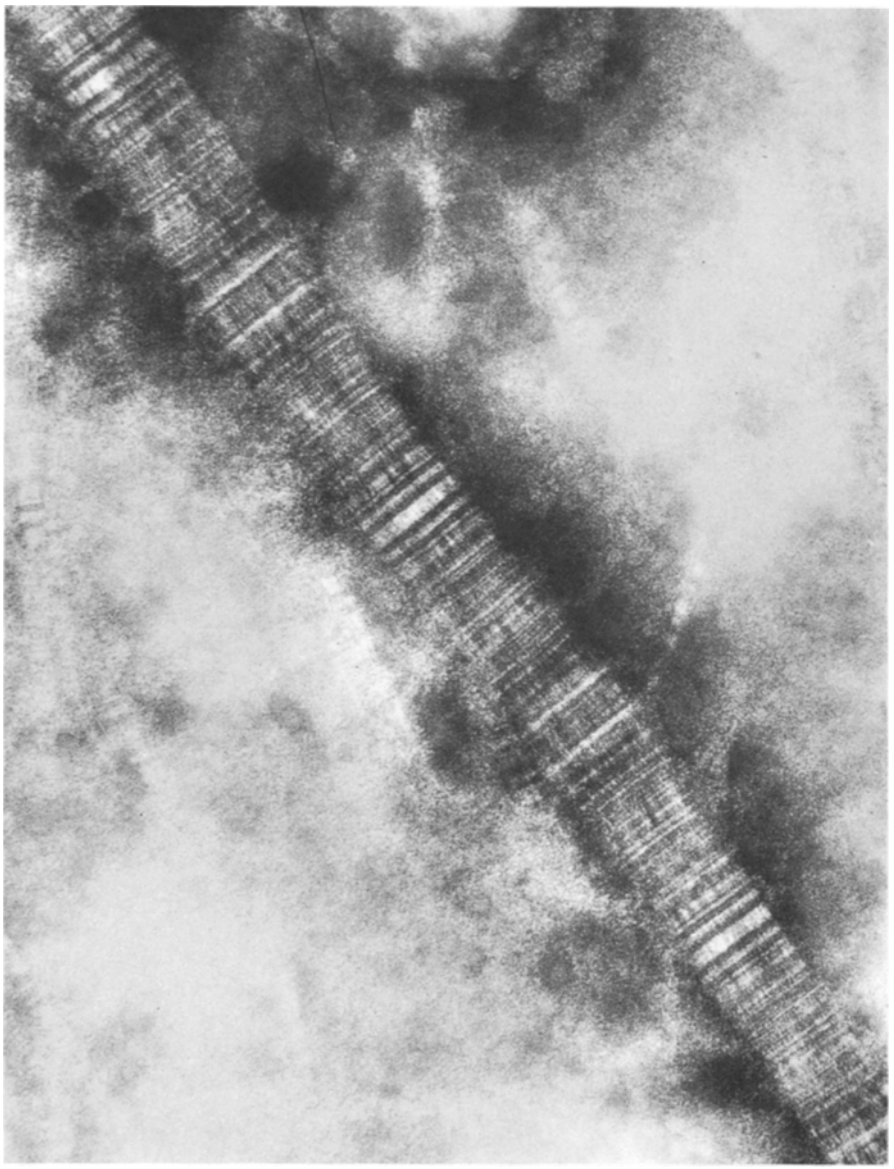


Fig. 6. Negatively stained (2% phosphotungstic acid, pH 7,0) SLS fibril of type II collagen from chicken cartilage. Formation in the presence of Arteparon (0.03 mg/ml) for 3 h (+4° C). Overlapping areas: 900 Å (*C–C*) and 100 Å (*N–N*) (compare with Fig. 5b, c). ×140,000

chicken cartilage. These fibrils resulted after 3 h influence of Arteparon (0.03 mg/ml). They show a tail-to-tail (*C–C*) and head-to-head (*N–N*) connection. The *C*-terminals overlap with 900 Å, the N-terminals with 100 Å. Positive staining: 0.5% phosphotungstic acid (pH 3.5), 1% uranyl acetate (pH 4.0). ×74,000. **c** Schematic arrangement of the collagen molecules from b

(4) In vitro Fibril Formation by Dialysis (+4° C). After dialysis against phosphate buffer the higher doses of all GAG (with the exception of hyaluronic acid) generated mostly SLS collagen and sometimes a few native collagen fibrils. The lower doses of the normally sulfated substances formed mostly fibrils with a native cross-striation pattern. Using lower concentrations of the highly sulfated SP54 and Arteparon, SLS collagen and native fibrils could be seen, whereas SMC mainly resulted in native fibrils. The presence of high doses of hyaluronic acid (final concentration 0.6 mg/ml) always resulted in fibrils which showed the cross-striation of native collagen. In the electron microscope these fibrils revealed a poor contrast, which was also noticed in fibrils from control experiments where no GAG were added. All native fibrils formed in the presence of GAG showed a good contrast.

The SLS aggregates formed revealed the same end-to-end joining and overlapping pattern as described (Results (3)). Thus collagen type I, type II from chondrosarcoma and type II from chicken cartilage showed identical cross-striation patterns as seen after 3 h incubation of the highly sulfated GAG and collagen solutions.

Discussion

Addition of various GAG, especially the highly sulfated SP54 and Arteparon, to collagen solutions in vitro results in unusual collagen structures. These highly sulfated substances influence the collagen molecules to aggregate in a way not normally observed. This is probably due to the firm electrostatic forces between the highly sulfated GAG and the polar groups of the collagen molecules (Kühn et al. 1959; Öbrink et al. 1975; Oegema et al. 1975). Segment-long-spacing collagen (SLS) with asymmetrical cross-striation is formed. Differing end-to-end connections and lengths of overlapping of single crystallites, forming longer aggregates, result in various cross-striation patterns. We could see no FLS aggregates with symmetrical cross-striation. It has been reported that addition of GAG to collagen solutions after dialysis results in FLS (Kühn and Zimmer 1961; Kühn 1962; Highberger et al. 1959). In our experiments both type I and II collagens produced SLS aggregates. When precipitating type I collagen with ATP (Stark and Kühn 1968, modified) we observed almost exclusively SLS fibrils (Fig. 3) and hardly any single SLS crystallites. These fibrils displayed the same cross-striation pattern as was seen after the addition of GAG (Fig. 2b). Thus, head-to-tail (C–N) polymers with an overlapping length of 300 Å were observed. This may be explained by the extraction procedures of type I collagen: the non-soluble collagen was treated with pepsin, which breaks the intermolecular side-to-side bonds but not the head-to-tail cross-linkages (Kühn et al. 1966). These cross-linkages connect the terminal regions of the collagen molecules in native fibrils and are located in the area of the 300 Å overlap of the molecules (Kühn et al. 1966; Zimmermann et al. 1970). Thus, the collagen solution prepared contains polymeric collagen molecules, which, when dialysed against ATP (or GAG), aggregate like monomeric molecules, i.e. in a parallel fashion and with their ends in line. Since the head-to-tail bonds are not broken, SLS fibrils with an overlapping length of 300 Å are formed. Dialysis of type II collagen

against ATP always resulted in single SLS crystallites. In the presence of the various GAG, collagen type II molecules formed SLS aggregates but never FLS. We do not know the explanation for this phenomenon. The origin of collagen and the different extraction procedures possibly play a role.

After centrifugation at $165{,}000 \times g$ for 1 h (+4° C) the freshly dissolved collagen solutions (starting material) always exhibited thin filaments without cross-striation electron microscopically. As diameters between 40 Å and 180 Å were measured, one can assume that molecules in monomeric form are hardly present. It has been shown that only lathyritic collagen can be dissolved into monomer tropocollagen molecules, whereas non-lathyritic preparations always revealed polymeric forms in spite of prolonged centrifugation (Öbrink 1972; Trelstad et al. 1976b). In our experiments type II collagen from rat chondrosarcoma showed filaments of the same width as the other preparations, although the rats were fed with β-aminopropionitrile (βAPN).

After 3 h incubation (at +4° C) of collagen solutions with the various GAG, we showed that only the synthetic, highly sulfated GAG change the aggregation pattern of the collagen molecules. Thus, SLS collagen is formed, whereas naturally occurring, normally sulfated GAG produce only thin filaments. In the short time of 3 h, well aggregated longer units are formed. These findings correspond to electron microscopic observations in limb bud cultures. It has been shown that addition of SP54 and Arteparon result in fibril-long-spacing collagen (FLS) after only a few hours (Merker et al. 1978). In the formation of SLS aggregates after 3 h, the degree of sulfatation of GAG seems to play an essential role. This is supported by the observation that, with lower doses of highly sulfated GAG, more thin filaments and less SLS were seen. This effect seems to be dependent upon the degree of sulfatation and not upon the size of the molecules. SP54 has a molecular weight of 2,000, the non-sulfated hyaluronate $2 \times 10^5 - 8 \times 10^5$ and chondroitin-4-sulfate about $2 \times 10^4 - 5 \times 10^4$.

The results obtained after dialysis against phosphate buffer also stress the importance of the negative charges of the sulfate. Despite the addition of high doses of hyaluronic acid, the collagen molecules aggregate normally and form native fibrils. Thus, the fibril formation is directed exclusively by polar groups along the collagen molecules. The normally sulfated GAG do not produce SLS after 3 h incubation at +4° C; these unusual collagen structures appear only after dialysis. As in the native fibrils the aggregation of the molecules can be attributed to the slow increase in pH and to the low ionic strength of the buffer used. As mentioned before, the formation of SLS collagen is also directed by the positive collagen molecules and the negative GAG molecules.

Using low doses of GAG, native fibrils and SLS aggregates are produced after dialysis. The negative charges of the sulfate groups are not sufficient to aggregate more than a limited number of collagen molecules to SLS. Thus the rest of the molecules form native fibrils. A dose-dependent decrease in the number of SLS aggregates could be observed, as was the case after a 3 h influence of GAG.

It has been shown electron microscopically that the intensity of the contrast of fibrils and aggregates reflects the aggregation behaviour of collagen molecules (Kühn et al. 1964). Clearly contrasted collagen forms are well aggregated, where-

as poorly contrasted ones are more loosely packed. The native fibrils from our control experiments revealed a poor contrast after dialysis as compared with GAG-influenced native fibrils. Thus the tropocollagen molecules in the untreated fibrils are improperly aggregated. GAG appear to be important for well aggregated native fibrils. Thus GAG seem to have a stabilizing influence on the in vitro fibril formation. Nevertheless, addition of highly sulfated GAG or high doses of normally sulfated GAG change the aggregation pattern of collagen molecules so that abnormal structures are formed.

In the SLS different types of overlapping and end-to-end connections of monomeric segments can be observed. The terminal contact areas of the collagen molecules (C and N terminals) overlap with different lengths. Type I collagen forms tail-to-hed (C–N) polymers with 300 Å overlapping areas. Kühn and Zimmer (1961) have shown SLS fibrils with C–N and N–N connections and an overlapping length of 250–300 Å. Type II collagen resulted in head-to-head (N–N) and tail-to-tail (C–C) aggregates. The overlapping lengths were measured to be 850–900 Å (C–C) and 1,150–1,200 Å (N–N) in type II from rat chondrosarcoma, 900 Å (C–C) and 100 Å (N–N) in type II from chicken cartilage. These two forms have not been reported before. The formation of these different aggregates from type II collagen may be due to the different origin of the material or to different extraction procedures. Not only the various type of end-to-end arrangements but also the different lengths of overlapping cause different cross-striations in the contact regions (Fig. 4).

Many authors have reported on long-spacing collagen formed in pathological tissues in vivo (Luse 1960; Ramsey 1965; Cravioto and Lockwood 1968; Mollo and Monga 1971, Edwards 1975; Imura et al. 1975; Fenoglio and McAllister 1976). These altered collagen structures may be explained by a changed proteoglycan pattern in pathological organs. Thus, these structures would reflect the chemical constitution of the milieu in which they are formed.

Our in vitro experiments may indicate that PG and GAG also in vivo participate in the formation of the morphology of collagen fibrils. Thus the two main components in the extracellular matrix, collagen and PG, may form a functional unit.

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