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The Proteoglycan Content and the Axial Periodicity of Collagen in Tendon

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The glycosaminoglycan content and the axial periodicity of collagen was determined in various regions of the rabbit flexor digitorum profundus tendon. This tendon, which passes from the calf to the toes round the inner side of the ankle, contains a thickened sesamoid-like pad where it is subjected to friction and pressure. Other regions of the tendon are subject only to longitudinal tension. In tensional areas the axial periodicity of collagen was of the order of 62nm and the tissue contained less than 0.2% proteoglycan on a dry weight basis. In the sesamoid-like region, however, the axial periodicity was a significant 13–15% less, and the proteoglycan constituted about 3.5% of the dry weight. Also, in the tensional areas the predominant glycosaminoglycan was dermatan sulphate, whereas in the sesamoid the predominant glycosaminoglycan was chondroitin sulphate. The possible interrelationships between collagen axial periodicity and proteoglycan content in this tissue are discussed.

Previous publications from our laboratories have shown that a relationship exists between the longitudinal tension imposed on collagen fibres and the availability of fixed positive charges on their surface to negatively charged stains of the Masson trichrome sequence (Flint *et al.*, 1975; Flint, 1976*a*,*b*). A further relationship has also been demonstrated between the tension imposed on the collagen fibres and their axial periodicity when observed electron-microscopically (Flint & Merrilees, 1977). It has been postulated that such conformational changes, and the resultant differences in charge distribution on the collagen molecule, may act as part of a homoeostatic feedback mechanism, which controls the type and amount of proteoglycan synthesized by adjacent cells (Flint *et al.*, 1975; Flint, 1976*a*).

To investigate this hypothesis further, the tendon of the flexor digitorum profundus of the rabbit was studied. This tissue contains discrete regions of both taut collagen and relaxed collagen *in vivo*. It is involved in flexion of the hind-limb toes and extends from muscle in the calf, beneath a bony pulley under the medial side of the ankle, and continues on under the sole of the foot.

When histological sections of the flexor digitorum profundus tendon were stained with the Masson trichrome sequence, it was found that certain regions (A, B and C, Fig. 1*a*) stained red, indicating fibres under tension, whereas the S region (Fig. 1*a*) stained green, indicating that the collagen fibres were relaxed (Flint, 1976*a*). These histological results support a theoretical analysis of the physical forces acting on this tendon, previously published by Ploetz (1938). He deduced that *in vivo*, tension is transmitted longitudinally along the tendon, but that the tendon is subject to pressure on the inner surface as it passes around the bony pulley below the ankle, and that longitudinal tension is greatest on the outside curve of the pulley and least on the inner surface (regions B and S respectively, Fig. 1*a*).

The glycosaminoglycan content and the axial periodicity of the collagen were characterized in the various regions of this tendon.

Materials and Methods

Biological material

For chemical analysis, the flexor tendons were obtained from 27 6–8-month-old New Zealand white rabbits of both sexes, divided into three groups of six and one group of nine. After removal of the paratenon, each tendon was divided into the four regions shown in Fig. 1(*a*). The gastrocnemius component of the tendo Achilles, a tendon subject to tensional forces only, was removed from the same animals for comparative analysis. The flexor tendons were also removed from a further group of six animals and the thickened sesamoid region was divided longitudinally into its three distinct anatomical layers (M. H. Flint & M. J. Merrilees, unpublished work) as shown in Fig. 1(*b*). The S_1 sample was taken from the inner side of the curve at the point of maximum compression, the B_1 sample from the back of the curve, which was tension-transmitting, and the third sample, M, taken from the middle of the sesamoid between the former two.

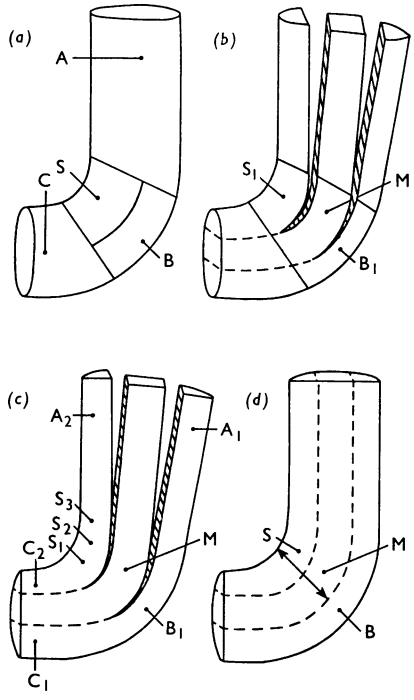

Fig. 1. *Segments of the flexor digitorum profundus tendon of the rabbit used in this study*

Segments shown in (*a*) and (*b*) were analysed for glycosaminoglycan content (*a*, see Tables 1 and 2; *b*, see Table 3). The axial periodicity of collagen fibrils was determined at the positions shown in (*c*) (see Table 4). (*d*) The collagen periodicity was also determined at 13 positions on a single section on a single grid across the S and M regions (↔)and also on a section taken from the B region of the same tendon (see Fig. 2).

Chemicals

Glucosamine hydrochloride, galactosamine hydrochloride, hexamethyldisilazane, trimethylchlorosilane, hyaluronic acid (grade 1), glucuronolactone, Dowex 1 (X2; Cl^- form; 200–400 mesh), Dowex 50 (X12; H^+ form; 50–100 mesh), *Proteus vulgaris* chondroitin ABC lyase and bovine testicular hyaluronidase (type V) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sorbitol was obtained from Chem Service Ltd., Westchester, PA, U.S.A. Pig skin dermatan sulphate was obtained from Hoffman–La Roche, Basel, Switzerland. Chondroitin sulphate was prepared from bovine nasal cartilage by papain digestion and cetylpyridinium chloride precipitation (Scott, 1960). The inert support, Diatoport S (80–100 mesh) and the stationary phase, UC W982, used in g.l.c., were obtained from Hewlett–Packard, Avondale, PA, U.S.A. All other chemicals and reagents were of analytical grade.

Methods

Proteolytic digestion of tissues and isolation of glycosaminoglycans. Samples for biochemical analysis were dried to constant weight at 105–110°C and digested with papain (EC 3.4.22.2) as described previously (Gillard *et al.*, 1975). After centrifugation at 2000rev./min on a bench centrifuge, the supernatant was dialysed against running tap water for 24h, followed by two changes of distilled water over a further 24h. Then 5M-NaCl was added to give a final concentration of 0.15M.

Fractionation of glycosaminoglycans on Dowex 1 (X2; Cl^- form). The glycosaminoglycans were fractionated by a modification of the method of Schiller *et al.* (1961). Columns (2–2.5ml bed-volumes) in Pasteur pipettes were equilibrated with 0.2M-NaCl. After application of the samples, the columns were washed with 20ml of 0.2M-NaCl, and initial effluents and 0.2M-NaCl washings were discarded. The glycosaminoglycans were eluted with 2×2ml of 0.75M-NaCl overnight, followed by 3×2ml of 0.75M-NaCl the next morning. These five 2ml batches were collected as a single fraction. This was repeated with 3M-NaCl. Fractions were dialysed as above. Overall recovery of hexuronic acid was over 80%.

Electrophoresis of glycosaminoglycans on cellulose acetate strips. Electrophoresis of fraction contents was carried out in unbuffered 0.2M-$ZnSO_4$ on cellulose acetate strips in a Beckman micro-zone electrophoresis system, and bands of glycosaminoglycan were detected with 1.0% Alcian Blue by the method of Breen *et al.* (1970).

Digestion with chondroitin ABC lyase. Up to 3μg of glycosaminoglycan digested with 0.01 unit of chondroitin ABC lyase (EC 4.2.2.4) in 10μl of Tris/HCl buffer, pH7.8 (0.2M with respect to Tris) at 37°C for 3h.

Digestion with testicular hyaluronidase. Up to 3μg of glycosaminoglycan was digested with 6.25N.F. units of hyaluronidase (EC 3.2.1.35) in 10μl of sodium acetate buffer, pH5.0 (0.05M with respect to acetate, and containing 0.15M-NaCl) at 37°C for 3h. (N.F. units are defined in The National Formulary, 12th edn., p. 96, published by the American Pharmaceutical Association Committee on National Formulary.)

Analytical methods. Hexuronic acid was determined by the method of Bitter & Muir (1962) by using glucuronolactone as standard. To obtain approximate proteoglycan contents, glucuronolactone values attributed to hyaluronic acid were multiplied by 2.15, and those attributed to chondroitin sulphate and dermatan sulphate by 3.3.

Dermatan sulphate was determined by the method of Di Ferrante *et al.* (1971). Pig skin dermatan sulphate was used as standard, and it was assumed that this dermatan sulphate gave the same colour yield

as that of tendon. It was also assumed that the dermatan sulphate was homogeneous throughout the tendon.

Hexosamine analyses were carried out after hydrolysis of samples in 4M-HCl at 100°C for 8h in tubes lightly sealed with marbles. After hydrolysis, acid was removed on a Buchler Rotary Evapomix at 40°C, and the samples were taken up in 1.3ml of water. A portion of this was used for total hexosamine assay by the method of Cessi & Piliego (1960), with galactosamine hydrochloride as standard.

The relative contents of glucosamine and galactosamine were determined by g.l.c. with a modification of the method of Braund *et al.* (1975). A portion of the aqueous solution of the acid hydrolysate containing 5–10μg of hexosamine was dried over P_2O_5 and NaOH pellets *in vacuo* and taken up in 3ml of water. Solutions were run on to columns (2ml bed-volume) of Dowex 50 (X12; H^+ form) previously washed with 2M-HCl and equilibrated with water. The columns were washed with 20ml of water and the hexosamines eluted with 3×1ml of 2M-HCl over a 2h period. As an internal check on retention times, sorbitol (2.5μg) was added. The acid was removed with a Buchler Evapomix and the mixture finally dried over P_2O_5 and NaOH pellets *in vacuo* for 24h. The silyl derivatives were prepared by addition of 25μl or 50μl of a 1:1:1 (by vol.) mixture of hexamethyldisilazane, trimethylchlorosilane and pyridine. After 30min at room temperature (20–30°C) up to 5μl was loaded on to a column (1.2m×3mm) of 3.8% UC W982 on Diatoport S (80–100 mesh) in a Hewlett–Packard 7610A gas chromatograph. A temperature gradient of 140–165°C was used at a rate of change of temperature of 0.5°C/min, with N_2 as carrier gas at a rate of 24ml/min. The system was calibrated by using standard mixtures of glucosamine hydrochloride and galactosamine hydrochloride. Total recoveries of hexosamine were variable, but ratios of the two components were within 5% of the known ratios of the original standard mixtures.

Electron-microscopic methods. The hind limbs of three rabbits were removed at autopsy and fixed *in toto* in a dorsiflexed position for 2 weeks in 10% formalin [4% (w/v) formaldehyde] containing 0.5% (w/v) cetylpyridinium chloride (Pearse, 1968). After fixation, one flexor tendon from each rabbit was removed and thin slivers of tendon were dissected from nine locations on each tendon (Fig. 1*c*). A block of tissue and a thin sliver (S↔M and B respectively, Fig. 1*d*) were also obtained from a fourth flexor tendon. Specimens were further processed in 1% (w/v) OsO_4 for 2h, dehydrated through increasing concentrations of ethanol and propylene oxide and embedded in Epon resin. The blocks were subsequently trimmed to a suitable size and sections were cut across the main axes of the fibre bundles. They were then mounted on 300-mesh grids, stained with either phosphotungstic acid or uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. The axial periodicity of the collagen fibrils was determined by measuring the length of ten axial periods from electron micrographs of constant magnification of 160000×, and calibrated against a catalase crystal standard of periodicity 8.12nm. Periodicity measurements were also made on collagen fibrils prepared from the tendo Achilles from the same animals for comparison.

Results

Qualitative results

Electrophoresis. Micro-zone electrophoresis of fractions from various tendon segments eluted from Dowex 1 (X2) with 0.75M-NaCl revealed a single band comparable in mobility with authentic hyaluronic acid as the only Alcian Blue-staining component in these fractions. This band was not apparent after digestion of the sample with either chondroitin ABC lyase or testicular hyaluronidase. The 3M-NaCl fraction contained a sharp band comparable with authentic dermatan sulphate and a more diffuse band comparable with chondroitin sulphate. The former band remained after hyaluronidase digestion, but neither band was apparent after digestion with chondroitin ABC lyase. No other band of Alcian Blue-staining material was present.

Hexosamine analysis. Hexosamine analyses were undertaken on the fractions eluted from Dowex 1 (X2) from the three groups each of six animals. Molar ratios of hexosamine to hexuronic acid are given in Table 1. Hexosamine analysis by g.l.c. of the 0.75M-NaCl fraction revealed that over 80% of the hexosamine in this fraction was glucosamine. In the 3M-NaCl fractions, traces of glucosamine were present, and hexosamine ratios of these fractions are included in Table 1.

Thus the major glycosaminoglycan containing hexuronic acid in the 0.75M-NaCl fractions was hyaluronic acid, although, from hexosamine analysis, traces of chondroitin and undersulphated chondroitin sulphate could not be ruled out. The hexuronic acid-containing glycosaminoglycans in the 3M-NaCl fractions were chondroitin sulphate and dermatan sulphate and the relative amounts of these were determined by the assay for dermatan sulphate described in the Materials and Methods section. However, from hexosamine analysis, there is a possibility of traces of heparin and/or keratan sulphate in these fractions.

Quantitative results

Results of the hexuronic acid and dermatan sulphate assays carried out on the Dowex 1 (X2)

Table 1. *Hexosamine analysis of the glycosaminoglycans obtained from rabbit tendon segments*
The glycosaminoglycans were prepared from rabbit Achilles tendon (TA) and segments of flexor digitorum profundus (FDP) tendons by papain digestion and eluted from Dowex 1 (X2) resin with 0.75M- and 3.0M-NaCl. Results are molar ratios.

	Total hexosamine / hexuronic acid		Glucosamine / galactosamine	
Sample	0.75M-NaCl fraction	3.0M-NaCl fraction	0.75M-NaCl fraction	3.0M-NaCl fraction
TA	0.91	0.85	8.1	0.12
FDP-tendon segment* A	1.06	0.98	9.0	0.14
B	1.01	0.99	8.1	0.05
C	0.98	0.91	6.7	0.14
S	1.03	0.99	4.3	0.06

* See Fig. 1(*a*).

Table 2. *Hexuronic acid-containing glycosaminoglycans of rabbit Achilles tendon (TA) and segments of flexor digitorum profundus (FDP) tendon*
Samples were prepared by papain digestion and Dowex-1 (X2) chromatography. Total hexuronolactone was determined be summation of the fraction contents. The tendons from 27 rabbits were analysed in four groups (see the Materials and Methods section).

		Percentage of total hexuronolactone attributable to:		
Sample	Total hexuronolactone (μg/mg dry wt.) (±S.D.)	Hyaluronic acid	Chondroitin sulphate	Dermatan sulphate
TA	0.563±0.047	30	13	57
FDP-tendon segment* A	0.487±0.046	33	22	45
B	2.26 ±0.032	55	22	23
C	1.16 ±0.017	55	24	21
S	7.56 ±0.90	22	58	20

* See Fig. 1(*a*).

Table 3. *Hexuronic acid-containing glycosaminoglycans of the sesamoid-like region of the rabbit flexor digitorum profundus tendon*
Samples were prepared by papain digestion and Dowex-1 (X2) chromatography. Total hexuronolactone was determined by summation of the contents of the fractions.

		Percentage of total hexuronolactone attributable to:		
Sample*	Total hexuronolactone (μg/mg dry wt.)	Hyaluronic acid	Chondroitin sulphate	Dermatan sulphate
B_1	1.41	61	23	17
M	3.56	47	33	20
S_1	11.85	20	60	20

* See Fig. 1(*b*).

fractions of the various regions of the flexor tendon (see Fig. 1*a*) are summarized in Table 2 and compared with the results of similar analyses of the tensional segment of the tendo Achilles. The total hexuronic acid content of the tissues was obtained by summation of the contents of the 0.75M- and 3.0M-NaCl fractions. The values in Table 2 demonstrate the similarity in the type and content of glycosaminoglycan in the main tensional segment (A region) of the flexor tendon and the tendo Achilles both in total hexuronic acid content and in the predominance of dermatan sulphate as the major glycosaminoglycan. However, in the thickened sesamoid region there was a 15-fold increase in hexuronic acid content, and the predominant glycosaminoglycan was chondroitin sulphate.

When the sesamoid region was studied in detail by analysis of its three longitudinal component parts (Fig. 1*b*), it was found that the highest concentration of glycosaminoglycan was in the inner pressure-bearing area (S_1) and the least in the tension-transmitting segment (B_1, Table 3). Chondroitin sulphate was the predominant glycosaminoglycan in the pressure-bearing area (S_1), but the proportion in the tension-transmitting segment (B_1) was equivalent to that of the normal tendon.

Table 4. *Periodicity of collagen fibrils sampled from regions of the flexor digitorum profundus (FDP) tendon and the tendo Achilles (TA)*

Electron micrographs were taken at a constant magnification of 160 000×, and a catalase crystal of periodicity 8.12 nm was used for calibration. Results are means ± S.D. for the numbers of determinations in parentheses.

Region of FDP tendon*	Axial periodicity of collagen fibrils (nm)
A_1	61.5 ± 1.8 (76)
A_2	61.8 ± 1.7 (83)
C_1	60.2 ± 3.0 (95)
C_2	62.0 ± 3.3 (132)
B_1	63.1 ± 2.8 (137)
S_1	53.7 ± 3.2 (165)
S_2	56.2 ± 3.5 (79)
S_3	59.4 ± 3.4 (154)
M	59.3 ± 2.6 (111)
TA†	62.1 ± 2.7 (146)

* See Fig. 1(*c*).

† Results from various locations in the TA have been grouped together.

Collagen-fibril periodicity

Results of collagen-fibril periodicity in various regions of the flexor tendon (Fig. 1*c*) and the tendo Achilles are shown in Table 4. In all tensional regions of the flexor tendon and the tendo Achilles, collagen-fibril periodicity approached the normally accepted value of 64 nm. In the sesamoid, however, there was a marked and significant decrease in periodicity, with a mean of 53.7 nm in fibrils sampled from the S_1 region. Further, the gradual and progressive transition from the tensioned fibrils at the back of the sesamoid (adjacent to region B_1 Fig. 1*c*) to the more relaxed fibrils of the inner zone of the sesamoid was accompanied by a progressive decrease in fibril periodicity (Fig. 2). Statistical analysis, by independent unpaired *t* tests, of the axial periodicity values used to plot Fig. 2 confirmed that the differences in periodicity between B and M regions and M and S

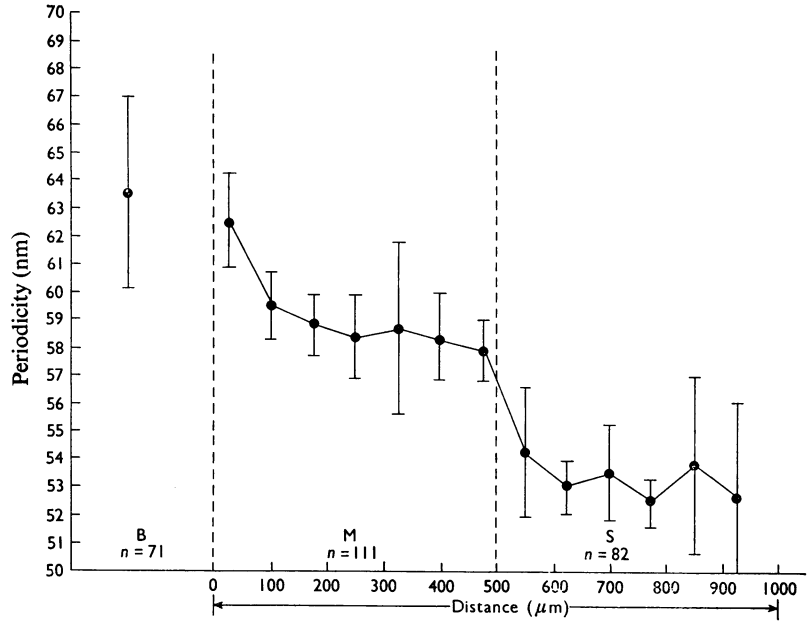

Fig. 2. *Axial periodicity of collagen fibrils sampled across the S ↔ M region of the flexor tendon*

Referring to Fig. 1(*d*), the B region of the tendon was separated from the outer curve of the tendon and measurements of axial periodicity were begun at 25 μm from the new edge of the tendon so produced. Thereafter, periodicity measurements were made at 75 μm intervals across the tendon. Measurements were made from electron micrographs taken at 160 000× magnification and calibrated against a catalase crystal standard of periodicity 8.12 nm.

regions (and, of course, B and S regions) were highly significant ($P<0.001$ in all cases).

Discussion

The tightly packed longitudinally running collagen fibres which form the bulk of the extracellular matrix of tension-transmitting tendon are normally associated with a small amount of proteoglycan (less than 0.2% of the dry weight in adult rabbit tendon and 0.5% in pig and calf tendon; Meyer *et al.*, 1956). The flexor digitorum tendon of the rabbit, however, contains a thickened sesamoid-like region which is also subject to compression and frictional forces where the tendon is in contact with the medial surface of the heelbone as it turns forward from the calf under the sole of the foot. In this thickened region of pressure contact, the proteoglycan content increased to approx. 3.5% of the dry weight of tissue. There is also a significant change in the type of proteoglycan produced by the cells of the pressure area; whereas in the tensional area dermatan sulphate is the predominant proteoglycan, in the pressure-bearing sesamoid segment there is a two- to three-fold increase in the proportion of chondroitin sulphate and a concomitant decrease in the proportion of dermatan sulphate.

It seems likely that the differences in the type and proportion of proteoglycan in the tension- and pressure-bearing segments of the tendon are directly related to the functional needs of the tissue. In the tensional segments, thick collagen fibres of high tensional strength are associated with a small amount of dermatan sulphate-containing proteoglycan, whereas in the pressure-bearing segment of the tendon, the cells produce a proteoglycan-rich matrix which, with its greater water-inclusion properties, is better suited to protect both the cells and collagen from pressure as the tendon is compressed against the bone during movement.

The results of the present investigation show that the proteoglycan of the sesamoid region is similar to, but not identical with, cartilage. Rabbit articular cartilage, for example, contains up to 9% proteoglycan on a dry-weight basis, the major glycosaminoglycans being chondroitin 4- and 6-sulphates and keratan sulphate (Lowther & Gillard, 1976); it contains very little hyaluronic acid (Gillard *et al.*, 1975). However, although the proteoglycan of the pressure-bearing area of the tendon contains predominantly chondroitin sulphate, there are significant amounts of both dermatan sulphate and hyaluronate. The differences in chemical composition between the cartilage-like matrix of the sesamoid segment and articular cartilage are probably related to their difference in function, for, although articular cartilage is almost purely pressure-bearing, the adaptive cartilage-like matrix of the thickened sesamoid segment of the flexor tendon is subject to friction, pressure and some tensional forces as well.

Similarly, although the C region of the flexor tendon (Fig. 1*a* and Table 2) at first seems anomalous in that it contains more proteoglycan than a normal tendon, even though the tendon is straight and collagen fibres transmit tension in a straight line, this part of the tendon is located beneath the foot and is subject to compressive forces during normal weight-bearing. Hyaluronic acid is present in all samples and in the B and C regions (Fig. 1*a* and Table 2), it is the predominant glycosaminoglycan. In paratenon, hyaluronic acid represents about 50% of the relatively large amount of glycosaminoglycan (Reid & Flint, 1974) and it is probably involved in the role of this tissue as a gliding surface. Considering the anatomical location of the B and C regions of the tendon, the hyaluronic acid may have a physiological role similar to that of the paratenon.

Electron microscopy has demonstrated that there are significant differences in the axial periodicity of collagen fibrils in different regions of the tendon. In tensional areas of the flexor tendon and tendo Achilles, where the proteoglycan content is least, the collagen fibres have an axial periodicity of about 62–64 nm, whereas in the pressure-bearing segments, where the proteoglycan content is markedly greater, there is a significant 13–15% decrease in the periodicity of collagen. These differences in axial periodicity also demarcate the regions that were under tension *in vivo*, since Flint & Merrilees (1977) have shown that collagen under longitudinal tension has a longer axial periodicity than relaxed collagen.

Collagen of stretched dermis or normally taut tendon has a greater preponderance of positively charged amino groups available for active dye-binding than has the relaxed collagen of normal dermis or surgically relaxed tendon. It is postulated that changes in the distribution of freely available fixed charges, which are reflected in the differences in axial periodicity and staining, could also be responsible for the piezoelectric effect previously reported (Bassett & Becker, 1962; Bassett, 1971; Fukuda & Yasuda, 1957, 1964) and perhaps for the changes in cellular metabolic activity which were observed to follow the release of tensional forces on collagen fibres in the rabbit tendo Achilles (Flint, 1972, 1976*a*,*b*; Reid & Flint, 1974).

The evidence presented here supports the hypothesis that mechanical factors can influence the organization of the extracellular matrix and the metabolic activity of the constituent connective-tissue cells. We suggest that the collagen fibres themselves may act as electrochemical transducers to the adjacent cells, transmitting information, originally manifested as a mechanical force, to initiate changes in cellular metabolism and maintain the glycosaminoglycan composition of the matrix.

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References

Bassett, C. A. L. (1971) in *Physiological Basis of Rehabilitation Medicine* (Downey, J. & Darling, R. E., eds.), pp. 283–316, Saunders, Philadelphia

Bassett, C. A. L. & Becker, R. O. (1962) *Science* **137**, 1063–1064

Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

Braund, K. G., Ghosh, P., Taylor, T. K. S. & Larsen, L. H. (1975) *Anal. Biochem.* **64**, 10–17

Breen, M., Weinstein, H. G., Anderson, M. & Veis, A. (1970) *Anal. Biochem.* **35**, 146–159

Cessi, C. & Piliego, F. (1960) *Biochem. J.* **77**, 508–510

Di Ferrante, N., Donnelly, P. V. & Berglund, R. K. (1971) *Biochem. J.* **124**, 549–553

Flint, M. H. (1972) *J. Embryol. Exp. Morphol.* **27**, 481–495

Flint, M. H. (1976*a*) in *The Ultrastructure of Collagen* (Longacre, J. J., ed.), chapter 4, pp. 60–66, Charles Thomas, Springfield, IL

Flint, M. H. (1976*b*) in *The Ultrastructure of Collagen* (Longacre, J. J., ed.), chapter 14, pp. 191–198, Charles Thomas, Springfield, IL

Flint, M. H. & Merrilees, M. J. (1977) *Histochem. J.* **9**, 1–13

Flint, M. H., Lyons, M. F., Meaney, M. F. & Williams, D. E. (1975) *Histochem. J.* **7**, 529–546

Fukuda, E. & Yasuda, I. (1957) *J. Phys. Soc. Jpn.* **12**, 1158–1162

Fukuda, E. & Yasuda, I. (1964) *Jpn. J. Appl. Physiol.* **3**, 117–121

Gillard, G. C., Caterson, B. & Lowther, D. A. (1975) *Biochem. J.* **145**, 204–213

Lowther, D. A. & Gillard, G. C. (1976) *Arthritis Rheum.* **19**, 769–776

Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956) *Biochim. Biophys. Acta* **21**, 506–518

Pearse, A. G. E. (ed.) (1968) in *Histochemistry*, 3rd edn., vol. 1, pp. 603–604, J. and A. Churchill, London

Ploetz, E. (1938) *Z. Orthop.* **67**, 212–234

Reid, T. & Flint, M. H. (1974) *J. Embryol. Exp. Morphol.* **31**, 489–495

Schiller, S., Slover, G. A. & Dorfman, A. (1961) *J. Biol. Chem.* **236**, 983–987

Scott, J. E. (1960) *Methods Biochem. Anal.* **8**, 145–197