J. Anat. (1981), **133**, 3, pp. 351–357
With 4 figures
Printed in Great Britain

Collagen fibril diameters and elastic fibres in the annulus fibrosus of human fetal intervertebral disc

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(*Accepted* 16 *October* 1980)

INTRODUCTION

The annulus fibrosus of intervertebral disc has a lamellar structure. Each lamella consists of parallel collagenous fibres which are tilted with respect to the axis of the spine; the direction of tilt alternates in successive lamellae (Peacock, 1952; Horton, 1958). Compression, torsion and flexion of the disc increase the tensile stress in these fibres; since the fibres can resist this stress they reinforce the disc and allow it to withstand distortion (Hickey & Hukins, 1980*a*). This same network of collagenous fibres is present in fetal disc (Peacock, 1951; Walmsley, 1953; Hickey & Hukins, 1980*b*). However, the mechanical properties of the network depend not only on the arrangement of the fibres (Hickey & Hukins, 1980*a*, *b*) but also on their internal structure. Such ultrastructural features include, in particular, the size distribution of the collagen fibrils and the presence of elastic fibres.

Connective tissues can be considered as fibre-reinforced composite materials; the collagen fibrils act as reinforcing fibres so that the tissue is able to withstand tension in the directions in which they are oriented. The initial response to deformation involves yielding of the matrix surrounding the fibres. The yielding matrix increases the tension in the fibres. But the tension at the ends of the fibres is zero and it only reaches its maximum value along a critical length. The critical length is proportional to the radius of the fibres; for a tough material, i.e. one which can withstand high stress and does not fail suddenly, this critical length should be as great as possible (Cottrell, 1964). Thus the mechanical properties of a tissue depend, in part, on the diameters of its collagen fibrils; further details are given by Hukins (1981).

Elastic fibres will also influence the behaviour of the annulus fibrosus in tension and hence the way in which the intervertebral disc responds to deformation. They consist of microfibrils surrounded by apparently amorphous elastin; in developing tissues there is little amorphous material but it increases with fetal age until, at maturity, it represents more than 90% of the fibre (Ross, 1973). Elastin is much less stiff than collagen and the combination of the two proteins introduces considerable variety into the mechanical properties of connective tissues (Minns, Soden & Jackson, 1973). Buckwalter, Cooper & Maynard (1976) observed elastic fibres in the annulus fibrosus of humans whose ages ranged from 12 hours to 25 years – despite the many previous electron microscopic studies which had failed to detect them and unsuccessful attempts to stain for elastin for examination of feline annulus fibrosus in the light microscope (reviewed by Buckwalter *et al.*).

Examination of fetal material by electron microscopy has provided information on the growth of collagen fibrils and the development of elastic fibres. Furthermore,

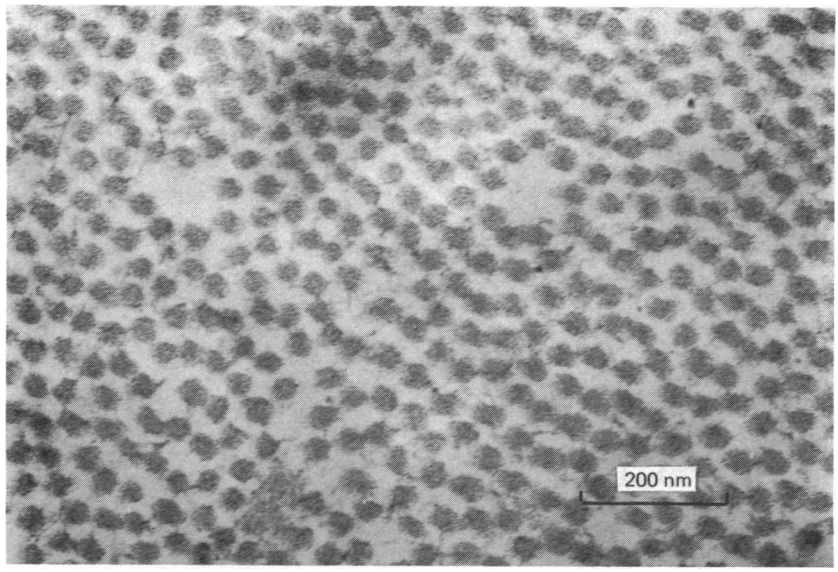

Fig. 1. Electron micrograph of a section of annulus fibrosus (stained with uranyl acetate and phosphotungstic acid) showing the roughly circular appearance of the transversely sectioned collagen fibrils.

the results should be of value in identifying degenerative changes in the adult tissue because the fetal disc is expected to be free from mechanical damage. The observations of Buckwalter *et al.* (1976) have also been confirmed, and extended, by finding elastic fibres in human fetal annulus fibrosus.

MATERIALS AND METHODS

The L5–S1 discs from the same 25 fetuses used in our earlier studies of fetal annulus fibrosus (Hickey & Hukins, 1980*b*) were used. Fetuses were obtained fixed in formol saline and their ages, 'conceptual' rather than 'menstrual' were estimated from crown–rump extended lengths (Bagnall, Jones & Harris, 1975). Specimens for electron microscopy were dissected from the anterior annulus fibrosus and embedded in Araldite as described previously (Hickey & Hukins, 1980*b*). Silver coloured sections were cut with an LKB 8801A ultramicrotome and stained with the following, either separately or in combination: uranyl acetate (Stempak & Ward, 1964), phosphotungstic acid (1 % w/v in water), lead citrate (Reynolds, 1963). Micrographs were obtained, using an AEI EM6B operated at a nominal magnification of 80000 from those areas in which collagen fibrils were sectioned transversely so that they appeared as discs of roughly circular cross section. The microscope was calibrated from micrographs of diffraction gratings.

Micrographs were displayed on the video monitor of a Magiscan Image Analysis System (Joyce-Loebl, Gateshead, England) and collagen fibril diameters indicated by a light pen (for details of the instrument see Taylor & Dixon, 1976; the computer programme for making the measurements was written by Mr S. M. W. Grundy. The fact that fibrils were unlikely to be sectioned exactly perpendicular to their long axes was allowed for by measuring the minor axes of those cross sections which appeared

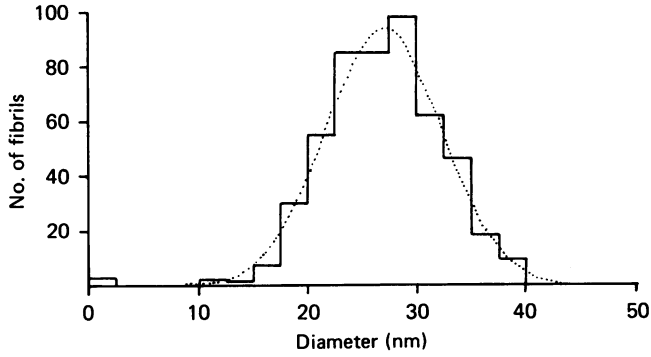

Fig. 2. Histogram showing the distribution of fibril diameters in the annulus fibrosus of an 11 weeks old fetus; the dotted curve shows the corresponding calculated normal distribution.

elliptical. Measurements on elastic fibres were made from the original micrographs with a Mitutoyo Model PJ 250C Optical Comparator.

For each fetus diameters were measured for 500 collagen fibrils; from one fetus a further 500 diameters were measured but this addition made no appreciable difference to the subsequent results. A histogram showing the distribution of collagen fibril diameters for each fetus was plotted; the mean and standard deviation of each distribution were calculated. To test whether there was any correlation between either the mean or the standard deviation with age, the Pearson coefficient, r, was used (see, for example, Woodward, 1972); an r value of zero implies no correlation while values of ± 1 indicate complete correlation. In practice the modulus of r is invariably between the extreme values of 0 and 1; the probability of a correlation existing can then be calculated as described by Woodward.

RESULTS

Figure 1 shows a typical electron micrograph from a section of fetal annulus stained with a mixture of uranyl acetate and phosphotungstic acid. A typical histogram of fibril diameters, measured from micrographs like Figure 1, for a single fetus is shown in Figure 2. The dotted curve is the normal distribution calculated from the mean and standard deviation of the experimental results.

It was surprising that the mean fibril diameter and the standard deviation were not correlated with fetal age in the range 10 to 24 weeks. The scattergram of Figure 3(*a*) shows no correlation between mean diameter and age ($r = 0{\cdot}27$; i.e. the correlation is not significant, $P < 0{\cdot}01$). The values obtained from all fetuses yield a mean of 30·9 nm and imply a 95 % probability of any fibril diameter being in the range $30{\cdot}9 \pm 8{\cdot}5$ nm (see Spendley, 1972, for the method of calculation). Similarly the scattergram of Figure 3(*b*) shows no correlation ($P \ll 0{\cdot}05$) between the standard deviation of the distribution and the age of the fetus ($r = 0{\cdot}08$).

Figure 4 shows an elastic fibre (E) in a micrograph from a section of fetal annulus stained with a mixture of lead citrate and phosphotungstic acid. This fibre is typical of the several hundred that were observed and its appearance is characteristic of an immature elastic fibre in that it consists largely of a bundle of microfibrils (Greenlee, Ross, & Hartman, 1966; Ross, 1973; Jones, Sear & Grant, 1980). The elastic fibres were identified not only from their characteristic appearance, which is distinct from

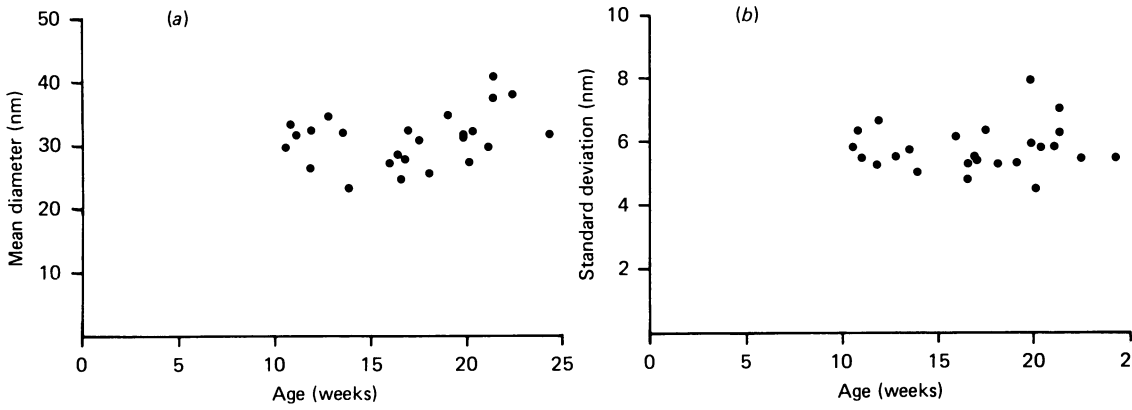

Fig. 3. Scattergrams showing (*a*) the mean diameter and (*b*) the estimated standard deviation of the diameters of the collagen fibrils in each of the fetuses investigated as a function of age.

that of collagen fibrils, but also from their dimensions; the measured centre-to-centre distance of the microfibrils was about 20 nm which is consistent with the 10 to 20 nm diameters of microfibrils in an elastic fibre (Greenlee *et al.* 1966; Jones *et al.* 1980).

DISCUSSION

Our results show that the collagen fibrils of the annulus do not continue to grow in diameter during the development of the fetus. Micrographs published by Walmsley (1953) indicated that collagen was first deposited in the annulus fibrosus when the fetus was about 10 weeks old. Yet we have found no increase in fibril diameter during the 10 to 24 weeks of development. The number of fibrils must increase, however, because we observed no decrease in fibril density as the disc grew. We have also found that the annular fibres have their characteristic tilt when the collagen is first deposited (Hickey & Hukins, 1980*b*). Thus, there appears to be no prenatal change in collagen fibril diameter or orientation after the fibrils are first formed within the first 24 weeks.

The true mean of the fibril diameters is almost certainly somewhat greater than the value of 30·9 nm measured from electron micrographs; fixation and dehydration in the specimen preparation procedures would be expected to shrink the fibrils. Storage of specimens in formol saline is known to draw the molecules of a collagen fibril closer together (Hickey & Hukins, 1979) presumably because of the known ability of aldehydes to cross-link collagen molecules (Bowes & Cater, 1968). Fixation in glutaraldehyde might be expected to have a similar effect. If the molecules become more tightly packed in a fibril its diameter will decrease. Dehydration is known to reduce the axial periodicity of the collagen fibril from 67 to 64 nm (Tomlin & Worthington, 1956). Air-drying also leads to molecules in elastoidin (fish fin-ray collagen) becoming more closely packed – leading to a decrease in fibril diameter (Woodhead-Galloway *et al.* 1978).

The diameters of the collagen fibrils in fetal annulus are similar to those in neonatal tissues of other species: about 30 nm in rat tail tendon (Torp, Baer & Friedman, 1975; Parry & Craig, 1977), about 40 nm in rat synovial sheath and flexor tendon (Greenlee & Ross, 1967) and 20 days old embryonic fowl metatarsal tendon (Fitton-

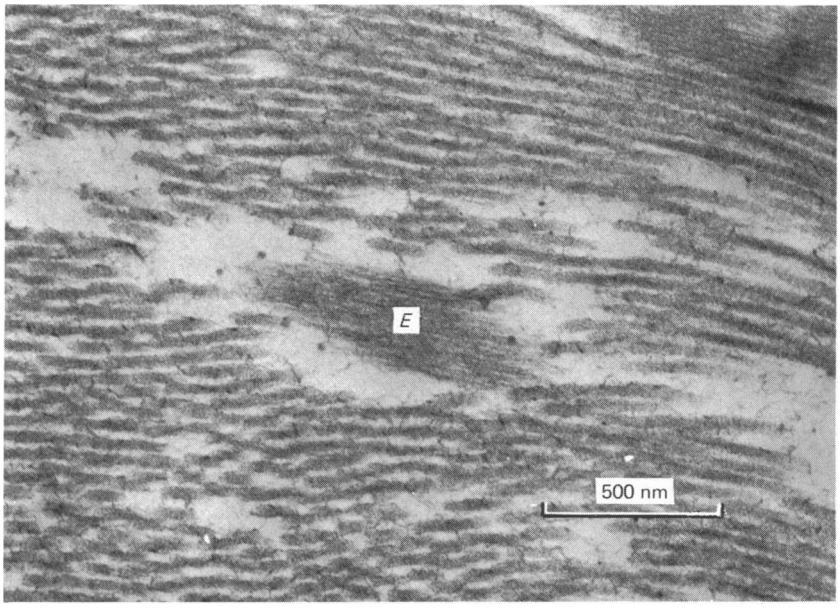

Fig. 4. Electron micrograph of a section of annulus fibrosus (stained with lead citrate and phosphotungstic acid) showing an elastic fibre (*E*) from a 20 weeks old fetus.

Jackson, 1956). In the fetal horse, however, some of the tendons and ligaments have much larger collagen fibrils (mean diameter 105 nm) (Parry, Craig & Barnes, 1978).

In other tissues, postnatal development includes increase in collagen fibril diameters; our preliminary results suggest that this fibrillar growth also occurs in the postnatal annulus fibrosus. Increase in mean fibril diameter and in the width of the distribution of diameters has been observed in rat tail tendon (Torp *et al.* 1975; Parry & Craig, 1977; 1978) and in horse tendons and ligaments (Parry *et al.* 1978). In a single strain of ageing mice, larger collagen fibrils appear in the annulus fibrosus and the distribution becomes markedly bimodal as the tissue ages (Hickey & Hukins, unpublished observations). The critical length over which the mechanical stress in a fibril can attain its maximum value depends on the strength of the surrounding glycosaminoglycan gel and its interactions with the fibrils as well as on fibril diameter (Hukins, 1981). Changes in the glycosaminoglycan content of the annulus during postnatal development (Adams & Muir, 1976) may then be associated with fibril diameter changes so that the tissue maintains the required mechanical properties.

Finally, the elastic fibres we have observed are typical of immature elastic fibres; as elastic fibres develop the microfibrils of Figure 4 become the minor constituent and surround an amorphous centre (Greenlee *et al.* 1966; Ross, 1973; Jones *et al.* 1980). The mature elastic fibres then more closely resemble those observed by Buckwalter *et al.* (1976) in adult human annulus fibrosus.

SUMMARY

Electron microscopy shows that the collagen fibrils of human fetal annulus fibrosus do not increase in diameter from the time they are first deposited, at an age of 10 weeks, to at least an age of 24 weeks. Their diameters, about 30 nm, in this age-range are comparable with values observed in other tissues from neonates of other species. Immature elastic fibres are associated with the collagen fibrils in the fetal annulus.

We thank Dr K. M. Bagnall for the material used in this study, Mr S. M. W. Grundy for the use of his computer programme to measure fibril diameters, Mrs C. Cummings for taking some of the electron micrographs and the Medical Research Council for the award of a research studentship to D.S.H.

REFERENCES

ADAMS, P. & MUIR, H. (1976). Qualitative changes with age of proteoglycans of human lumbar discs. *Annals of the Rheumatic Diseases* **35**, 289–296.

BAGNALL, K. M., JONES, P. R. M. & HARRIS, P. F. (1975) Estimating the age of the human fetus from crown–rump measurements. *Annals of Human Biology* **2**, 387–390.

BOWES, J. H. & CATER, C. W. (1968). The interactions of aldehydes with collagen. *Biochimica et biophysica acta* **168**, 341–352.

BUCKWALTER, J. A., COOPER, R. R. & MAYNARD, J. A. (1976). Elastic fibres in human intervertebral disc. *Journal of Bone and Joint Surgery* **58A**, 73–76.

COTTRELL, A. H. (1964). Strong solids. *Proceedings of the Royal Society* A **282**, 2–9.

FITTON-JACKSON, S. (1956). The morphogenesis of avian tendon. *Proceedings of the Royal Society* B **144**, 556–572.

GREENLEE, T. K. & ROSS, R. (1967). The development of the rat flexor digital tendon, a fine structural study. *Journal of Ultrastructure Research*, **18**, 354–376.

GREENLEE, T. K., ROSS, R. & HARTMAN, J. L. (1966). The fine structure of elastic fibers. *Journal of Cell Biology* **30**, 59–71.

HICKEY, D. S. & HUKINS, D. W. L. (1979). Effect of methods of preservation on the arrangement of collagen fibrils in extracellular matrices: an X-ray diffraction study of annulus fibrosus. *Connective Tissue Research* **6**, 223–228.

HICKEY, D. S. & HUKINS, D. W. L. (1980*a*). Relation between the structure of the annulus fibrosus and the function and failure of the intervertebral disc. *Spine* **5**, 106–116.

HICKEY, D. S. & HUKINS, D. W. L. (1980*b*). X-Ray diffraction studies of the arrangement of collagenous fibres in human fetal intervertebral disc. *Journal of Anatomy* **131**, 81–90.

HORTON, W. G. (1958). Further observations on the elastic mechanism of the intervertebral disc. *Journal of Bone and Joint Surgery* **40B**, 552–557.

HUKINS, D. W. L. (1981). Biomechanics of collagen. In *Collagen in Health and Disease* (ed. M. I. V. Jayson & J. B. Weiss). Edinburgh: Churchill Livingstone.

JONES, C. J. P., SEAR, C. H. J. & GRANT, M. E. (1980). An ultrastructural study of fibroblasts derived from bovine ligamentum nuchae and their capacity for elastogenesis in culture. *Journal of Pathology* **131**, 35–53.

MINNS, R. J., SODEN, P. D. & JACKSON, D. S. (1973). The role of the fibrous components and the ground substance in the mechanical properties of biological tissues. A preliminary investigation. *Journal of Biomechanics* **6**, 153–165.

PARRY, D. A. D. & CRAIG, A. S. (1977). Quantitative electron microscope observations of the collagen fibrils in rat-tail tendon. *Biopolymers* **16**, 1015–1031.

PARRY, D. A. D. & CRAIG, A. S. (1978) Collagen fibrils and elastic fibres in rat-tail tendon: an electron microscopic investigation. *Biopolymers* **17**, 843–855.

PARRY, D. A. D., CRAIG, A. S. & BARNES, G. R. G. (1978). Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibres as a function of age. *Proceedings of the Royal Society* B **203**, 293–303.

PEACOCK, A. (1951). Observations on the pre-natal development of the intervertebral disc in man. *Journal of Anatomy* **85**, 260–274.

PEACOCK, A. (1952). Observations on post-natal structure of intervertebral disc in man. *Journal of Anatomy* **86**, 162–179.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

ROSS, R. (1973). The elastic fiber. A review. *Journal of Histochemistry and Cytochemistry* **21**, 199–208.

SPENDLEY, W. (1972). Statistical inference. In *Statistical Methods in Research and Development*, 4th ed. (ed. O. L. Davies & P. L. Goldsmith), pp. 58–68. Edinburgh: Oliver & Boyd.

STEMPAK, J. G. & WARD, R. T. (1964). An improved staining method for electron microscopy. *Journal of Cell Biology* **27**, 697–701.

TAYLOR, C. J. & DIXON, R. N. (1976). Quantitative image analysis. In *Medical Images: Formation, Perception and Measurement* (ed. G. A. Hay), pp. 295–307. Bristol: Institute of Physics.

TOMLIN, S. G. & WORTHINGTON, C. R. (1956). Low-angle X-ray diffraction patterns of collagen. *Proceedings of the Royal Society* A **235**, 189–201.

TORP, S., BAER, E. & FRIEDMAN, B. (1975). Effects of age and of mechanical deformation on the ultra-

structure of tendon. In *Structure of Fibrous Biopolymers* (ed. E. D. T. Atkins & A. Keller), pp. 223–250. London: Butterworth.

WALMSLEY, R. (1953). The development and growth of the intervertebral disc. *Edinburgh Medical Journal* **60**, 341–365.

WOODHEAD-GALLOWAY, J., HUKINS, D. W. L., KNIGHT, D. P., MACHIN, P. A. & WEISS, J. B. (1978). Molecular packing in elastoidin spicules. *Journal of Molecular Biology* **118**, 567–578.

WOODWARD, R. H. (1972). Correlation. In *Statistical Methods in Research and Development*, 4th ed. (ed. O. L. Davies & P. L. Goldsmith), pp. 229–235. Edinburgh: Oliver & Boyd.