ULTRASTRUCTURAL STUDIES ON HORSE LIGAMENTS

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DEDICATION

This thesis is dedicated to my supervisor Professor D.A.D. Parry

ABSTRACT

This thesis has been devoted to studying one of the largest structural units present in most connective tissues - the collagen fibril. Diameter distributions of collagen fibrils from horse ligaments have been investigated as a function of age. A fairly complete age-related study of collagen fibril diameter distribution was targeted during this work. The ligaments (lateral collateral ligament, medial collateral ligament, radioscaphoid ligament, lateral pisoformometacarpal ligament and scaphocapitate ligament) were sampled from horses of ages one year, two-and-half year, five year, six year and eleven year. Electron microscopy methods were employed and corresponding electron micrographs were obtained from transverse sections. These were used to calculate a mean diameter and mass-average diameter of the collagen fibrils. Individual histograms were plotted showing the frequency and mass distribution of the fibrils versus the diameter at each of the ages studied. The data obtained have been related to the mechanical properties of the ligaments and their mode of growth. The diameter distributions obtained clearly reflect the mechanical needs of the ligaments during various stages of maturation. Results from a previous study of these ligaments at one particular age (four years) have been compared and found to be compatible with the results obtained during the course of this study. The effect of training on collagen fibril diameter distribution of horse ligaments has also been discussed.

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Chapter 1. INTRODUCTION

The shape and structure of the animal body is mainly determined by the composition and spatial arrangement of the structural entities that comprise connective tissue. The family of connective tissues include tendon (which links muscle to bone), ligament (which links bone to bone), skin, cornea, bone, teeth, cartilage and vitreous humour (Harkness, 1961; Bailey and Robins, 1976; Parry and Craig,1984).

All connective tissues are composed of three microscopic constituents - cells, fibrous components and extracellular matrix or ground substance. In loose connective tissues (which lie subcutaneously and attach the skin to the underlying tissues) the cells are quite numerous when compared to other tissues such as tendon, ligament, skin etc. where the cell density is much less, and where closely packed fibres separated by relatively small quantities of matrix make up the majority of the intercellular matrix. The dominant structural material present in connective tissue is protein, of which collagen (a fibrous protein) is the most important and widely distributed. Indeed collagen is a characteristic structural protein of the soft tissues of all animals. In vertebrates, however, it is the dominant one, and accounts for about 25% of all body protein (Harkness, 1961). Consequently it has been very widely studied. Elastin, another important protein in connective tissue, is present as an amorphous core surrounded by a containing cluster of elastin microfibrils. The elastin fibres lie in parallel with the collagen fibrils and are responsible for much of the elasticity of the tissue. A further key constituent of connective tissues is the proteoglycans. These consist of protein cores covalently linked to glycosaminoglycans i.e. polysaccharides containing amino sugars and acidic groupings. Minerals and water are also present in characteristic proportions in different connective tissues and play an important part in specifying the mechanical properties of the tissue. The fibrous framework formed by the collagen and elastin fibres within a tissue is embedded in a matrix containing the cells, proteoglycans, minerals, elastin, and water. Some specialized tissues such as Wharton's jelly in the umbilical cord are

essentially devoid of fibrous masses and the polysaccharide provides a homogenous jelly-like consistency. In bone, the proteinaceous matrix becomes mineralized to provide the rigid structures required for skeletal stability.

The cellular components of collagenous tissues differ greatly and may contain migratory cells such as monocytes, lymphocytes, eosinophils, neutrophils or mast cells, or stationary cells such as the mesenchymal cells, macrophages, adipocytes, and fibroblasts. The fibroblasts, which are the predominant types of cells in denser connective tissues like tendon, cartilage, bone are also known as tenocytes, chondrocytes, and osteocytes respectively. The relative proportions of cells and matrix vary with the function of the individual tissue.

1.1 Molecular Architecture Of The Collagen Molecule:

The molecular architecture of collagen is such that three α-chains assemble to form a triple-helical structure. Each α-chain adopts a hydroxyproline-type left-handed helix with three residues per turn. The three α-chains then coil about a common axis to form a right-handed triple helix with ten repeating units in three turns. This coiled-coil structure has a supercoil pitch length of ~8.7 nm (Ramchandra & Kartha, 1955; Fraser *et al* , 1979, 1983), a width of 1.5 nm and a length of ~300 nm. Much of the sequence of the α-chains is of the form $(\text{glycine - X - Y})_n$ (where n = 338 for tendon collagen). Glycine is the smallest of all the amino acids and accounts for about one-third of the total residues in the molecule. Approximately 25% of the X and Y residues are either proline or hydroxyproline, which are imino acids. In all tendon collagen molecules short non-helical extensions termed telopeptides are found at the amino- and the carboxy- terminal ends of the chains respectively. These are involved in the formation of intermolecular covalent crosslinks and are responsible for much of the structural integrity of the collagen fibrils.

The nature of the three-fold helix of an individual α-chain is such that the glycine residues all lie along one edge. When the three chains assemble, the glycine side-chains (which consist

of but a single hydrogen atom) are all directed towards the core of the molecule, thus facilitating chain packing, and in doing so allowing the formation of a hydrogen bond network to stabilise the molecular structure.

Distinguished by their physico-chemical properties (amongst other things) at least 18 types of collagens have been chemically categorised. Table 1.1 lists 13 types of collagens and their distribution. With a few minor exceptions (Parry & Craig, 1981) all of the collagens have at least a part of their structure in the classical triple-helical conformation, similar to the one explained above. Nonetheless the extent of triple-helical structure varies very greatly, with types I, II and III having the highest percentages. Some of the interspecific differences can be summarised as follows :

(a) The number of triplets in types I, II, III, IV collagen are 338, 340, 341 and 430 respectively.

(b) The chain composition of types I, II, III and IV collagen molecules are $[\alpha(I)]_2\alpha2$, $[\alpha1(II)]_3$, $[\alpha1(III)]_3$ and $[\alpha1(IV)]_2\alpha2(IV)$.

(c) The carbohydrate content of types I, II, III, IV collagens are ~ 0.4%, 4%, 0.4% and 12% respectively.

(d) Type IV collagen molecules, in contrast to Types I, II and III, are non-fibril forming collagens and are found in basement membranes in the form of a meshwork.

Other types of collagens (types V, VI, VII, VIII, IX, X, XI, XII and type XIII) are generally found in small amounts. Their functional importance, however, is not to be underestimated. From these and other data it is now well established that different tissues contain collagen molecules of one or more types. It follows that these may have different lengths, degree of hydroxylation, glycosylation and cross-linking ability. Indeed it is rare to find a tissue

TABLE 1.1

COLLAGEN TYPES	DISTRIBUTION
Type I	Skin, bone, tendon, cornea, dentin, fibrocartilage, large vessels, intestine, uterus.
Type I-trimer	Dentin, dermis, tendon.
Type II	Hyaline cartilage, vitreous humour, *nucleus pulposus*, notochord.
Type III	Large vessels, uterine walls, dermis, intestine, heart valve, gingiva.
Type IV	Basement membranes.
Type V	Cornea, placental membranes, large vessels, hyaline cartilage, gingiva.
Type VI	Descemet's membranes, skin, *nucleus pulposus*, heart muscle.
Type VII	Skin, placenta, lung, cartilage, cornea.
TypeVIII	Produced by endothelial cells, Descemet's membrane.
Type IX	Cartilage.
Type X	Produced by hypertrophied chondrocytes during the process of endochondral ossification.
Type XI	Hyaline cartilage, intervertebral disc, vitreous humour.
Type XII	Chick embryo tendon, bovine periodontal ligament.
Type XIII	Foetal skin, bone, intestinal mucosa.

From "Collagens: Biochemistry and Pathophysiology" by E. Kucharz, 1992

containing a single molecular species of collagen. It should be noted that collagen type often varies with age.

1.2 The Collagen Fibril :

Electron microscope investigation of collagen fibrils shows a regular cross-striated (banded) appearance with the period of banding being designated as " D " (Hall *et al*, 1942; Schmitt *et al*, 1942; Wolpers, 1943).Using X-ray diffraction studies on native hydrated connective tissues D is ~67 nm in tendon (Bear, 1942) but is lower (~65.5 nm) in skin (Brodsky and Eikenberry, 1981) and cornea (Inouye and Worthington, 1983). The appearance of the D-banding in the collagen fibrils as observed by electron microscopy is dependent on the method of staining. Negative staining results in one predominantly dark-staining band and a second predominantly light-staining band, each about D/2 long (Tromans *et al*, 1963; Olsen, 1963; Hodge and Petruska, 1963). Alternatively, positive staining produces a polarized D-period containing 12 or 13 narrow darkly-staining bands (Bruns and Gross, 1974). Schmitt *et al* (1955) proposed that the molecules were staggered by a distance D with respect to their immediate neighbours. Early work had suggested that the molecules were in end-to-end contact and that the length of each molecule was 4D. Hodge and Petruska (1963), however, found that the length of the collagen molecule was close to 4.4D. This implies that gaps of ~0.6D (40 nm) exist between molecules staggered axially with respect to one another by 5D. Thus the D-periodic collagen fibril has an overlap region of 0.4D and a gap region of 0.6D, and thus accounts for the alternate light and dark bands seen in the electron micrographs of negatively stained fibrils. The work of White *et al* (1977) refined these parameters and proposed that the length of the collagen molecule was 4.47D, taking into account the telopeptides (the terminal non-helical regions). Values for the gap regions and the overlap regions are therefore 0.47D and 0.53D respectively.

1.3 Packing Of Collagen Molecules In Fibrils:

The simplest arrangement of collagen molecules with a D-period will contain five molecules in transverse section, each contributing a different segment to the D-period (Miller 1976). Smith (1968) had already recognized this feature and incorporated it in his model of the sub-fibrillar structure of collagen - the five-stranded microfibril. Miller (1971) predicted on the basis of X-ray diffraction studies that such a structure would have a diameter of 3.8 nm. Miller and Parry (1973) after studying improved X-ray diffraction patterns, proposed that the molecules comprising the five-stranded microfibril were supercoiled and that the microfibrils were packed together on a square lattice. Thus the substructure of the collagen fibrils could be accounted for by tetragonally arranged groups of four microfibrils forming a unit cell of side ~ 7.6 nm. (2 x 3.8 nm.). A variety of other models have been suggested, which include a two-stranded model (Woodhead-Galloway, 1975), a four-stranded model (Veis and Yuan, 1975), an eight-stranded model (Hosemann *et al*, 1974), a liquid crystal model (Hukins and Woodhead-Galloway, 1977), the quasi-hexagonal model (Hulmes and Miller, 1979; Miller and Tocchetti ,1981, Fraser and MacRae,1981, Fraser *et al* 1983), and the five-stranded compressed model (Trus and Piez, 1980 ; Piez and Trus, 1981). The quasi-hexagonal model for packing of collagen molecules is now more generally accepted. This proposes that in the collagen fibrils the molecules are tilted with respect to the fibril axis and packed on a quasi-hexagonal lattice that is both laterally and axially sheared, thus having the character of a molecular crystal. This model however does not eliminate the possibility that a distinct pattern of crosslinks exist which defines a microfibril similar to that proposed by Smith (1968). Indeed there is much evidence to suggest that a structure such as this is required to explain a variety of other experimental observations (Parry and Craig, 1981).

1.4 The Mechanical Properties Of Connective Tissues :

Connective tissues play a significant role in maintaining the form and integrity of the animal body. Notable progress has been made of late in perceiving the relationship between the

mechanical role of connective tissues and the chemical constituents present in the tissue. As a part of these studies, Parry and Craig have collected, analyzed, and compared a considerable range of electron microscope data on the lateral dimensions of collagen fibril size as a function of age and mechanical role in a wide variety of tissues including tendon, skin and cornea. A distinctive trend was observed in all cases, where the foetal tissues contained small, but uniform diameter fibrils. The fibril diameters were less than 50 nm but were close to multiples of 8 nm (Parry and Craig , 1979; Parry *et al*, 1980). As the tissue developed the collagen fibril diameter distribution was found to broaden (Craig and Parry , 1981a; Eikenberry *et al* , 1982a,b). With further maturity the distributions broaden still more. However in specialized tissues such as cornea, the distribution remained uniform throughout life. At maturity, the maximum mass average diameter was generally reached. However, a slightly different trend has been observed in passive skins (skins having a passive mechanical role) from precocious animals (animals capable of locomotory function within a few hours of birth). Collagen fibrils in such skins has been found to increase rapidly in mean diameter during foetal development and reach a maximum value at a time close to birth. At adulthood, the mean fibril diameter decreases. Thus, in such animals the collagen fibrils have their largest diameter at birth (or at an age close to birth), reflecting the high degree of development of these animals. Degenerative trends in many connective tissues are observed at late maturity or senescence, whereby the mass average diameter of the fibrils generally decreases. This was also seen in the electron micrographs in the form of altered or irregular fibril morphology (Parry and Craig, 1983).

The diverse mechanical roles executed by connective tissues have been found to be related to the distribution of fibril sizes and to the ultrastructural organization of those fibrils in the tissue. It has been shown that the largest diameter fibrils are present in tissues suffering the highest stress levels, like tendons, while tissues which are subjected to lower stress levels, contain a high percentage of smaller diameter fibrils. Tissues subjected to low stress levels often have the functional requirement of resisting permanent creep (Parry and Craig, 1977,

1978; Parry *et al*, 1978a; Craig and Parry, 1981a,b; Parry and Craig, 1983). The above mentioned observations have been accounted for by Parry *et al* (1978). The percentage of covalent cross-links between collagen molecules is predicted to increase with fibril diameter (up to about 100 nm) and hence lead to fibrils of greater strength (Parry *et al*, 1978). Molecules lying within the peripheral layer of a fibril are unable to form the full complement of covalent crosslinks, in contrast to those molecules lying within the body of the fibril. Large diameter fibrils which have a smaller peripheral layer per unit mass of collagen will thus have higher crosslink densities than small fibrils, where the peripheral layer forms an appreciable portion of the total mass. Since the surface area of the fibrils per unit mass of collagen increases as the fibril diameters decrease, it follows that the possible number of interactions between the matrix and fibrils can be maximized by decreasing the diameters of the fibrils, which implies that there is a concomitant increase in the number of fibrils. Thus a larger diameter fibril would be predicted to be stronger than a smaller fibril, whereas smaller fibrils would provide the best opportunity for the tissue to resist permanent creep. Some evidence supporting this concept may be found in Parry *et al* (1980).

The fact that the most abundant connective tissues have a fibril-matrix structure is also directly related to their mechanical properties. Such a composite structure not only has collagen fibrils to provide the tensile strength of the tissue but also matrix in the form of hydrated glycosaminoglycans to resist compression (Scott, 1975). Together this fibril-matrix structure provides the tissue with a mechanism to arrest fracture and crack propagation. This basic concept of design is found in other biological tissues (e.g. wool fibres) and is mimicked by man in his construction of reinforced concrete and fibre composites. In this way stresses are more evenly distributed over the tissue.

Relatively small diameter collagen fibrils (mean diameter ~90 nm) and a narrow distribution of sizes are a characteristic of low tensile tissues like skin, while bimodal distributions are

observed for tissues like tendons which require large diameter fibrils for strength and smaller diameter fibrils to avoid permanent creep. However specialized skins such as those from rat-tail and trout, which also have an additional locomotory role, have fibril diameter distributions intermediate between those of body skin and tendon. Such skins are said to have an "active" role and are usually "exotendinous" (Flint et al , 1984). Again it follows that mechanical properties can be correlated with the collagen fibril diameter distributions.

Studies of rat-tail tendon with polarized light (Rigby *et al* , 1959) have shown that unstretched tendon has a wavy appearance which, when strained by 3% or more, will disappear. This specialized collagen structure, which has been observed by Diamant *et al* (1972) and Gathercole and Keller (1975) as a macroscopic crimp, has also been observed in vertebrate tendons from different sources which are normally subject to rapid application of high stress levels. The crimp length and angle were found to be typically 1 - 100 μm and 5° - 25° respectively. Such a macroscopic crimp provides a compliance mechanism enabling the tissue to undergo an increase of about 3% in length before the individual molecules are stretched, thus helping to prevent the tissue from sustaining permanent damage.

1.5 Role Of Glycosaminoglycans In Collagen Fibril Development :

Extensive data on glycosaminoglycans (GAG) composition and collagen fibril diameter distribution has been collected and studied for a diverse range of tissues by Parry *et al* (1982). It was shown that tissues with the smallest diameter collagen fibrils (mass average diameter < 60 nm) have a high concentration of hyaluronic acid. As the tissue matured, the concentration of hyaluronic acid decreased while the concentration of chondroitin sulphate generally increased. Tissues which undergo rapid application of high stress levels were found to have a high concentration of dermatan sulphate, and the diameters of the collagen fibrils were found to be large (mass average diameter ~ 200nm). A hypothesis has been put

forward that an excess concentration of hyaluronic acid has an inhibitory role on the lateral dimensions of collagen fibrils. This inhibitory effect may be removed by an increasing concentration of chondroitin sulphate and or dermatan sulphate. In turn chondroitin sulphate may inhibit fibril growth beyond a mass average diameter of ~150 nm. This inhibition may be eliminated by an increasing concentration of dermatan sulphate, so that it becomes the dominant GAG present in the tissue. All foetal tissues display a high concentration of hyaluronic acid, while mature tissues frequently show dermatan sulphate as the dominant GAG.

1.6 Diameter Distribution Of Collagen Fibrils As A Function Of Age:

An extensive study by Parry *et al* (1978a, 1978b), has shown that there is a distinctive trend in the diameter distribution of collagen fibrils as the tissue develops from the foetal stage to senescence. An analysis of the data collected on the collagen fibril diameter distributions from connective tissues led the authors to note a few general characteristics which are briefly restated below:

1) Unimodal diameter distributions were observed in the case of collagen fibrils from connective tissues at birth, and in the foetal stage of development.

2) Collagen fibrils from tissues of precocious animals (animals capable of performing locomotory function within a few hours of birth) were found to have broad diameter distributions, while altricious animals (animals incapable of locomotion at birth and for a considerable period afterwards relative to their lifespan) were found to have sharp collagen fibril diameter distribution. Thus the form of the collagen fibril diameter distribution reflects the degree of development of the animal.

3) The mass average diameter of collagen fibrils in all tissues except those from corneal stromal lamella increases from birth to maturity.

4) Most orientated type I collagenous tissues (tendons, ligaments) which are subjected to long-term high stress levels have a bimodal distribution of collagen fibril diameters at maturity. Tissues subjected to short-term stress usually have unimodal distributions. Distributions are broad or sharp corresponding to high stress or low stress respectively.

5) Mass average diameters of collagen fibrils at late maturity or senescence are smaller than at the onset of maturity.

1.7 Scope And Aim Of This Thesis :

Tendons and ligaments consist of bundles of collagen fibres and parallel rows of fibroblasts (tenocytes). In large animals such as horses, there are no muscle groups from the carpal and hock joints distally. This renders the tendon relatively unprotected and consequently it can suffer a wide range of injuries including the complete severing or rupture of the tendon. In horses trained for the racing industry, the tendons and ligaments undergo severe strain when excessive and prolonged stress is applied to them. Such injuries result in collagen fibre slippage and possible rupture. This in turn may give rise to an altered collagen fibril diameter distribution, related to the changing mechanical properties of the tissue.

Although a complete study of injured tendons and ligaments is beyond the scope of this thesis, an attempt has been made at an ultrastructural study on five ligaments from the carpal region from horses of ages one, two and a half, five, six and eleven years. It should be noted, however that only one horse from different age groups was used during this study. These tissues represent a clinically significant group. It is hoped that this will allow some further insights into the mechanical properties of the tendons and ligaments to be gained. Depending upon the type of distribution, it may be possible to obtain new information regarding the stress levels to which these ligaments are subjected.

Chapter 2. METHODS AND MATERIALS

Horse ligaments were obtained from a variety of sources. Of the five horses used, three (two-and-a-half year, five year, and eleven year) were obtained from an equine export slaughter-house near Hamilton. The amputated forelegs (left foreleg from each of the horses) were flown to Palmerston North and brought to the Massey University post-mortem facility in the Veterinary Science Faculty, where sampling was carried out. The six-year-old horse was euthanased at Marton for behavioural problems, and the amputated forelegs brought to Massey University where the tissues were excised. The one year old horse was euthanased after emergency exploratory surgery at Massey University in the Equine Clinic.

Although the exact histories of the horses are not available, as far as possible the choice of horses was restricted to those without any detectable disease or anomalies in the carpal region, so that the tissues under consideration would be free from any major defects. For ethical reasons, horses in good health were not put down for the sole purpose of obtaining the required tissues.

2.1 Sampling Fixation And Embedding Of The Tissues :

Excision of the tissues was carried out by Professor Elwyn Firth (Veterinary Clinical Sciences), Mrs. Helen Hodge (Veterinary Clinical Sciences), Associate Professor Alex Davies (Physiology and Anatomy) and myself. The sampled tissues were removed from the carpal region within three hours of death. Some of these tissues had previously been studied at one particular age by Deane (1991) (lateral collateral ligament, medial collateral ligament, lateral pisoformometacarpal ligament, palmar carpal ligament, scaphocapitate ligament, radioscaphoid ligament) and these results were included in the following analysis to allow comparison of results and to facilitate a complete age study.

The ligaments were excised *in toto* using a scalpel and forceps and blocks (1 x 0.5 x 0.5 cm where appropriate) were then removed from the centre of each of them. The order in which the tissues were sampled is given in Table 2.1. and the location sites are detailed in

Figure 2.1. The blocks were further dissected (1.5 x 1.5 x 10 mm) and immediately placed into a small labelled bottle containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3), which served as the primary fixative. This kills the tissue, while keeping the structural, biochemical and molecular features of the living entity in as near a life-like state as possible. Glutaraldehyde is the preferred fixative as it is known for the speed with which it fixes a tissue, for its penetration into the tissue, and for its completeness of fixation. After two hours in fixative, each sample was further dissected with a double-edged razor blade into fine longitudinal slivers (5 mm long and less than 1 mm thick) and kept overnight in buffer solution. The tissue slivers were placed in serialized baskets and loaded onto the Lynx™ Tissue Processor. The tissue processor was programmed to follow the schedule given in Table 2.2. Buffer washes have been used to minimize alteration of cellular components due to pH shock, which would otherwise arise from the initial plunging into the primary fixative. Osmium tetroxide is a quick and complete fixative, but its usefulness is limited by its very poor penetration, and hence it has been used here only as a post-fixative. It is also known to impart contrast to the tissue by selective deposition of heavy metal.

Water-immiscible resin was used in preparing the samples for electron microscopy hence necessitating the removal of water from the tissue. This was achieved through a series of AnalaR grade ethanol dehydration steps. Dehydration was carried out using increasing concentrations of ethanol to ensure that the constitution of the tissue was as little changed as possible. It is also worth noting that technical problems will arise in the vacuum column of the electron microsope if the tissue is not dehydrated. In vacuum, water will boil and this will not only introduce water molecules into the vacuum column but also destroy the specimens. Propylene oxide was used as an infiltrating agent to ensure that penetration of the resin is complete throughout the resin.

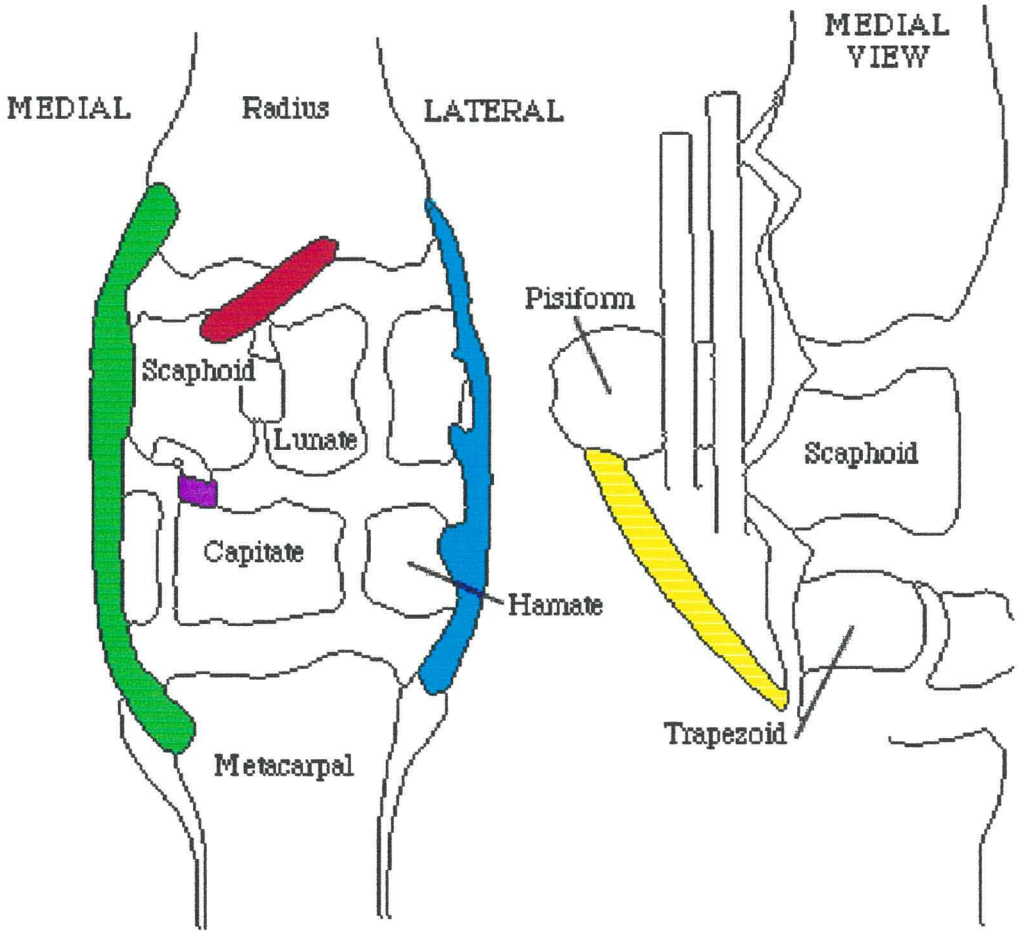
After the processing was complete, tissues were removed from the baskets and embedded in 100% resin. Rubber moulds were used to embed the tissues in the form of blocks. Care was taken to label each appropriately by inserting small paper strips with

TABLE 2.1

TABLE SHOWING THE LIGAMENTS SAMPLED

Identification	Sampling order	Tissue and sample location
A	1	Lateral collateral ligament - at the level of the middle carpal joint.
B	2	Medial collateral ligament - at the level of middle carpal joint.
C	3	Lateral pisoformometacarpal ligament - from the lateropalmar aspect.
D	4	Radioscaphoid ligament - in the radiocarpal joint approached through the palmar carpal ligament.
E	5	Scaphocapitate ligament - approached from the dorsal aspect of the middle carpal joint

Figure 2.1
Dorsopalmar and mediolateral views of the equine carpus illustrating the sites of the ligaments.



Lateral Collateral Ligament
Medial Collateral Ligament
Radioscaphoid Ligament
Lateral Pisoformometacarpal Ligament
Scaphocapitate Ligament

TABLE 2.2

TABLE SHOWING THE PROGRAM FOLLOWED BY THE LYNX^{TM} TISSUE PROCESSOR

VIAL NO.	TIME	TEMP.	FUNCTION.
1	2 hours	4°C	Primary fixation in glutaraldehyde
2	10 mins.	4°C	Buffer wash
3	1 hour	4°C	Buffer wash
4	1 hour	4°C	Buffer wash
5	2 hours	4°C	1% osmium tetroxide
6	10 mins.	4°C	Buffer wash
7	30 mins.	4°C	Buffer wash
8	20 mins	4°C	25% Ethanol
9	20 mins	4°C	50% Ethanol
10	20 mins	4°C	70% Ethanol
11	20 mins	4°C	95% Ethanol
12	20 mins	4°C	Absolute Ethanol
13	20 mins	4°C	Absolute Ethanol
14	20 mins	4°C	Propylene oxide
15	20 mins	4°C	Propylene oxide
16	18 hours	10°C	Propylene oxide / Resin (2:1)
17	24 hours	10°C	Propylene oxide / Resin (1:2)
18	48 hours	23°C	Resin

details of the tissue and its age. Polymerization was achieved by curing the blocks for 72 hours in an oven set at a constant temperature of 62°C. At this time the blocks were firm and could be stored at room temperature in labelled bottles.

2.2 Sectioning Methods :

The blocks were trimmed by hand on a Reichert-Jung "Ultracut-E" ultramicrotome using a quarter of a double-edged razor blade, mounted on a grooved rubber and fitted onto an aluminium grip. The block face was polished with the help of a glass knife. In each case light microscopy was undertaken to ensure that the specimens were orientated longitudinally, so that sectioning would result in true transverse micrographs. A 3mm DDK diamond knife mounted on the Reichert-Jung ultramicrotome was used to cut sections which in all cases showed an interference pattern of pale gold to silver (section thickness of ~80 nm). The sections, stretched using chloroform vapours to reduce contraction effects, were collected on 400 mesh unsupported copper grids, which had been immersed in a chloroform-sellotape cement prepared by dissolving sellotape adhesive in chloroform. This was done to ensure that the sections were not washed away during staining. The grids were routinely stored in grid boxes prior to staining.

2.3 Staining Methods :

Standard staining procedures have been used in this work. An alcoholic solution of uranyl acetate was prepared using saturated uranyl acetate in 50% ethanol. Lead citrate stain was prepared using the method of Sato (1967). Droplets of stain were put onto parafilm and the grids were then floated on the surface of the droplets. Each grid was stained in uranyl acetate for ten minutes, washed with 50% ethanol to remove excess uranyl acetate, and then given a secondary wash with distilled water to remove excess alcohol. Post-staining was achieved using lead citrate as a secondary stain for ten minutes. This was followed by

a wash with distilled water, to remove excess lead citrate. The grids were then allowed to air dry before they were examined in the electron microscope.

2.4 Electron Microscopy :

Electron micrographs were obtained using a Philips TEM 201C transmission electron microscope at the electron microscopy laboratory in the Horticulture and Food Research Institute of New Zealand, Palmerston North (see Figure 2.2).

Electron microscopy has significant advantages over light microscopy in many biophysical investigations since it employs an incident beam of much shorter wavelength than light and hence a greater inherent resolving power is obtained. Although light microscopes and optical processing can, in principle, give a comparable level of magnification as the electron microscope, light microscopy cannot provide the same resolution. Commercial electron microscopes commonly use a tungsten filament as a thermionic electron source as a part of an electron gun, whose emission is controlled by a Wehnelt cylinder. The electron beam is produced by accelerating electrons with anode applied voltages ranging from 50-100 kV in a high vacuum column maintained at 10^{-5} torr or ~1Pa. Electromagnetic lenses are preferred to electrostatic lenses in commercial electron microscopes, as they can be manufactured having smaller imaging defects than the latter. The magnetic fields used to focus the electrons are strong, highly localized and coaxial with the column. Although electrons travel in a helical path through these magnetic lenses, this motion does not affect the focusing ability of the lens since the "envelope" of electrons emerging from the lens is analogous to the "light-envelope" emerging from a converging lens (Siegel, 1965). The ray diagrams for electron beam passing through the system of magnetic lenses are thus similar to those of light beams passing through optical lenses, and the formulae used in light-optics are equally applicable to electron-optics (see Figure 2.3).

The magnification of the electron microscope was consistently set at 11X throughout any particular series of electron micrographs. However after each set of micrographs was

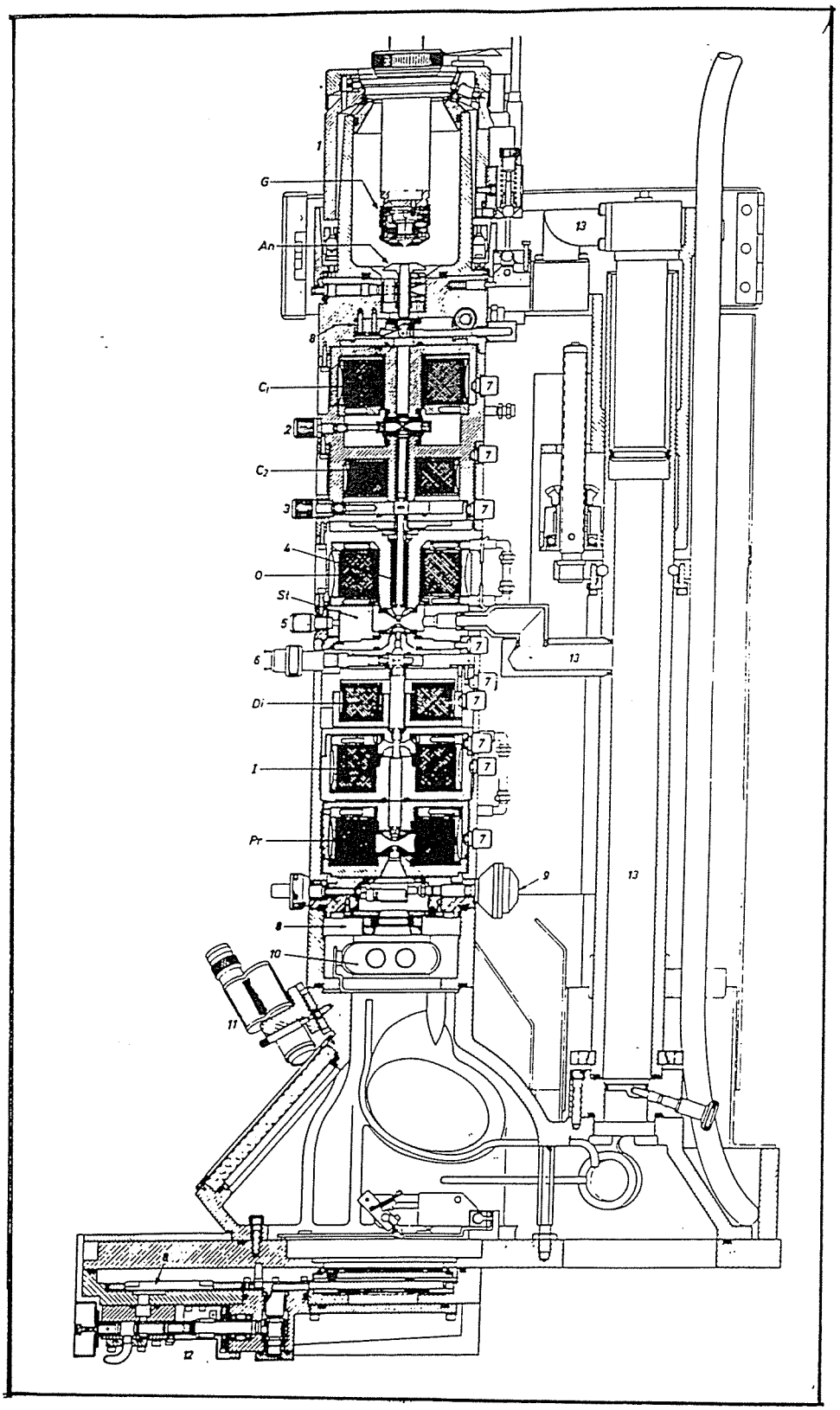


Figure 2.2 Detailed section of a Philips electron microscope column. G-gun; An-anode; C1 and C2-condenser lenses; O-objective lens; St-specimen stage; Di-diffraction lens; I-intermediate lens; Pr-projector lens; l-gimbal ring; 2, 3, 4, 5 and 6-diaphragm alignment controls; 4-supplementary objective lens coils for oblique illumination and focussing; 7-alignment controls for lens pole pieces; 8-vacuum valves; 9-shutter; 10-roll-film camera; 11-binocular microscope; 12-plate camera; 13-vacuum manifold.

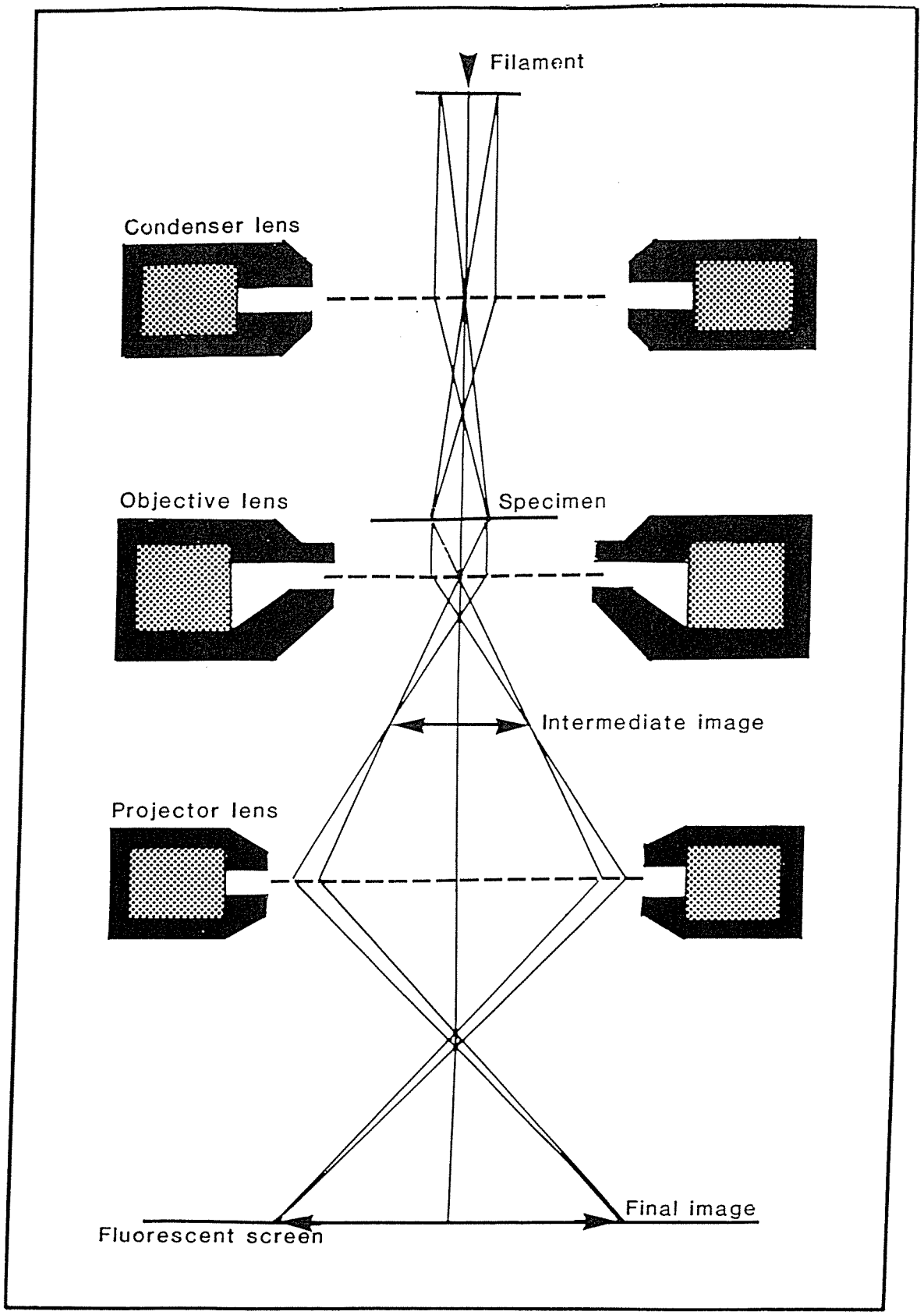


Figure 2.3 Simplified ray diagram for an electron microscope. The condenser lens forms part of the illuminating system and focuses the electron beam on to the specimen. The objective lens magnifies the intermediate image which is further magnified by subsequent lenses (only one shown here) to form an image which can be viewed on a fluoroscent screen.

obtained, an additional micrograph of a 2160 lines/mm diffraction cross-grating replica was taken as a magnification reference. This was done without altering the set magnification, thus eliminating any possible changes in magnification caused by the fluctuations in high tension voltage or changing currents in the electromagnetic lenses of the microscope. All the transmission electron micrographs were recorded on 35mm fine grain positive film (Agfa Copex PET-10), and developed in a high contrast developer (Kodak D19 - modified to D19B).

2.5 Mensuration Methods :

Micrographs were chosen which displayed arrays of collagen fibrils and minimal cellular and non-collagenous components. All collagen fibril diameter measurements were made from thin sections of transversely sectioned fibrils. As far as possible, four measurements were taken (no less than two) from the grating replica micrographs (two pairs at right angles), and the mean of these measurements was recorded as a magnification reference. Fibrils appearing elliptical in a micrograph, were considered to be obliquely cut and the minor diameter was measured. Diameters were measured directly from the micrographs magnified 76 000 times. An engraved metallic rule was used to measure the collagen fibril diameters to the nearest 1 mm. Narrow distributions of the scaphocapitate ligament were obtained by measuring the collagen fibrils diameters directly off the negative under a dissecting microscope, fitted with an eye piece graticule. This was essential as the fibrils were too small to be measured accurately from the prints, and an erroneous distribution could have resulted. The number of fibril diameter measurements necessary to obtain a distribution that would not change significantly in form with increasing numbers of measurements was determined using the guidelines set down by Parry et al (1978), which are as follows,

d_m/d	Number of fibril diameter measurements required
1	100
1.05 - 1.2	300
1.3 or more	1000

where "d_m" is the mass-average diameter and "d" is the mean diameter.

Hence, for sharp unimodal distribution of diameter a minimum of 100 measurements were recorded, and for broad or multimodal distributions, upwards of 1000 measurements were taken to ascertain the true form of the distribution. From these distributions the mean diameter and the mass-average diameter were calculated using the expression

$$d = \Sigma n_i.d_i / \Sigma n_i \quad \text{----------(1)}$$

$$d_m = \Sigma n_i \, . \, d_i^3 / \Sigma n_i \, . \, d_i^2 \quad \text{----------(2)}$$

"n_i" is the number of fibrils whose diameter was measured as "d_i". Histograms of mass average diameter vs diameter, and number of fibrils vs diameter was plotted using Cricket Graph™ on an Apple Macintosh computer.

Chapter 3. RESULTS

Tendon and ligament collagen have an important function in providing mechanical strength to connective tissue as discussed in Chapter 1. This function is achieved only after collagen molecules have aggregated into fibrils, and fibrils into fibres. Intermolecular crosslinking is also of crucial importance in stabilizing the fibrils. Electron micrographs of collagen fibrils from the ligaments studied in this work have been prepared along with the corresponding collagen fibril diameter distribution and their mass-average diameter histograms. This has allowed age-related observations to be made on the variations in the collagen fibril diameter distributions, and for the related mechanical properties to be assessed. The values for the mean diameter, standard deviation and the mass-average diameter of the collagen fibrils are given in Table 3.1.

3.1 Lateral Collateral Ligament :

The ligament from the one-year-old horse shows a broad bimodal collagen fibril diameter distribution. A significant percentage (~65%) of fibrils in the larger diameter mode are seen here (see Figure 3.1a, 3.2a). In the two-and-a-half year old horse the distribution remains bimodal (Figure 3.1b, 3.2b), but the largest diameter fibrils previously seen are no longer present. At five years of age the larger diameter fibrils again seem to have decreased a little in number, though it is not clear that this observation is statistically significant (Figure 3.1c, 3.2c). At six years of age the collagen fibril diameter distribution is still bimodal, but there is a marked decrease in the number of fibrils in the larger diameter mode. (Figure 3.1d, 3.2d). At eleven years of age the fibril diameter distribution is comparable to that seen for the six-year-old specimen (Figure 3.1e, 3.2e). Fibrils, however, often appear to be irregular in shape.

TABLE 3.1

TABLE SHOWING THE MEAN DIAMETER, MASS-AVERAGE DIAMETER AND THE STANDARD DEVIATION OF THE COLLAGEN FIBRILS.

Ligament Type	Age (years)	Mean Diameter (nm)	+/-Standard Deviation	Mass Average Diameter (nm)
Medial collateral ligament	1	127.8	56.4	169.4
	2.5	100.9	69.3	180.6
	4*	124	68.5	190.1
	5	116.3	69.7	184.3
	6	88	64.8	168.7
	11	83.3	39.1	115.5
Lateral collateral ligament	1	123.9	60.1	175.9
	2.5	80.1	48.5	131.8
	4*	89.9	58.7	161.3
	5	74.4	47.2	127.7
	6	59.7	38.7	109
	11	64.5	53.7	145.7
Radioscaphoid ligament	1	74.7	39.1	108.4
	2	122.5	60.4	169.3
	4*	128	55.9	168.8
	(5	55.1	38	96.6)
	6	100	58.1	149.5
	11	82.9	37.8	109.5
Lateral pisoformo metacarpal	1	100.3	40.5	127.6
ligament	2.5	94.6	54.7	146.4
	4*	91.4	48.4	138
	5	74.2	50.6	129.8
	6	88.4	46.4	125.6
	11	67.8	46.7	121.3

(continued)

Ligament Type	Age (years)	Mean Diameter (nm)	+/-Standard Deviation	Mass Average Diameter (nm)
Scaphocapitate ligament	1	41.2	7.2	43.8
	2.5	54.5	20.7	68.4
	4*	53.7	13.7	61.1
	5	52	10.7	56.4
	6	58.6	17.5	69.4
	11	53.6	18.9	67.4

* The values for the 4 year old horse have been obtained from N. J. Deane's M.Phil thesis, 1991.

Parentheses indicate a value which does not fit the pattern established by other results.

Reasons for the anomaly are discussed in the text.

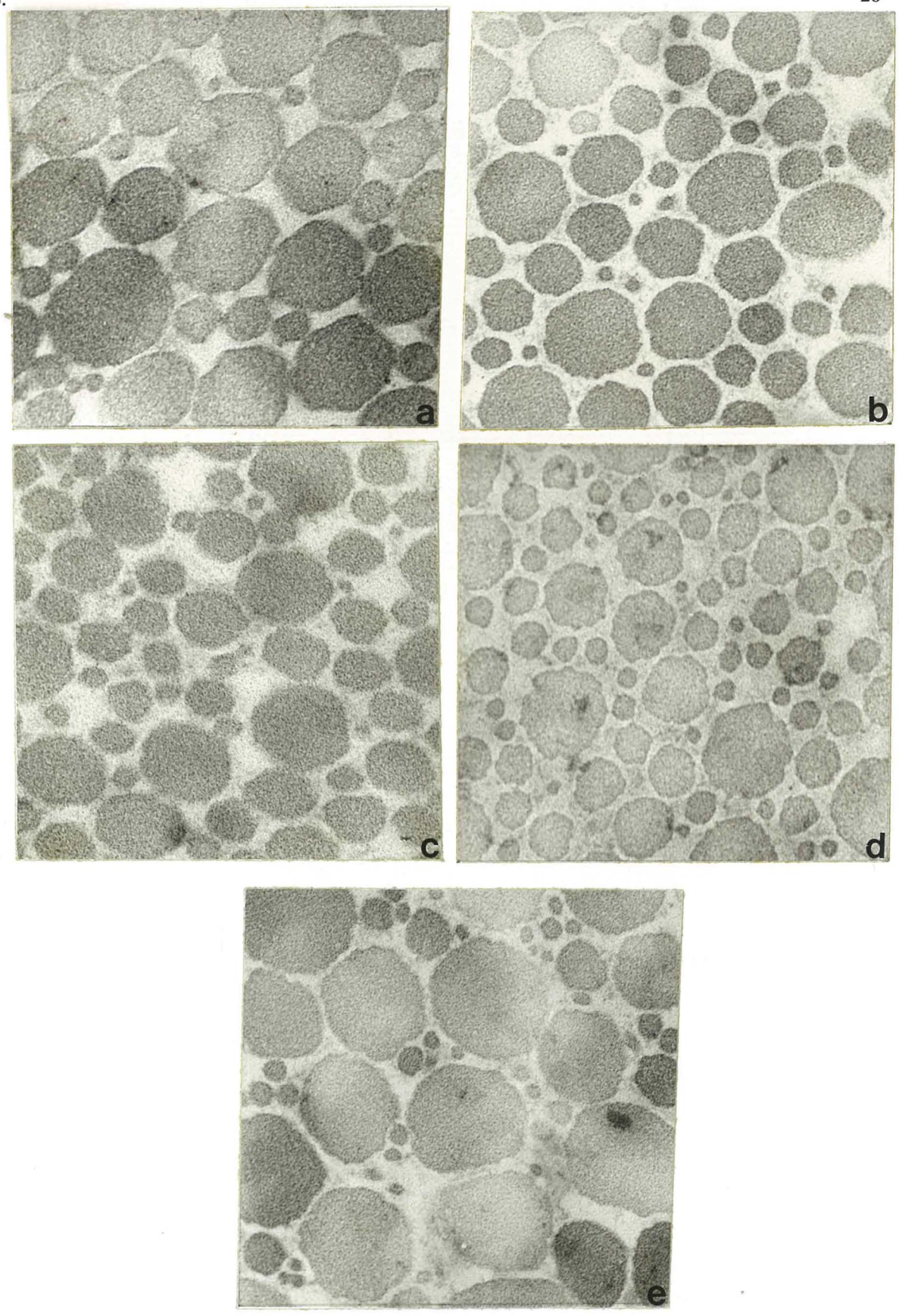


Figure 3.1: Transverse section of collagen fibrils from the lateral collateral ligament from horse at the ages of (a) 1 year (b) 2.5 years (c) 5 years (d) 6 years (e) 11 years. The micrographs show the changing collagen fibril diameter distributions reflected by the histograms in Figure 3.2 . Magnification : 76 000X

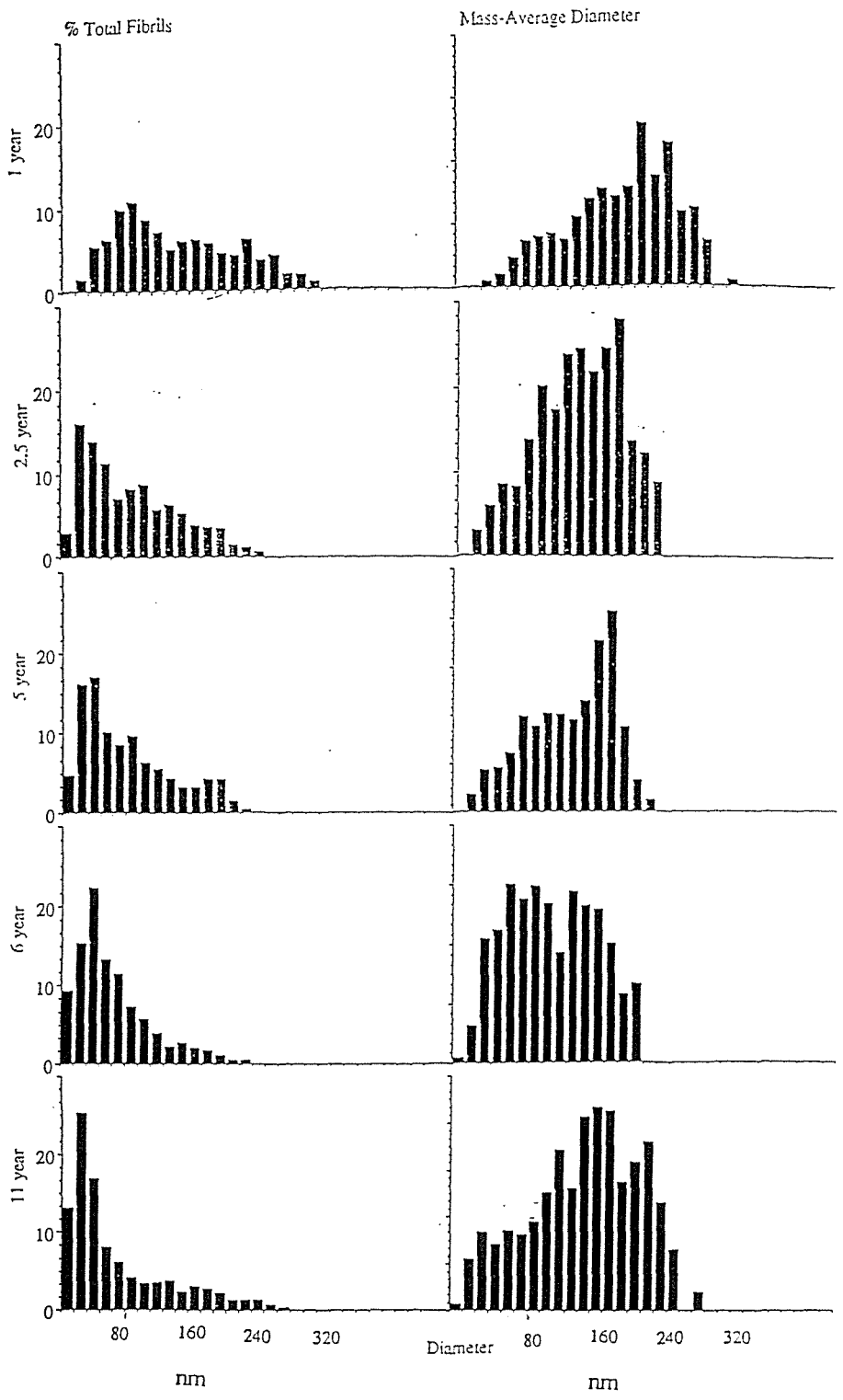


Figure 3.2. Collagen fibril diameter distribution and mass-average diameter distribution for the lateral collateral ligament at the ages (a) one year, (b) two and a half year, (c) five year, (d) six year and (e) eleven year.

3.2 Medial Collateral Ligament :

A unimodal distribution of fibril diameters is observed at the age of one year though very few fibrils have a diameter > 100 nm (see Figure 3.3a, 3.4a). At two and a half years of age, however, there is a large percentage of collagen fibrils whose diameter is greater than 100 nm. The distribution becomes bimodal and is very broad at this stage (Figure 3.3b, 3.4b). At five years of age, when the horse can be considered as mature, a further percentage increase in the number of larger diameter fibrils is observed, the distribution remaining both bimodal and broad (Figure 3.3c, 3.4c). The collagen fibril diameter distribution from the six-year-old horse shows a decrease in the percentage of larger diameter fibrils (and a corresponding higher percentage of smaller diameter fibrils). The fibril diameter distribution at this stage is still bimodal, although it is less broad than it was previously (Figure 3.3d, 3.4d). At eleven years of age the fibril diameter distribution is unimodal and is narrower than at two and a half, five or six years of age. The form of the distribution may be compared to the distribution at one year (Figure 3.3e, 3.4e). It seems that at old age there is a significant breakdown of the larger collagen fibrils into smaller units, similar to the one discussed by Parry *et al* (1984).

3.3 Radioscaphoid Ligament :

A bimodal and a fairly broad distribution of collagen fibril diameters is observed in this one-year-old ligament from the horse (see Figure 3.5a, 3.6a). At two and a half years of age the distribution becomes more clearly bimodal and is noticeably broader than at one year (Figure 3.5b, 3.6b). A similar trend is observed in the case of the five-year-old ligament (Figure 3.5c, 3.6c) though the distribution is very clearly anomalous (see Table 3.1). At six years of age, however, the distribution is narrower than at two and a half years, but does remain bimodal in form (Figure 3.5d, 3.6d). The percentage of collagen fibrils in the smaller diameter mode at this stage is quite low. The large reduction in the percentage of the largest diameter collagen fibrils is very conspicuous at eleven years of age (Figure 3.5e, 3.6e).

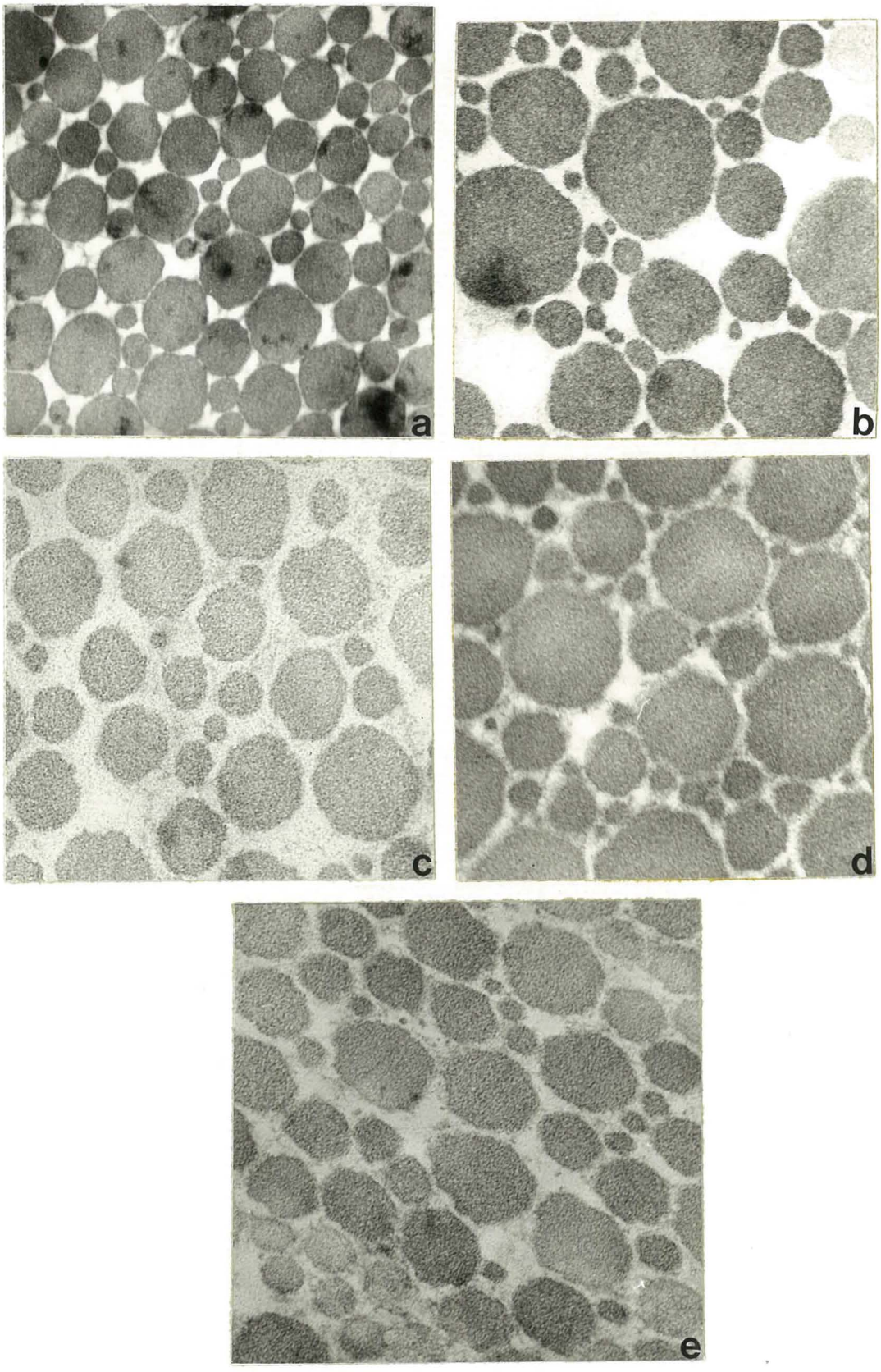


Figure 3.3: Transverse section of collagen fibrils from the medial collateral ligament from horse at the ages of (a) 1 year (b) 2.5 years (c) 5 years (d) 6 years (e) 11 years. The micrographs show the changing collagen fibril diameter distributions reflected by the histograms in Figure 3.4 . Magnification : 76 000X

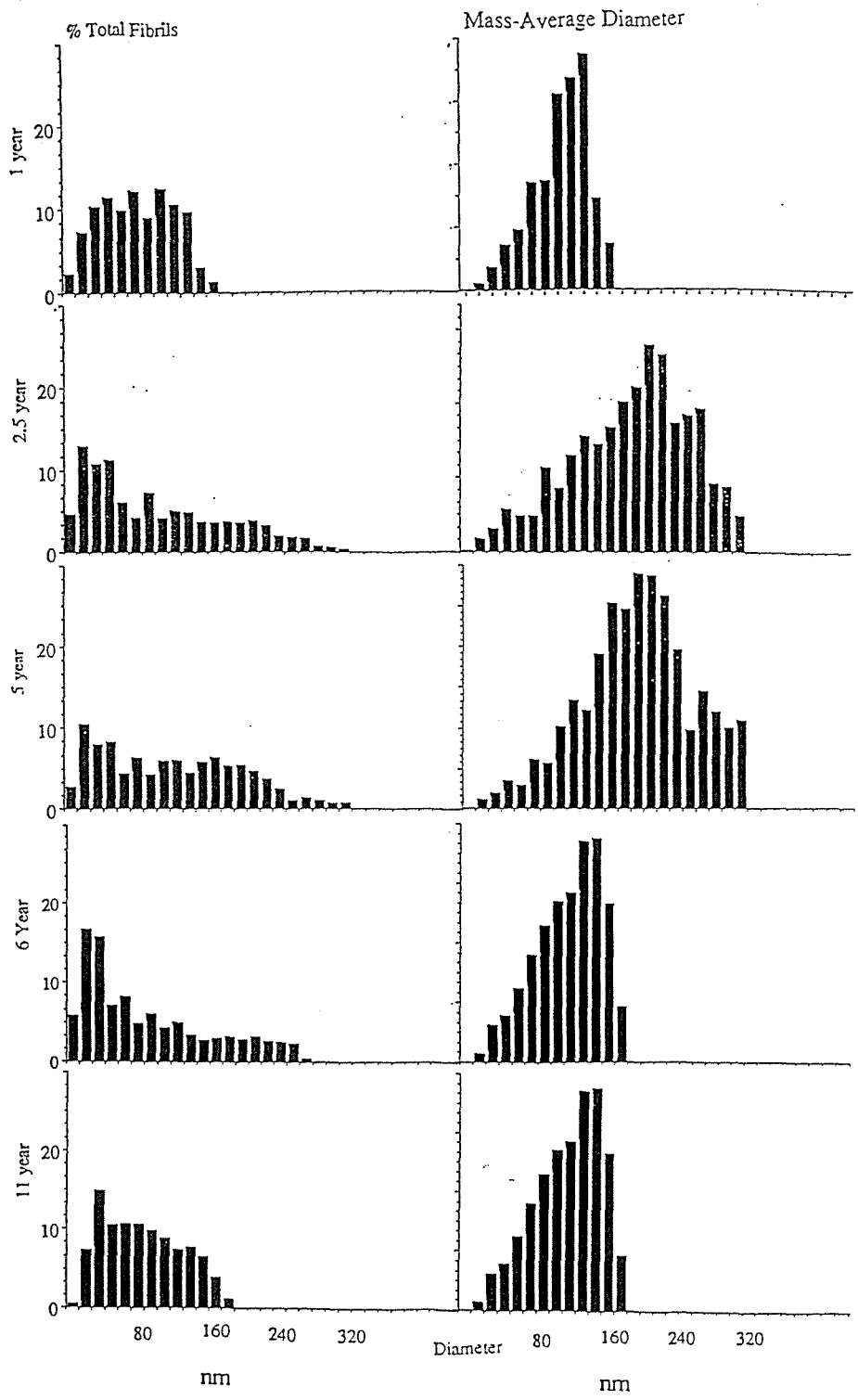


Figure 3.4. Collagen fibril diameter distribution and mass-average diameter distribution for the medial collateral ligament at the ages (a) one year, (b) two and a half year, (c) five year, (d) six year and (e) eleven year.

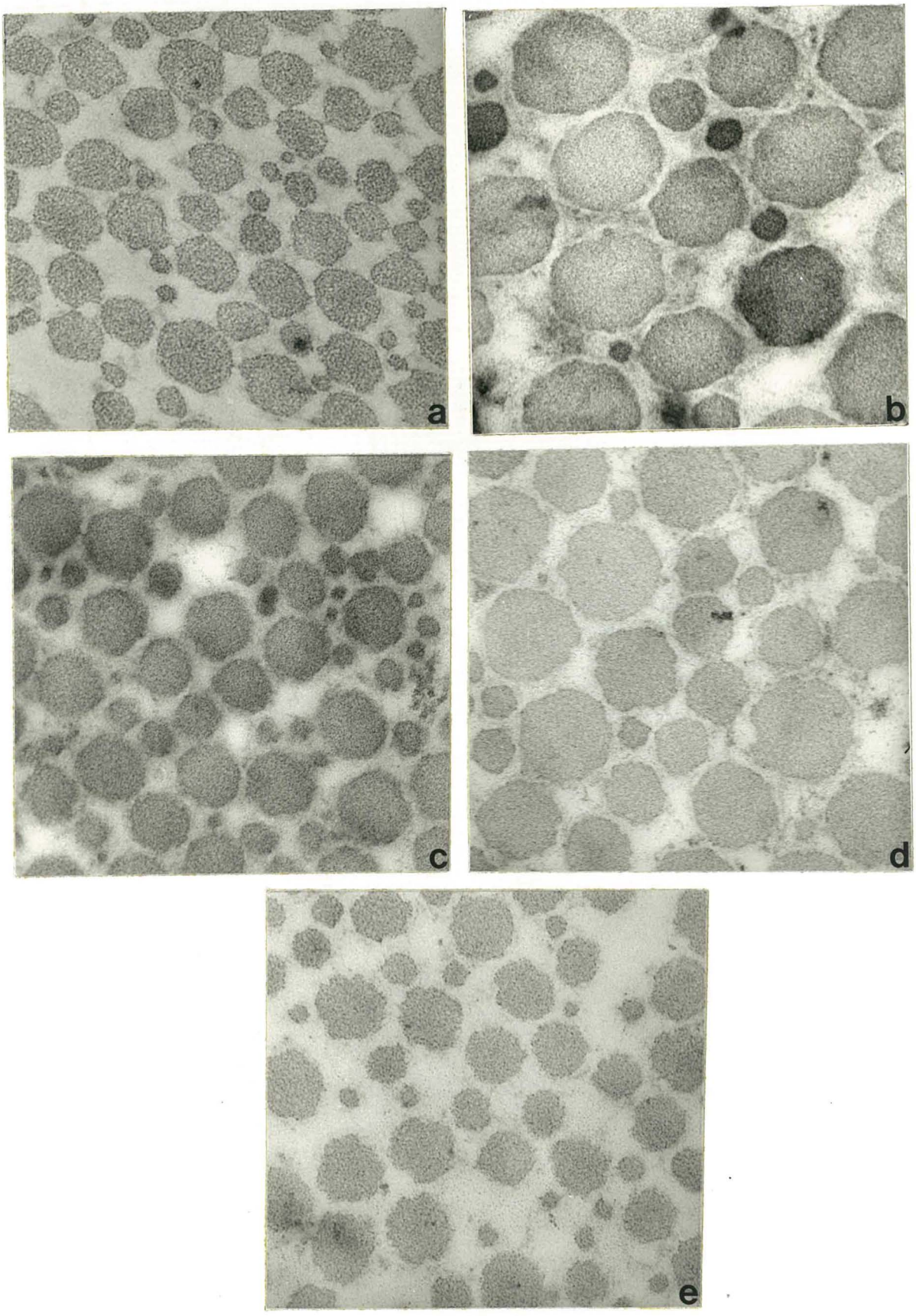


Figure 3.5: Transverse section of collagen fibrils from the radioscaphoid ligament from horse at the ages of (a) 1 year (b) 2.5 years (c) 5 years (d) 6 years (e) 11 years. The micrographs show the changing collagen fibril diameter distributions reflected by the histograms in Figure 3.6 . Magnification : 76 000X

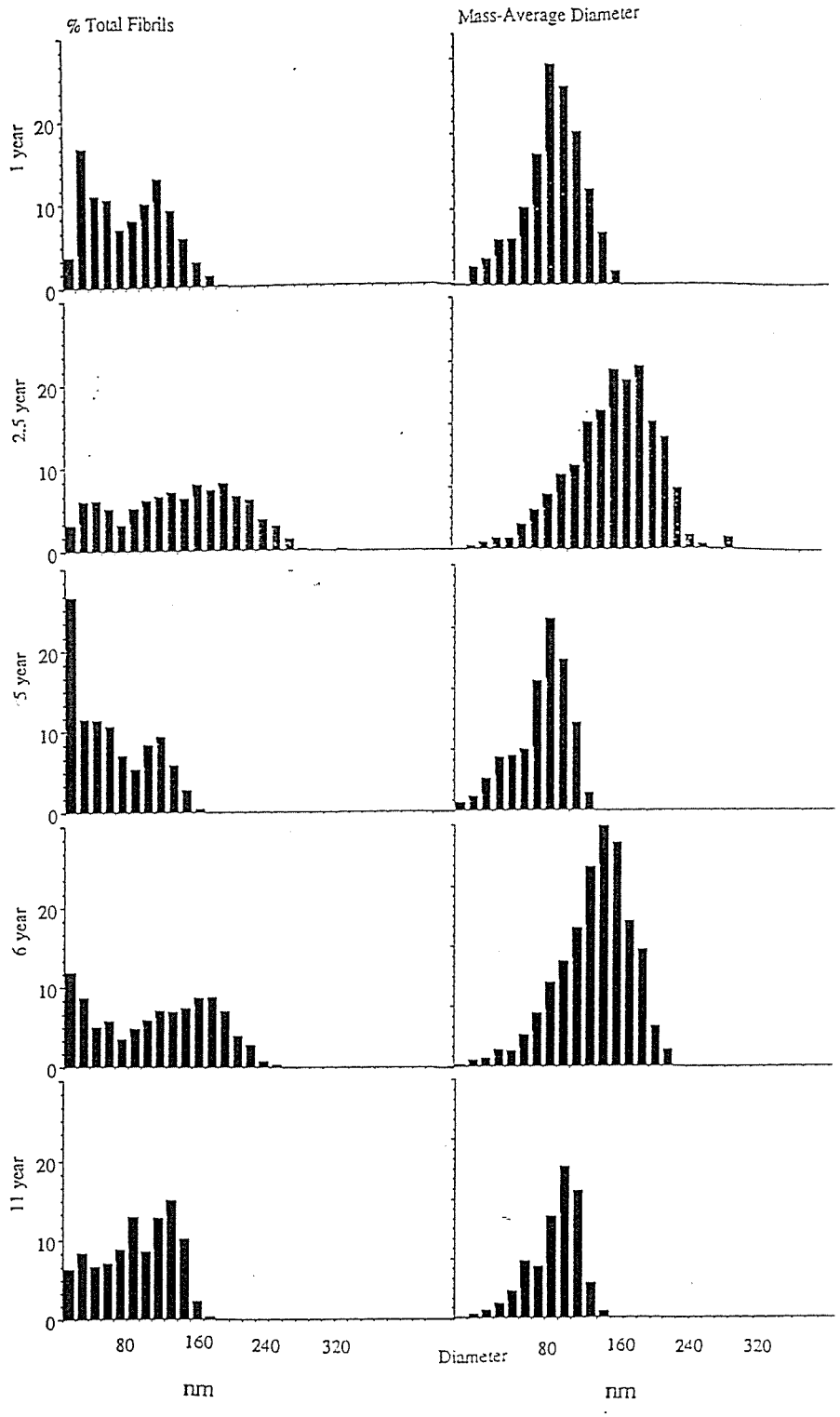


Figure 3.6. Collagen fibril diameter distribution and mass-average diameter distribution for the radioscaphoid ligament at the ages (a) one year, (b) two and a half year, (c) five year, (d) six year and (e) eleven year.

3.4 Lateral Pisoformometacarpal Ligament :

As expected, a unimodal collagen fibril diameter distribution is observed for this one-year-old equine ligament (see Figure 3.7a, 3.8a). Following the general trend established by some of the other ligaments, the distribution broadens and appears bimodal at two and a half years of age (Figure 3.7b, 3.8b). A significant population of fibrils in the larger diameter mode is observed. Similar observations were recorded for the ligament from the five-year-old horse (Figure3.7c, 3.8c). However at six years of age, the distribution is less obviously bimodal (Figure3.7d, 3.8d) though at eleven years of age the distribution is again quite clearly of this form (Figure 3.7e, 3.8e). A higher percentage of fibrils in the smaller diameter mode is found here when compared to the other ages studied. This may be attributed to the breakdown of large diameter fibrils into smaller units.

3.5 Scaphocapitate Ligament :

A different trend has been observed in this ligament. In this one-year-old ligament the collagen fibril diameter distribution was exceptionally sharp and unimodal (see Figure 3.9a, 3.10a). The mean diameter was found to be 53.7 nm. At two and a half years of age, the collagen fibril diameter distribution was broader, though not nearly as broad as the distribution for the other ligaments. (Figure 3.9b, 3.10b). The form of the distribution was still unimodal. A similar unimodal distribution is observed in the five and six year old ligaments though the distributions are broadened further as compared to previous ages (see Figures 3.9c, 3.10c and 3.9d, 3.10d). However at eleven years of age the fibril diameter distribution is little changed from that recorded at six years of age (Figure 3.9e, 3.10e).

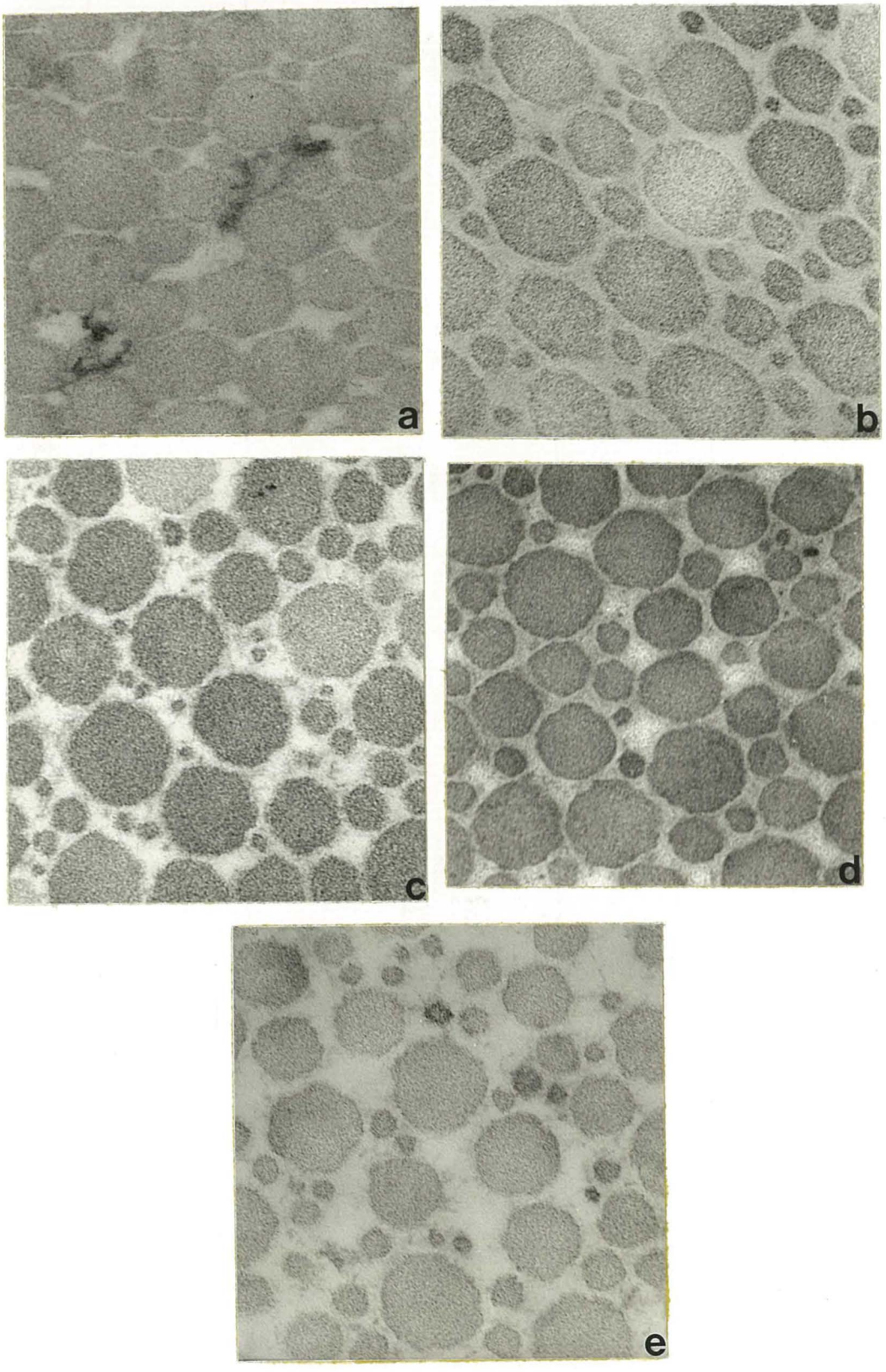


Figure 3.7: Transverse section of collagen fibrils from the lateral pisoformometacarpal ligament from horse at the ages of (a) 1 year (b) 2.5 years (c) 5 years (d) 6 years (e) 11 years. The micrographs show the changing collagen fibril diameter distributions reflected by the histograms in Figure 3.8 . Magnification : 76 000X

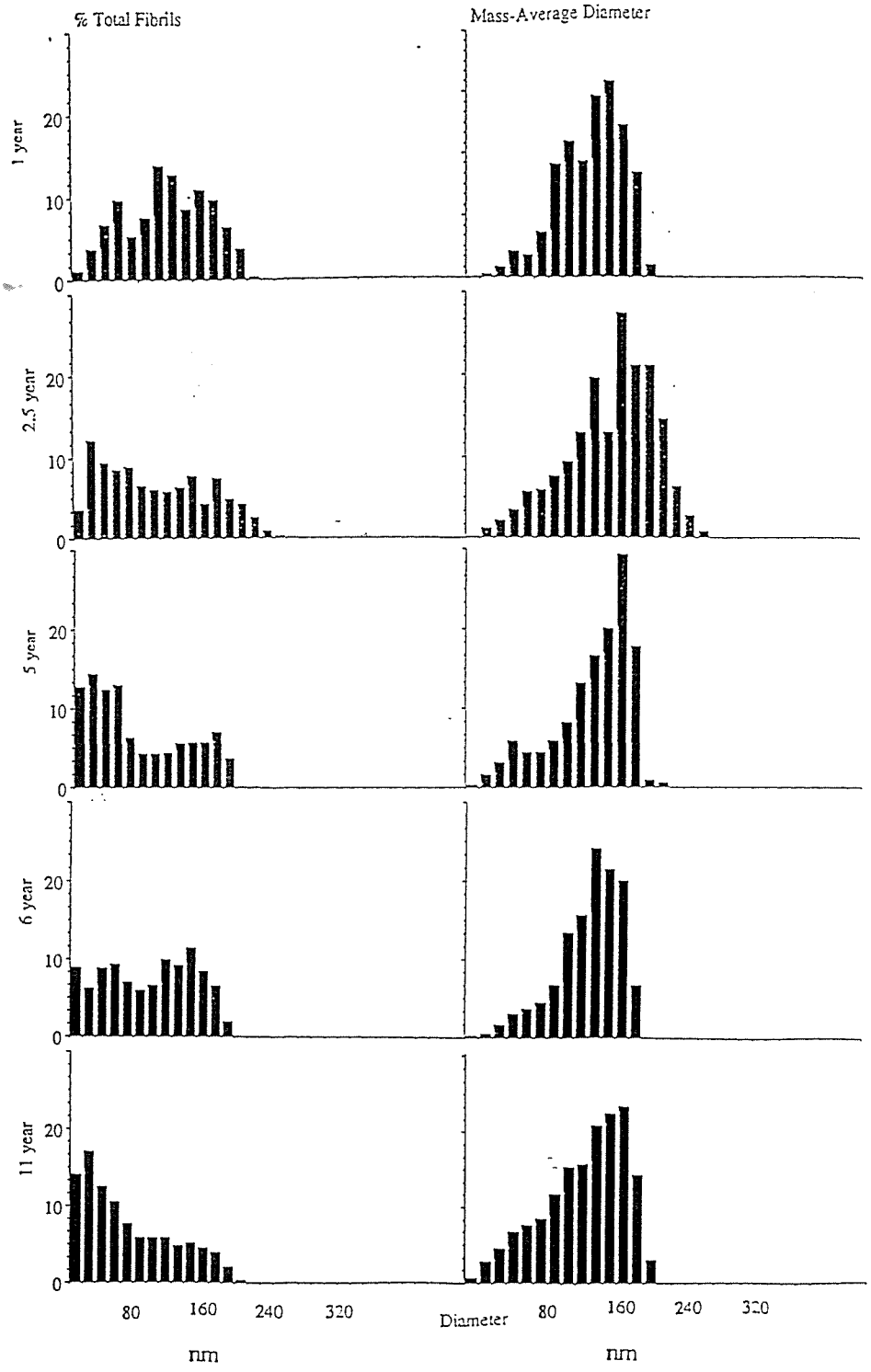


Figure 3.8. Collagen fibril diameter distribution and mass-average diameter distribution for the lateral pisoformometacarpal ligament at the ages (a) one year, (b) two and a half year, (c) five year, (d) six year and (e) eleven year.

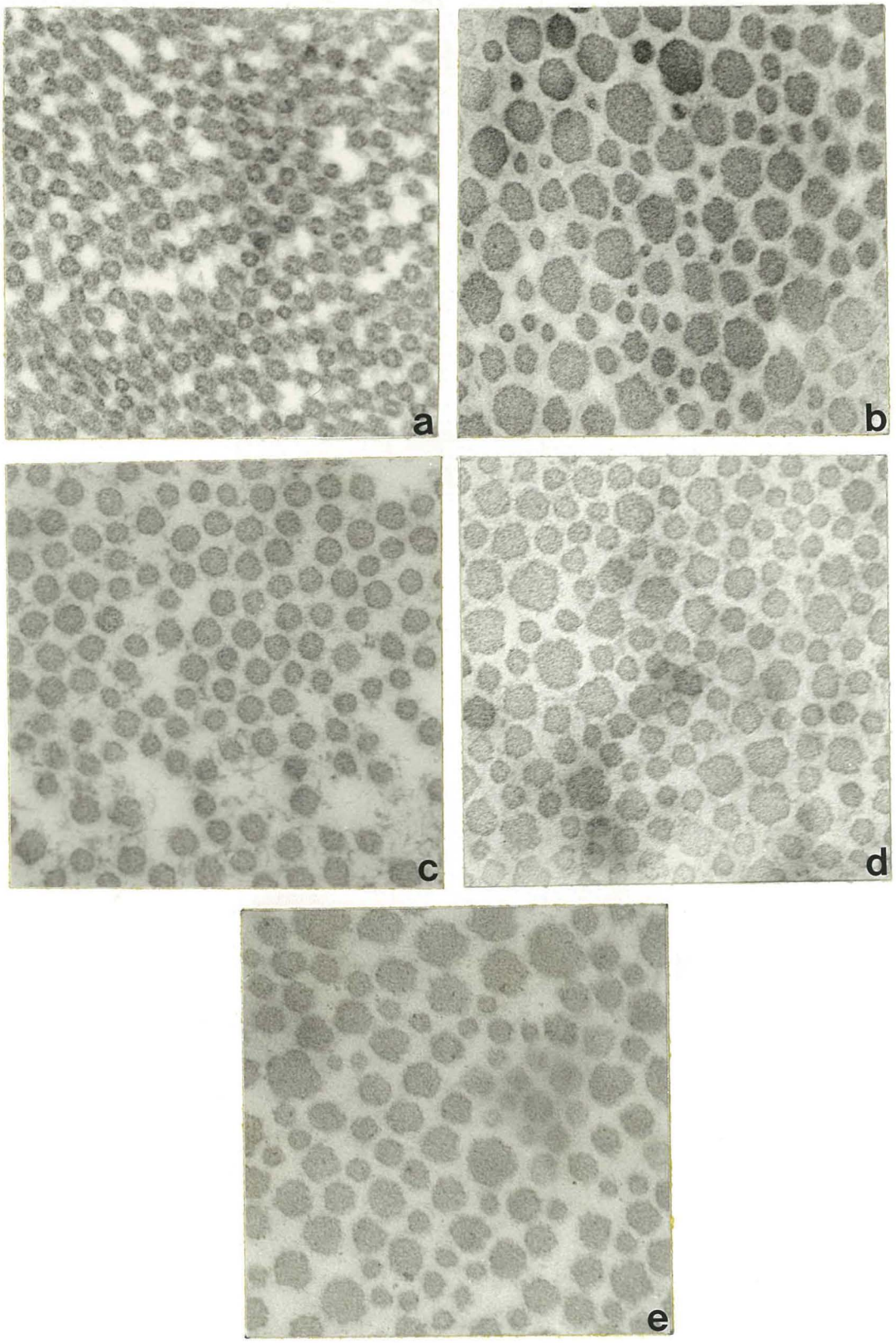


Figure 3.9: Transverse section of collagen fibrils from the scaphocapitate ligament from horse at the ages of (a) 1 year (b) 2.5 years (c) 5 years (d) 6 years (e) 11 years. The micrographs show the changing collagen fibril diameter distributions reflected by the histograms in Figure 3.10 . Magnification : 76 000X

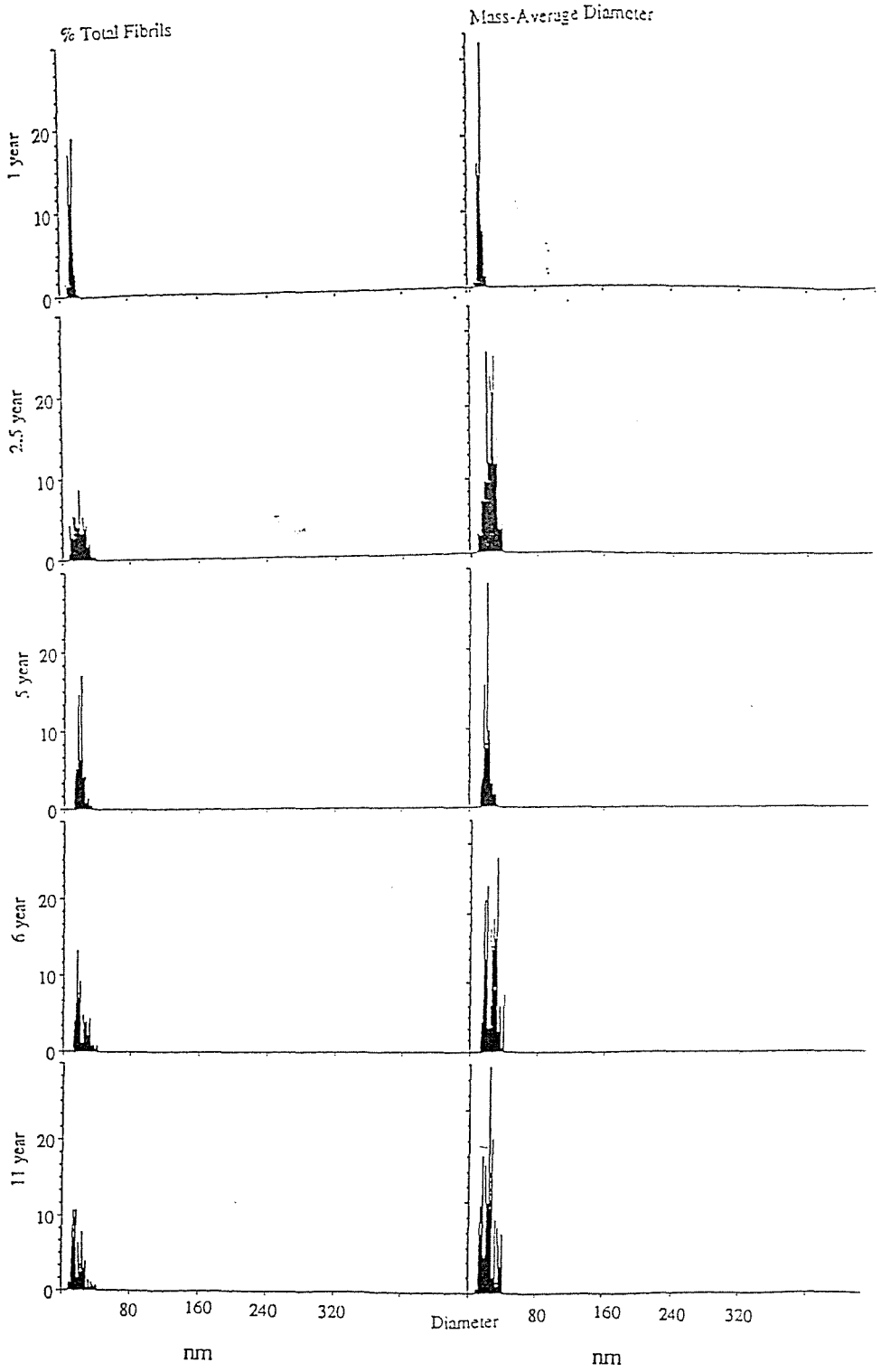


Figure 3.10. Collagen fibril diameter distribution and mass-average diameter distribution for the scaphocapitate ligament at the ages (a) one year, (b) two and a half year, (c) five year, (d) six year and (e) eleven year.

Chapter 4. DISCUSSION

The mechanical properties of connective tissues have been found to depend upon a number of factors such as the type of tissue, its age, nature of intramolecular and intermolecular covalent crosslinks, type and quantity of glycosaminoglycans associated with the collagen fibrils and the content of elastic fibres, water and minerals. It is thus difficult to attribute a precise role to any one of the components in such a complex system. However, since the most common constituent (other than water) in most connective tissues is collagen, its primary role has been more easily perceived. This work is mainly concerned with the interpretation of the distribution of transverse dimensions of collagen fibrils and its correlation with age. Where possible an attempt has also been made to obtain an idea about the stress levels to which the tissues under consideration are likely to be subjected.

Parry *et al* (1978a, 1978b) carried out an extensive age-related study on the transverse dimensions of collagen fibrils from tendons and ligaments of horse and rat-tail tendon. They were able to gain new insights regarding the mechanical properties of connective tissues by observing the form of the collagen fibril diameter distribution as the tissue aged from the foetal stage to senescence. A general trend was observed regarding the form of collagen fibril diameter distribution. In all tissues the distribution was unimodal at birth. At maturity the distributions of many tissues broadened and became bimodal. On the basis of experimental observations by Vogel (1974, 1978, 1979) Parry *et al* (1978) noticed a trend showing that the tensile strength of connective tissues increased manyfold between birth and maturity, but tended to decrease a little at senescence (see Figure 4.1). The number and mass distributions of the collagen fibril diameters were used to calculate a mean and a mass-average diameter respectively. Parry *et al* (1978a) put foward a hypothesis proposing that "the ultimate tensile strength of connective tissues are positively correlated with the mass-average diameters of the collagen fibrils". A similar approach has been used to form the basis of interpretation of results from this work. In a normal distribution, an increase in the

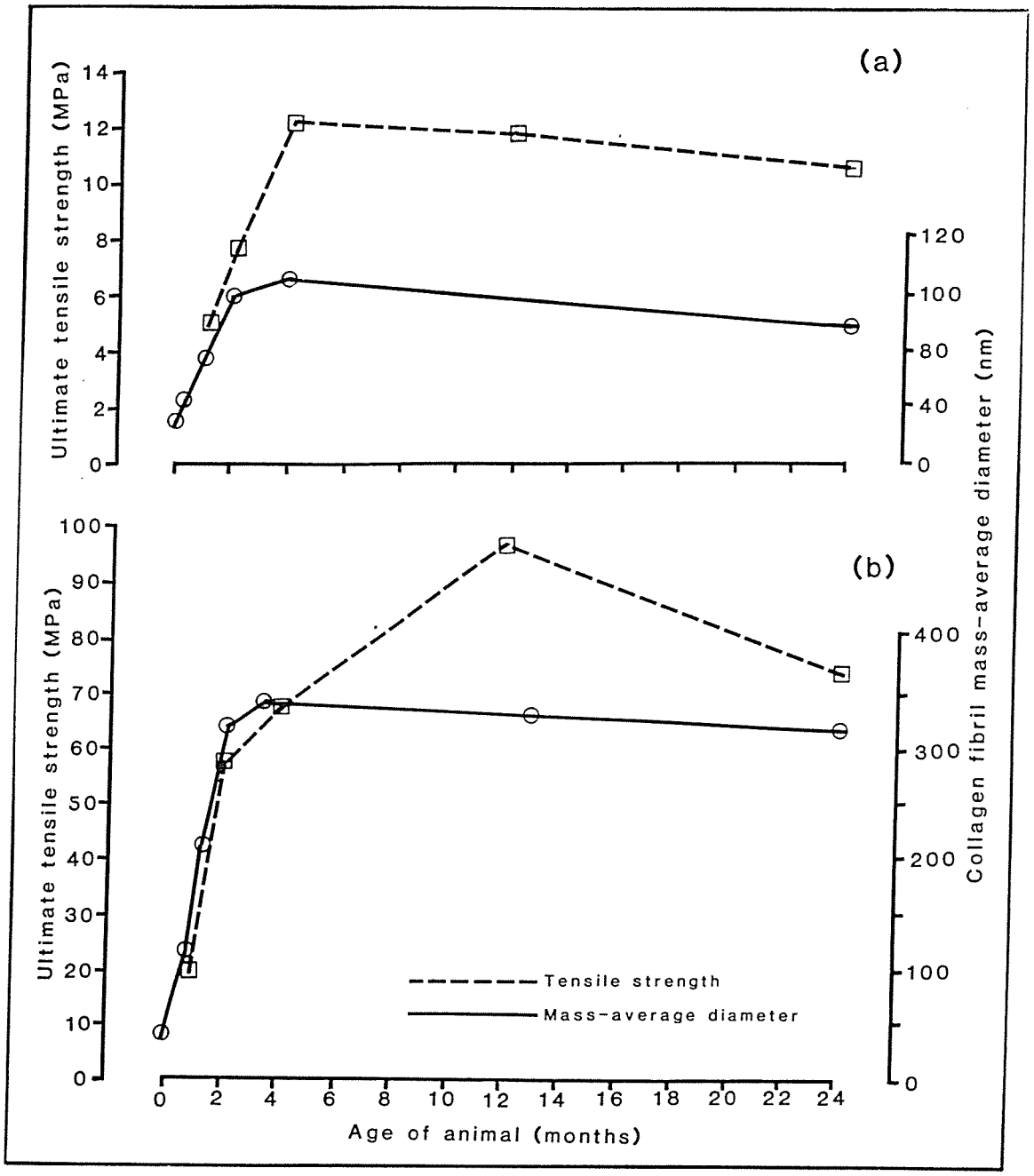


Figure 4.1 Graphs of (a) tensile strength and mass-average collagen fibril diameter versus age for rat skin, and (b) tensile strength and mass-average collagen fibril diameter versus age for rat-tail tendon.

mean diameter parallels an increase in the mass average diameter. This correlation, however, was not found to exist in distributions which are skewed or bimodal. Collagen fibril diameter distributions are a function of both the applied stress and its duration. Tissues that are subjected to long-term high stresses have been found to have a bimodal distribution of collagen fibril diameters. A unimodal form of distribution is observed where the tissues are subjected to short-term stresses. Distributions are broad if the range of stresses is high and narrow if the range of stresses is low. Barnes and Pinder (1974) had observed that the common digital extensor tendon from the horse endures short term stresses. Studies on the collagen fibril diameter distributions from the common digital extensor tendon of the horse by Parry *et al* (1978) showed that the form of distribution was unimodal. The suspensory ligament from horse, the rabbit *flexor digitorum profundus* tendon and the human Achilles tendon are all subjected to long term stress and in each case, the distribution of collagen fibrils was found to be bimodal.

In a tissue designed to withstand high stress levels, the tensile strength of most of the components in that tissue must be high. If larger diameter rather than smaller diameter collagen fibrils form the bulk of the collagenous mass in such a tissue, then the potential density of intrafibrillar covalent crosslinks is enhanced (Parry *et al*,1978). Hence it was proposed that the tensile strength of larger diameter collagen fibrils will be greater than that of an equivalent mass of collagen in the form of small diameter fibrils. Another important property that a tissue often requires is elasticity. This enables the tissue to return back to its original length and shape after the removal of applied stress. If a tissue subject to long-term stress suffers from irrecoverable creep, the functional role of the tissue would be jeopardised. Parry *et al* (1978) proposed that creep inhibition is achieved through the presence of smaller diameter collagen fibrils. Since the surface area of these fibrils is much higher than larger diameter collagen fibrils, the possible density of interactions (electrostatic or otherwise) between the collagen fibrils and the hydrated glycosaminoglycan matrix will be greater and the collagen fibrils will be bound together more tightly. The result of the

increased density of interactions between the collagen fibrils and the matrix will be to stiffen the tissue and this may help to prevent changes in shape after the removal of applied stress. Consequently the authors put forth the hypothesis that "the mechanical properties of a connective tissue are strongly correlated with the collagen fibril diameter distribution." Parry *et al* (1980) were able to support this hypothesis experimentally by stretching tendons until they ruptured. Electron micrographs of these ruptured tendons showed areas with varying degrees of fibril damage. It was observed consistently that the larger diameter collagen fibrils break down in preference to the smaller diameter collagen fibrils. This observation is also in agreement with the hypothesis of Parry *et al* (1978a) who proposed that at a given strain, the larger diameter collagen fibrils will suffer a higher stress than the smaller diameter collagen fibrils.

4.1 DISCUSSION OF RESULTS :

The discussion below pertains to the results obtained from this work. As expected, similar trends to those previously discussed have been observed for the collagen fibril diameter distributions from all five ligaments under consideration. In all cases (except the lateral collateral ligament and the radioscaphoid ligament) the collagen fibril diameter distribution was unimodal when the ligaments were one-year-old. As the tissues matured the distribution changed form and all (except the scaphocapitate ligament) became bimodal. This, presumably, reflected the use of the tissue and the stress levels to which they were subjected. Stress levels acting on the tissues are presented here qualitatively only as the scope of the thesis did not allow quantitative mechanical data to be obtained. The stress levels experienced by the tissues is directly dependent on their function and position within the carpus. In the following part of the discussion an attempt has been made to analyze the collagen fibril diameter distributions from these ligaments and possible information

regarding their mechanical properties has been obtained according to the work of Parry *et al* (1978a, 1978b).

a) **Lateral collateral ligament :**

The lateral collateral ligament extends distally from its attachment on the styloid process of the radius and provides stability to the carpus (along with the medial collateral ligament) during flexion of the radiocarpal joint and the middle carpal joint. The one-year-old lateral collateral ligament shows a bimodal distribution which is indicative that the tissue is already subjected to long-term stress. A comparison of the width of this distribution with those from other ligaments at the same stage of development indicates that a higher range of stress levels are present. At two-and-a-half years of age, the mass-average diameter dropped to about 130 nm (from 176 nm), whilst the distribution remained bimodal. This drop resulted from the fact that there has been a considerable reduction in the percentage of large diameter collagen fibrils in the diameter range of 210 to 300 nm between one and two and a half years of age. Stress levels may thus be expected to be lower than in the one-year-old ligament. At five, six and eleven years of age, the mass-average diameter remains at a similar value to that recorded at two and a half years of age, as does the general form of the distribution of diameters.There is, however, a small rise in the mass-average diameter at eleven years and a small drop at six years, but the cause for such variation probably lies within the sampling errors generally experienced in an electron microscope study of this kind. The four year old tissue reported by Deane (1991) also follows the expected pattern (see Table 3.1).

The general trend followed by the collagen fibril diameter distribution is similar to that of the suspensory ligament and the superficial digtal flexor tendon (Parry *et al* 1978a) which are known to experience long-term stresses of high levels. Hence this work indicates that the lateral collateral ligament would be expected to experience a similar range of stress levels.

b) **Medial collateral ligament :**

The medial collateral ligament extends from the medial styloid process of the radius and widens distally to attach to the proximal ends of the second and third metacarpal bones. This ligament, along with the lateral collateral ligament, governs the flexion of the radiocarpal joint and the middle carpal joint. The collagen fibril diameter distribution for this ligament at one year of age is unimodal. Such a distribution indicates that it is subjected to short term stress. The distribution appears to be neither very sharp nor very broad. This may indicate that a moderate range of stress levels are present at this stage of life. However there was a marked difference in the collagen fibril diameter distribution of this ligament in the two-and-half-year-old horse. The distribution was distinctly bimodal, and there was a small but significant increase in the mass-average diameter of the collagen fibrils. The distribution was quite broad with values ranging from 10 to 300 nm. The distribution characteristics imply that at this point of development the ligament is exposed to long-term stresses at higher levels than experienced at one year of age. At five and six years of age when the horse is mature, the mass-average diameter differs little from that recorded at two and a half years of age. The distribution remained bimodal and broad, implying that similar stresses are present on the ligament as at the previous age. At eleven years of age the mass-average diameter drops significantly and the distribution is similar to that obtained at one year of age. A high percentage of smaller diameter fibrils are now seen and these may arise from the breaking down of larger diameter collagen fibrils into smaller subunits. Note also the work of Scott and Parry (1992) which proposed that fibril growth resulted from the aggregation of subfibrils of about 10 nm in diameter. It is reasonable to assume that fibril breakdown may represent the reverse process. Values obtained for the mean and the mass-average diameter for a four-year-old medial collateral ligament (Deane, 1991) follow the trend established by the results presented here.

The trend followed by the collagen fibril diameter distribution of this ligament is again similar to that of the suspensory ligament and the superficial digital flexor tendon. This

increases the possibility that the type of stress levels present on the medial collateral ligament are similar to those experienced by the suspensory ligament and the superficial flexor tendon.

c) **Radioscaphoid ligament :**

The radioscaphoid ligament originates on the radius and onserts on the scaphoid distally. A bimodal distribution of moderate breadth is observed when this tissue is one year old. This would imply that moderate levels of stress are acting on the tissue at this stage of development. At two-and-a-half years of age, there was a significant increase in the breadth of collagen fibril diameter distribution, which became more distinctly bimodal. Higher stress levels of long-term duration would be expected to be present at this stage on the basis of the collagen fibril size distribution. An unexpected drop in the mass-average diameter is observed in the five year old tissue. This anomaly could be attributed to the tissue being diseased and hence non-representative, although this is uncertain. At six years of age the distribution of collagen fibril diameters became less broad though it remained bimodal, possibly indicating the presence of long-term moderate stress levels. There was a slight drop in the mass-average diameter. At eleven years of age the distribution appeared to be similar to that at one year of age. A marked drop in the mass-average diameter was observed. As expected, a high percentage of smaller diameter fibrils was observed, which may be a direct consequence of the break down of larger diameter collagen fibrils into smaller sub-units. The value obtained for this four-year-old ligament by Deane (168.8 nm) is in close agreement with the results obtained during this work. Thus long-term stresses are possibly present within this tissue, which is in keeping with the action of the carpus. This ligament is taut and under tension when the horse is standing (i.e. the carpus is extended) implying that long-term stresses are present.

d) **Lateral pisoformometacarpal ligament :**

This ligament is one of the set of four ligaments which supports the pisiform bone (accessory carpal bone). The unimodal distribution of collagen fibrils at one year of age is as expected for this ligament since short-term stresses of moderate levels would be expected to act on this ligament at this stage of development. At two-and-a-half years of age, a significant rise in the mass-average of the collagen fibrils was observed. As the distribution at this stage was bimodal the ligament would be predicted to be subjected to long-term stresses. Indications of high stress levels are also given by the fact that the distribution was broad. At five and six years of age there was slight drop in the mass-average diameter of the collagen fibrils which arises from the decrease in the percentage of larger diameter collagen fibrils. The collagen fibril diameter distribution at this stage was still bimodal. At eleven years of age there is a further drop in the mass-average diameter of the collagen fibrils. A high percentage of smaller diameter collagen fibrils were now seen, which may be a result of the breakdown of the larger diameter fibrils.The value for the mass-average diameter from the same tissue in a four-year-old horse obtained earlier by Deane (138 nm) is in close agreement with the values obtained from this work at a similar age.

The broad and bimodal nature of the collagen fibril diameter distribution indicates that the lateral pisoformometacarpal ligament may well be subjected to high stress levels of long-term duration. This agrees with carpal anatomy: the ligament is taut in the extended carpus of the standing horse.

e) **Scaphocapitate ligament :**

The scaphocapitate ligament lies close to the axis of rotation of the middle carpal joint. It originates distally from the scaphoid but spirals and inserts into the capitate bone. As can be seen from the diameter distribution histograms (see Figure 3.10) a very different trend is shown by this ligament as it matures. At one year of age, the distribution is exceptionally sharp and unimodal. It is probable that it is subjected to only intermittent stresses at low

levels. At two-and-a-half years of age, the mass-average diameter increased, but the breadth of the distribution did not increase in a comparable manner seen for the other ligaments. A small rise in the level of stress to which the ligament is subjected would be expected. The distribution at this stage remained unimodal. At five, six and eleven years of age the distribution remained unimodal without any significant change in the mass-average diameter of the collagen fibrils. The value obtained for the mass-average diameter from the four year old horse reported by Deane (61.1 nm) is in good agreement with the values obtained during this work.

The collagen fibril diameter distribution for the scaphocapitate is similar to the ones found in tissues such as skin and cornea. Such a distribution thus suggests that low stress levels of short-term duration may be present in this tissue. Study of the carpal anatomy suggested that the scaphocapitate ligament is relaxed on extension of the carpus and is under tension only when the carpus is flexed (Deane, 1991). The results from this work supports this observation.

4.2 EFFECT OF TRAINING :

Horses bred and trained for the racing industry are subjected to rigorous training programmes. During this process severe stresses are experienced by the tendons and ligaments in the limbs of the horse. Overloading frequently results in injuries to such tissues. These usually occur at sites with the smallest cross-sectional area, possibly because the stress will be highest at such points (Fackleman, 1973), although this view has been refuted (Riemersma and Schamhardt, 1985). However, Gillard *et al* (1977) and Merrilees and Flint (1980) have shown that the structure and composition of rabbit flexor tendons are markedly different for different regions of the tendon. In particular they showed that the portion of the tendon in contact with the bone had many of the characteristics of a cartilage with regard to the cell structure, glycosaminoglycan content and type, and fibril size

distribution. In contrast, the outermost portion of the tendon contained elongate cells, had reduced glycosaminoglycan content, and contained larger diameter fibrils. Sorokin and Efimov (1980) also demonstrated that the morphological, histological and biochemical constitution of tendons of humans and dogs vary along their length.

Parry *et al* (1978) demonstrated that the fibrillar collagen content was different for the superficial digital flexor tendon, suspensory ligament and the common digital extensor tendon of the horse. Furthermore, Reimersma and Schamhardt (1985) have shown that the collagen content (amount of collagen fibres present) in the suspensory ligament is much lower than in the superficial digital flexor tendon, and that the modulus of elasticity for the suspensory ligament is lower than that for the superficial digital flexor tendon. Consequently it can be concluded that variations in histological and biochemical constitutions of tissues reflect their adaptations for taking up tensile loads as well as withstanding compression. These observations, made by various authors, show that the collagen content in a tissue is to a significant extent governed by the level of loading to which that tissue is subjected. All of these data imply that the structure and composition of the tendons and ligaments of a horse will differ significantly depending on function.

Flint *et al* (1980) have shown that the glycosaminoglycan levels vary depending upon the stress levels in the tissue. The rabbit *flexor digitorum profundus* tendon was studied by these authors. This tendon is subject to longitudinal tension forces throughout its length. Also it is subjected to compressional forces at the points where it is in contact with bone. Biochemical analysis of this tendon showed that in the tensional zone, dermatan sulphate was the dominant glycosaminoglycan, while in the pressure zone the dominant glycosaminoglycan was found to be chondroitin sulphate. *In vivo* modification of the tendon was achieved by translocating the tendon anteriorly, which subsequently modified the physical forces acting on the tendon. It was found that there was a rapid loss of chondroitin sulphate from the pressure zone and a marked reduction of glycosaminoglycan content in that area. During remodelling of the tensional regions, the glycosaminoglycan

content was found to increase during relaxation. Re-establishment of tensional forces throughout the length of the tendon effected an increase in the level of glycosaminoglycans. In particular the level of dermatan sulphate was established at a level comparable to that found in purely tension-transmitting tendon. Replacement of the tendon into normal anatomical and functional position resulted in a re-establishment of the normal glycosaminoglycan distribution. Such dynamic behaviour exhibited by the glycosaminoglycans will almost certainly take place in the horse ligaments when subjected to stress. This change in the glycosaminoglycans may be linked with the property of limiting the lateral dimensions of collagen fibrils to a certain diameter, depending upon the precise stress levels to which it is subjected.

Parry *et al* (1980) have shown that the collagen fibril diameter distribution changes catastrophically when a tendon is stretched to the point of rupture. It was observed that the larger diameter fibrils break down into sub-fibrillar components. A similar effect may occur in the tendons and ligaments of the horse due to overtraining, since these tissues will be subjected to higher stresses than those which are normally present. Prolonged application of excessive loads which can occur when a horse is overtrained may also cause collagen fibre slippage and alter the collagen fibril diameter distribution. A degradation of larger diameter collagen fibrils may also occur. Since these larger diameter collagen fibrils are mainly responsible for the mechanical strength of the tissue there will be a significant decrease in the tensile properties of the tissue rendering the tissue temporarily or, in the long run, permanently incapacitated.

SUMMARY.

This work presents an age-related study regarding the collagen fibril diameter distribution of the five ligaments (lateral collateral ligament, medial collateral ligament, radioscaphoid ligament, lateral pisoformometacarpal ligament, and scaphocapitate ligament), the limitations being imposed by the fact that only one horse from different age groups was used during the course of this study. The general trend observed here is in good agreement with the work done earlier. The mechanical strength, as deduced from the collagen fibril diameter distribution, tends to increase as the ligaments age towards maturity, though appears to drop as the ligament approaches senescence. This evident from the trend shown by the diameter distributions, which become bimodal and broad (except the scaphocapitate ligament) and display increasing mass-average diameters with increasing maturity. Such a trend further strengthens the hypothesis of Parry *et al* (1978) that larger diameter fibrils have a higher tensile strength than smaller diameter fibrils.

Another observation which is in accordance with work done earlier is the apparent breakdown of larger diameter fibrils at senescence. This may be due to the fact that the tissues are constantly under stress, and that during the transition from maturity to senescence a 'fatigue mechanism' may be taking place within the tissue (Parry *et al*,1984). This 'fatigue mechanism' could involve a rupturing of some of the covalent crosslinks between the molecules and may explain why irregularly shaped fibrils are commonly seen in older tissues. Electron micrographs of the eleven-year-old ligaments presented in this thesis show a high percentage of irregular fibrils. Such fibrils are thought to be more susceptible to enzymatic attack rather than "normal" ones, and could hence break down further and more readily into a population of smaller diameter fibrils.

Tissues which undergo long-term high stress require large diameter collagen fibrils for strength and smaller diameter collagen fibrils for creep-inhibitive properties. Collagen fibril diameter distributions of such tissues was found to be bimodal and broad. However, tissues

which do not experience high stress levels and need not inhibit creep absolutely gave a unimodal form of collagen fibril diameter distribution. This is in good agreement with the work of Parry *et al*, 1978a.

Complete age related studies such as the one carried out during this work will enable us to delineate the clinical significance of the tissues individually and to gain new information regarding its role and the function.

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