THE FIBRILLAR ORGANIZATION OF COLLAGEN IN CONNECTIVE TISSUE

A thesis presented in partial fulfilment
of the requirements for the degree of
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DEDICATION

This thesis is dedicated to my wife - Wendy

and our children

Michael, Kim, Kirsten and Hadley.

ABSTRACT

Although certain aspects of connective tissue structure have been studied in considerable detail, comparatively little effort has been devoted to studying one of the largest structural units present in most tissues - the collagen fibril. In this thesis electron microscope observations have been made on the transverse dimensions of fibrils from tissues as diverse as cornea, skin and tendon. Collagen fibril diameter distributions have been measured for such tissues from a wide range of animals - predominantly mammals, but also fish, amphibians, reptiles and birds - at varying stages of development. These data have allowed the growth of collagen fibrils to be studied quantitatively and their size distributions to be related to their mechanical attributes. Diseased tissues or tissues containing anomalous fibril diameter distributions have also been studied and, where possible, the data have been related to the altered mechanical properties of the tissue and to its mode of growth and development. In a coordinated study with other research workers, the content of the individual glycosaminoglycans in a tissue have been shown to be related to the mass-average diameters of the collagen fibrils in those tissues. These results provide a basis for understanding the feedback mechanism by which fibril size distributions may be modified in line with changing mechanical needs and indicate the fundamental steps in the growth and development of fibrils.

In addition to these studies, two other specific problems were addressed. In the first, the ultrastructure of a specialized connective tissue - the cornea - was studied in detail. By maintaining precise experimental protocols and measurement procedures it was shown, contrary to the previous data of others, that the

collagen fibrils in mammals, birds, reptiles, amphibians and cartilaginous fish were similar to one another but significantly different to the corneal stromal fibrils of the bony fish. Further studies, which indicated that the fibrils were constant in diameter across the width of the stroma, clarified previous results which had indicated a gradual change in diameter with varying depth in the stroma. An age-related study of fibril diameters in the cornea was also undertaken. The second problem investigated was the degree of shrinkage introduced during the preparative procedures for electron microscopy. In collaborative studies with others, X-ray and electron microscope observations were made on the same tissue in hydrated and dehydrated states respectively. Analyses of these data indicated that significant lateral shrinkage does indeed occur in fibrils from foetal or immature tissues as well as in mature tissues containing only small diameter fibrils. Throughout the thesis possible sources of artefact introduced by the technique of electron microscopy have been considered and the data interpreted conservatively.

ACKNOWLEDGEMENTS

Many people have helped me in a variety of ways to get this thesis into its present form.

In my earlier years at DSIR Keith Williamson introduced me to, and gave me sound guidance in, the principles and techniques of electron microscopy. Subsequently my Director, Ray Bailey, gave me the encouragement and provided the impetus for me to embark on this present course of study.

Throughout this thesis the experimental results obtained by electron microscopy have, where possible, been related to biochemical and X-ray diffraction data kindly made available to me by Barbara Brodsky, Eric Eikenberry, Michael Flint, Gerry Gillard and Isabel Williams. Gary Thomas and Bob Fletcher supplied me with histogram plotting and population deconvolution programs and together provided me with oft-needed statistical advice. Doug Hopcroft has been responsible for the excellent maintenance of the electron microscopes and Ray Bennett printed the micrographs. I was assisted by many friends in typing the manuscript but it was June Tipoki who bore the lion's share of this chore.

Finally, and of greatest importance to me, my research colleague and supervisor, David Parry, not only instigated this project but displayed an un-ending enthusiasm for it. I am indebted to his necessary and continual encouragement throughout my writing-up.

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CHAPTER ONE

THE COLLAGENOUS COMPONENT OF CONNECTIVE TISSUES

1.1 Introduction

Collagen is a truly ubiquitous fibrous protein. It is the major protein constituent of all vertebrate species and has been estimated to account for about 25% of all the body protein in mammals (Harkness, 1961). Collagen occurs in a variety of macroscopic forms in tissues as diverse as skin, tendon, blood vessels, cartilage, bone, cornea, vitreous humour and basement membranes, and is present in the framework and interstices of all other tissues and organs with the exception of blood, lymph and the keratinous tissues. In most cases the dominant role of the collagen is to provide the tissue with a structural integrity; the most singular feature which permits collagen to play this supportive role is the high tensile strength of its fibrils. This property is due to its unique molecular conformation which is bestowed upon it by the regular repeating units in the amino acid sequence, the highly specific alignment and packing of the molecules in the fibrils in the extracellular matrix and the axial and lateral cohesion afforded by the formation of intermolecular covalent crosslinks. Knowledge of the structure of the collagen molecule is therefore of considerable importance if the mechanisms of fibrillogenesis, the modes of fibril growth and the properties of collagenous tissues are to be understood. There have been many extensive reviews of collagen structure and function to date (see for example: Traub and Piez, 1971; Miller, 1976; Fietzek and Kuhn, 1976; Ramachandran and Ramakrishnan, 1976; Glanville and Kuhn, 1979;

Bornstein and Traub, 1979; Fraser et al., 1979a; Bornstein and Sage, 1980; Bailey and Etherington, 1980; Brodsky and Eikenberry, 1982; Parry and Craig, 1984).

1.2 The Structural Hierarchy of Collagen

1.2.1. The Collagen Molecule

The collagen molecule is composed of three polypeptide α-chains. Each α-chain adopts a left-handed polyproline type helix with three residues per turn (Cowan and McGavin, 1955). The three similarly-directed α-chains coil about a common axis to form a right-handed triple-helical structure with a supercoil pitch length of ~8.6 nm (Ramachandran and Kartha 1955; Fraser et al., 1983; see Figure 1.1), a width of 1.5 nm and a length of ~300 nm. Short non-helical extensions of five to 25 amino acids are found at both the amino- and carboxy-terminal ends of the chains. One third of all the residues in the helical portions of the α-chains are glycine, such that the sequence can be represented by $(Gly\text{-}X\text{-}Y)_n$ (where n = 338 for Type I collagen) and 25% of the residues X and Y are accounted for by the imino acids proline and hydroxyproline. The nature of the three-fold helix of the individual α-chains is such that the glycine residues all lie along one edge. Consequently when the chains come together to form the triple-helical collagen molecule, the glycine side-chains (which consist of but single hydrogen atoms) are all directed towards the core of the molecule, thus facilitating chain-packing. In addition interchain hydrogen-bonds stabilize the structure thus adopted. Initial work suggested that the helix was stabilized by one interchain hydrogen-bond per triplet (Traub et al., 1969). It was also suggested that at least one water bridge per

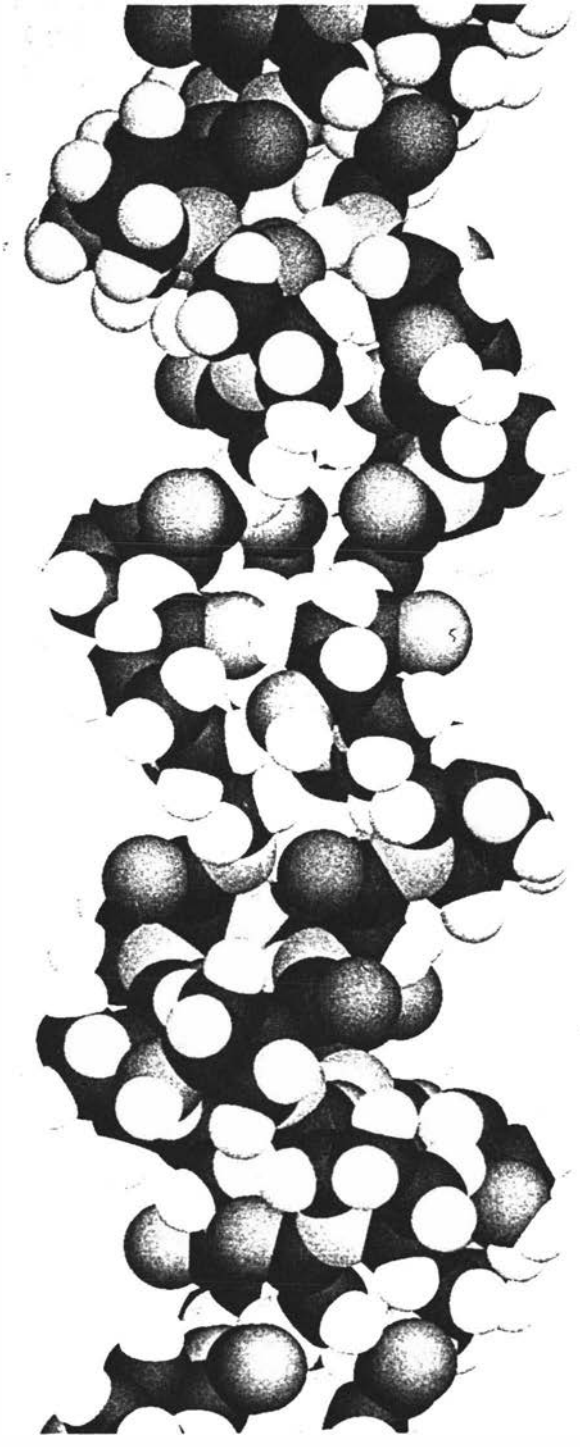

Figure 1.1 Space-filling model of the collagen molecule as refined by Fraser et al., 1983. Three similarly directed α-chains coil about the axis and form a right-handed triple-helical structure with a supercoil pitch length of the order of 8.6 nm. This figure has kindly been provided by Dr. R.D.B.Fraser, CSIRO Division of Protein Chemistry, Melbourne.

triplet could be formed and that this may involve hydroxyproline residues (Traub, 1974). Refinement of the molecular structure of collagen (Fraser et al., 1979a, 1979b) has confirmed the presence of a single interchain hydrogen-bond per triplet but has also indicated that hydrogen-bonded networks of water molecules between the carbonyl groups of the Y and glycine residues are involved in the stabilisation of the molecule.

Prior to helix formation there is a selective hydroxylation of many of the prolyl and lysyl residues. This is followed by glycosylation of many of the resulting hydroxylysine residues (Fessler and Fessler, 1978). In the helical portion of the molecule, the saccharides galactose or glucosylgalactose are attached via a glycosidic linkage to the hydroxyl groups of specific hydroxylysine residues. Preprocollagen molecules are synthesised not only with the terminal non-helical portions which exist in collagen molecules, but also with both carboxy- and amino-terminal extension peptides (see Figure 1.2) which are removed by specific peptidases in the extracellular processing prior to fibrillogenesis. The cellular biosynthesis of collagen molecules and their extracellular assembly into fibrils is summarized in Figure 1.3.

A number of genetically distinct "types" of collagen α-chain have been chemically characterized. The predominant form of collagen in connective tissues is known as Type I collagen. This molecular species contains two $\alpha 1(I)$ chains and one $\alpha 2$ chain and thus a molecular composition designated $[\alpha 1(I)]_2\ \alpha 2$. In contrast Type II and Type III molecules each contain three identical α-chains known as $\alpha 1(II)$ and $\alpha 1(III)$ respectively. The designations of these and further genetically distinct collagen types and a list of tissues in

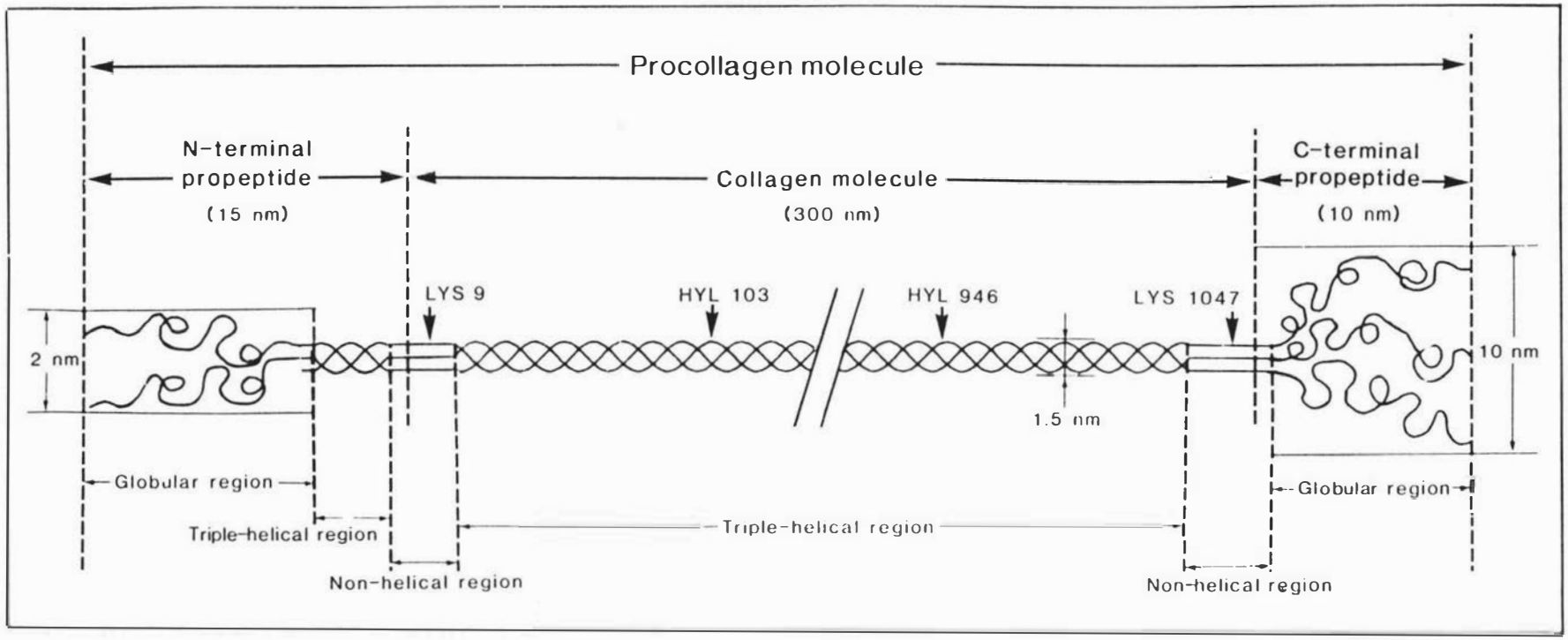

Figure 1.2 Schematic representation of the structure of the procollagen molecule (after Prockop et al., 1979). The symbols Lys 9, Hyl 103, Hyl 946 and Lys 1047 represent the sites of the lysine and hydroxylysine residues which take part in the intermolecular covalent crosslinks.

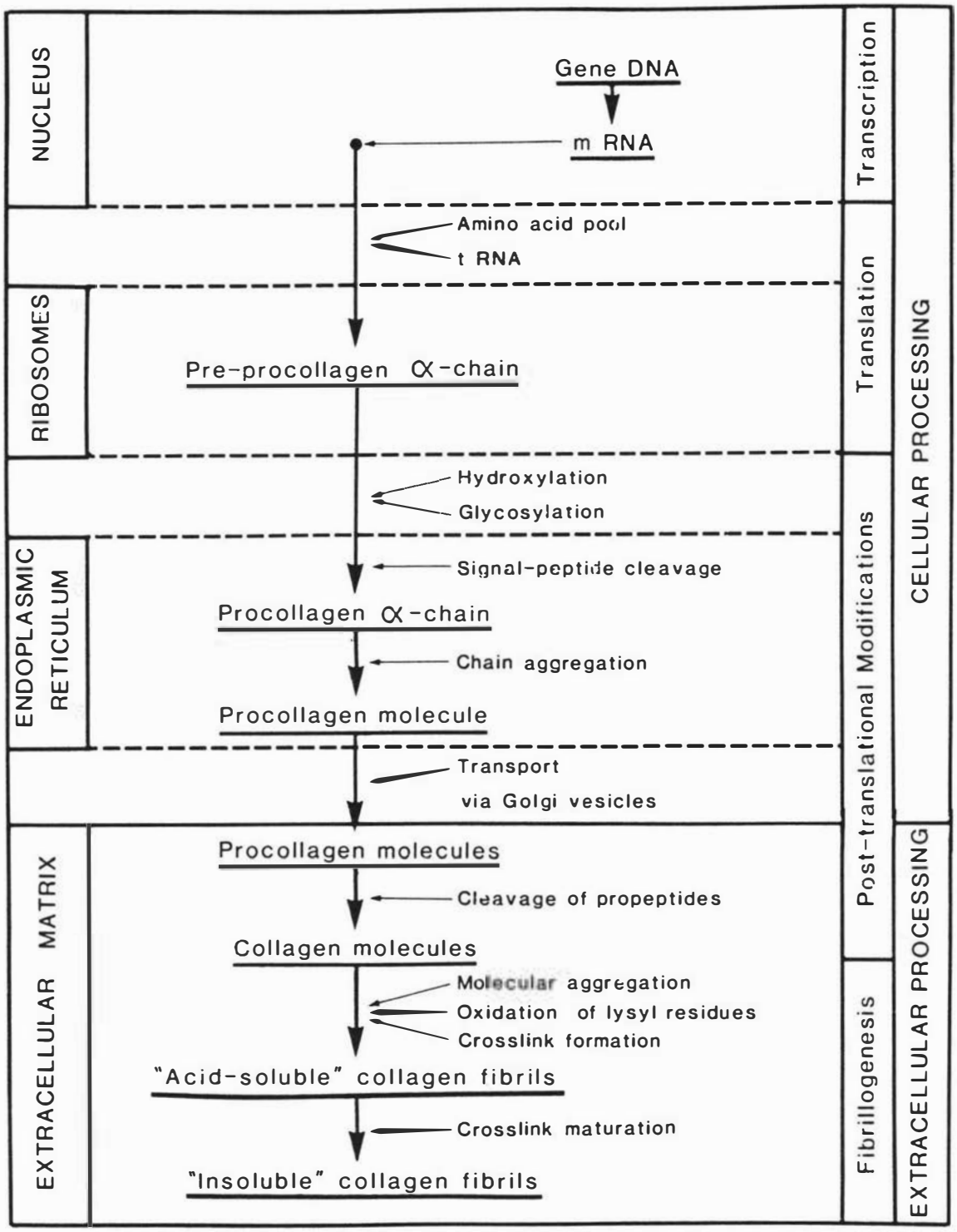

Figure 1.3 Schematic representation of collagen biosynthesis and fibril formation.

which they occur is given in Appendix 1.

The differing α-chains are characterized not only by their amino acid sequence but also by their differing chain lengths and differing degrees of glycosylation. Type I collagen has 1014 residues in the helical (triplet) regions of the α1(I) and the homologous α2(I) chains while Type III collagen has 1023 residues in the helical regions of its α1(III) chains. Type I and Type III collagens have a carbohydrate content of about 0.4% whereas there is about 4% carbohydrate in collagen Type II. In the latter case about 40% of the hydroxylysine residues are glycosylated (Miller, 1971). With the exception of the carboxy-terminal telopeptide in the α2(I) chain, the amino- and carboxy-telopeptide regions of the α-chains in Type I, II and III collagen molecules each contain a lysine residue involved in forming a covalent crosslink. In Type I collagen such crosslinks are formed between hydroxylysine 103 in collagen molecule 1 and the carboxy-terminal telopeptide lysine 1047 in molecule 2 and between hydroxylysine 946 of molecule 2 and the amino-terminal telopeptide lysine 9 of molecule 1 (see Figure 1.2; Glanville and Kuhn, 1979; Light and Bailey, 1979).

Type IV collagen has a much higher carbohydrate content (~10%) than the other molecular species. It also has a total triplet length which is greater than either Type I or Type III collagen but it is believed that the major triplet segment has frequent interruptions giving rise to discontinuities in the helix (Schuppan et al., 1980). Such interruptions will possibly cause greater flexibility of the molecule (Timpl et al., 1981) and may be responsible for its apparent lack of ability to form D-periodic fibrils. Timpl et al., (1981) using rotary shadowing have shown that Type IV collagen molecules

aggregate to form a lattice of side ~800 nm.

1.2.2. The Collagen Fibril

Collagen fibrils observed by electron microscopy have a regular cross-striated (banded) appearance (Hall et al., 1942; Schmitt et al., 1942; Wolpers, 1943) with the period of banding being designated by the symbol D (~67 nm). This D-period has been confirmed many times from X-ray diffraction studies on native hydrated connective tissues (see for example Bear, 1942; Miller and Parry, 1973; Brodsky and Eikenberry, 1982). The detailed electron microscope appearance of the D-period is dependent on the method of staining; negative-staining results in one predominantly dark staining band and a second predominantly light staining band each ~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 (Gross and Schmitt, 1948; Nemetschek et al., 1955; Bruns and Gross, 1974).

Using these data the arrangement of molecules within the collagen fibril was proposed by Schmitt et al., (1955); they suggested that the molecules were staggered axially by a distance D with respect to their neighbours. It was thought that the molecules were in end-to-end contact and that the length of the molecule was equal to 4D. This model formed the basis of the classical "quarter-stagger" hypothesis. Later, Hodge and Petruska (1963) determined the length of the molecule more accurately as 4.4D and this implied that gaps ~0.6D (40 nm) exist between molecules axially staggered with respect to one another by 5D. This model has a D-period containing an "overlap region" of 0.4D and "gap region" of 0.6D and this accounts well for

the light and dark bands seen in electron micrographs of negatively stained fibrils. More recent determinations of these parameters suggest that the molecule, including the telopeptides, is about 4.47D long and that the gap and overlap regions are about 0.47D and 0.53D respectively (White et al., 1977). The collagen molecule may thus be schematically represented as five segments, the first four being of length D and the fifth of length 0.47D. This D-periodic arrangement is illustrated in the negatively stained whole-mount of collagen fibrils in Figure 1.4.

Any arrangement of molecules having a projected axial period of D must contain equal numbers of each of the five segments in each D-period (Doyle et al., 1974a); thus "the simplest arrangement of collagen molecules with a true axial period of D will contain five molecules in transverse section, each contributing a different segment to the D-period" (Miller, 1976). This feature was indeed recognized by Smith (1968) who incorporated it in his model of the subfibrillar structure of collagen - the five-stranded microfibril. X-ray diffraction studies of Miller and Wray (1971) lent credence to such a structure which they predicted would have a diameter of ~3.8 nm. Miller and Parry (1973) suggested that such five-stranded microfibrils were supercoiled and 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). During the period 1973 to 1979 a large number of other models were also proposed. These included 2-, 4-, 5- and 8-stranded models as well as models in which the collagen molecules were packed in liquid crystalline arrays. References to these and other models are cited in Appendix 2.

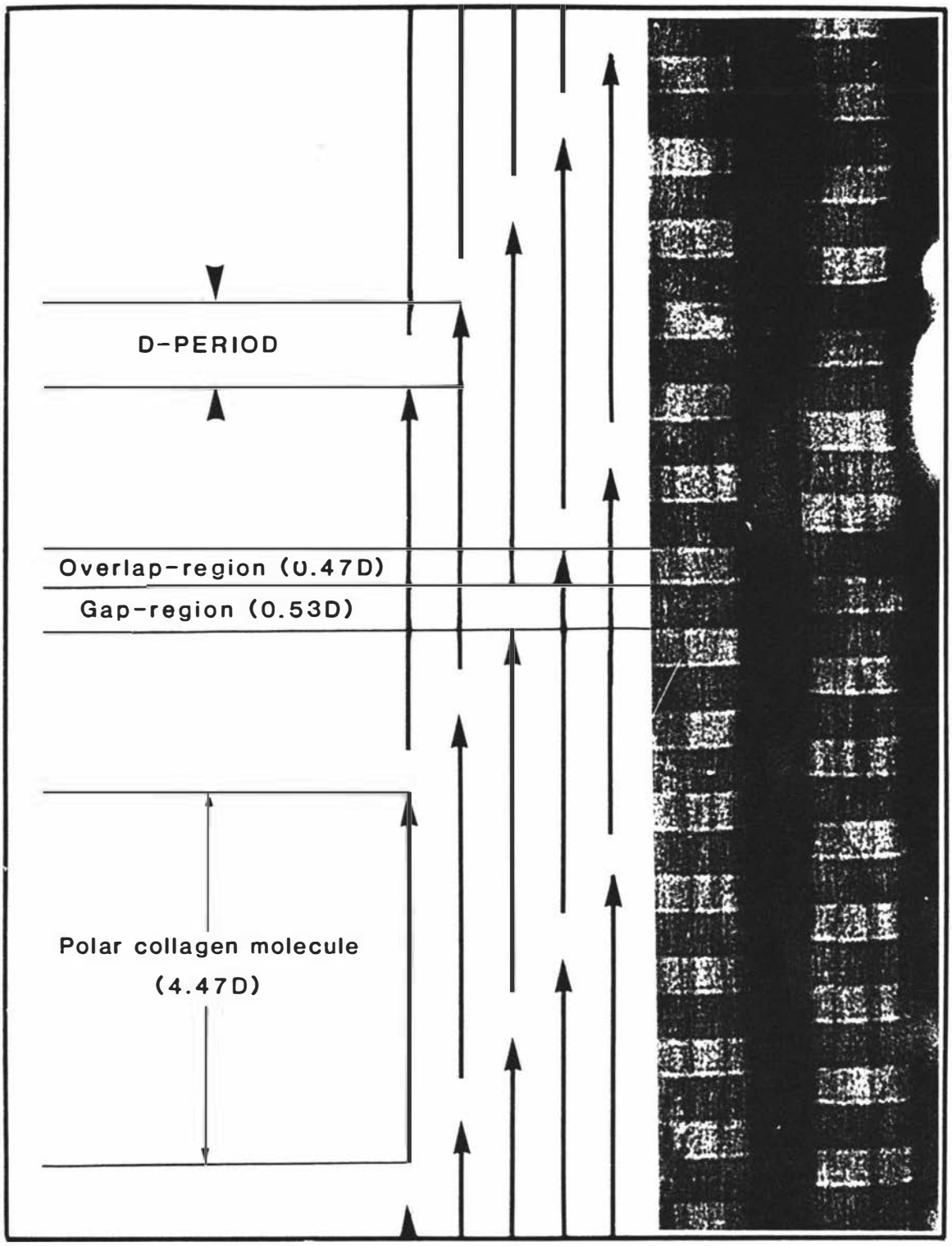

Figure 1.4 Negatively stained preparation of collagen fibrils showing D-periodic banding consisting of alternate dark (gap) and light (overlap) bands. This pattern is generated by staggering collagen molecules 300 nm long (4.47D) by multiples of D in a direction parallel to the fibril axis. This gives rise to a D-period ~67 nm and gap and overlap regions of 0.53D and 0.47D respectively. Magnification: 200 000 X.

It would thus seem evident from both X-ray diffraction and electron microscopy (Bear, 1942; Pease and Bouteille, 1971; Anderson and Sajdera, 1971; Lillie et al., 1977; Ruggeri et al., 1979; Parry and Craig, 1979; Squire and Freundlich, 1980) that the native collagen fibril has an ordered filamentous D-periodic substructure of some kind. The three-dimensional crystal model of Hulmes and Miller (1979), based on a reinterpretation of the X-ray data, is in conflict with the microfibrillar models. For instance it does not demand an ordered structure intermediate between the molecule and the fibril but rather suggests that single molecules are axially tilted (~5° in tendon) and packed in a "quasi-hexagonal" array. Further refinement of the X-ray data by Miller and Tocchetti (1981), Fraser and MacRae (1981) and Fraser et al., (1983) provide strong support for this model. Compressed microfibrillar models (Bailey et al., 1980; Trus and Piez, 1980; Piez and Trus, 1981) have also been offered as a compromise between the single molecule and microfibrillar schools of thought.

1.3 The Nature of Collagenous Tissues

1.3.1. The Cellular Components

Collagenous tissues have a cellular component, a fibrous component and an extracellular matrix. However, the relative composition of the tissue changes significantly with age (see Figures 1.5 and 1.6). The cells of collagenous (connective) tissues are diverse, and may be described as either "fixed" or "wandering". The migratory cells include monocytes, lymphocytes, plasma cells, eosinophils, neutrophils and mast cells and are present for a variety of functions including phagocytosis of effete and dying cellular elements and also antibody and histamine production for the

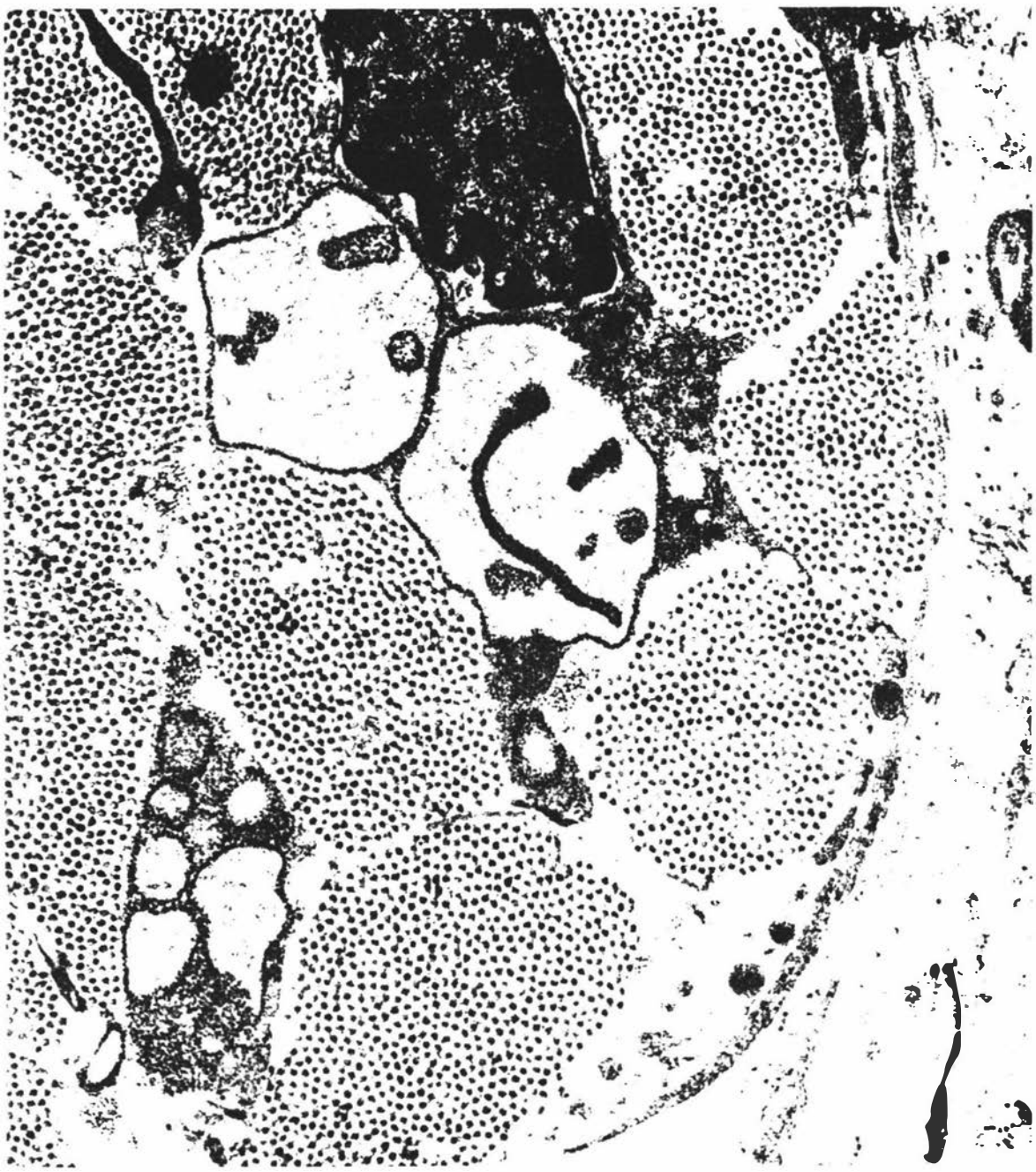

Figure 1.5 Electron micrograph showing the collagen fibrils in developing (18 day foetal) rat tail-tendon. The fibrils have a sharp unimodal distribution of diameters (mean ~25 nm) and are grouped into what will later become large collagen "fibres". The cellular elements of the connective tissue are still very evident at this stage of development. Magnification: 40 000 X.

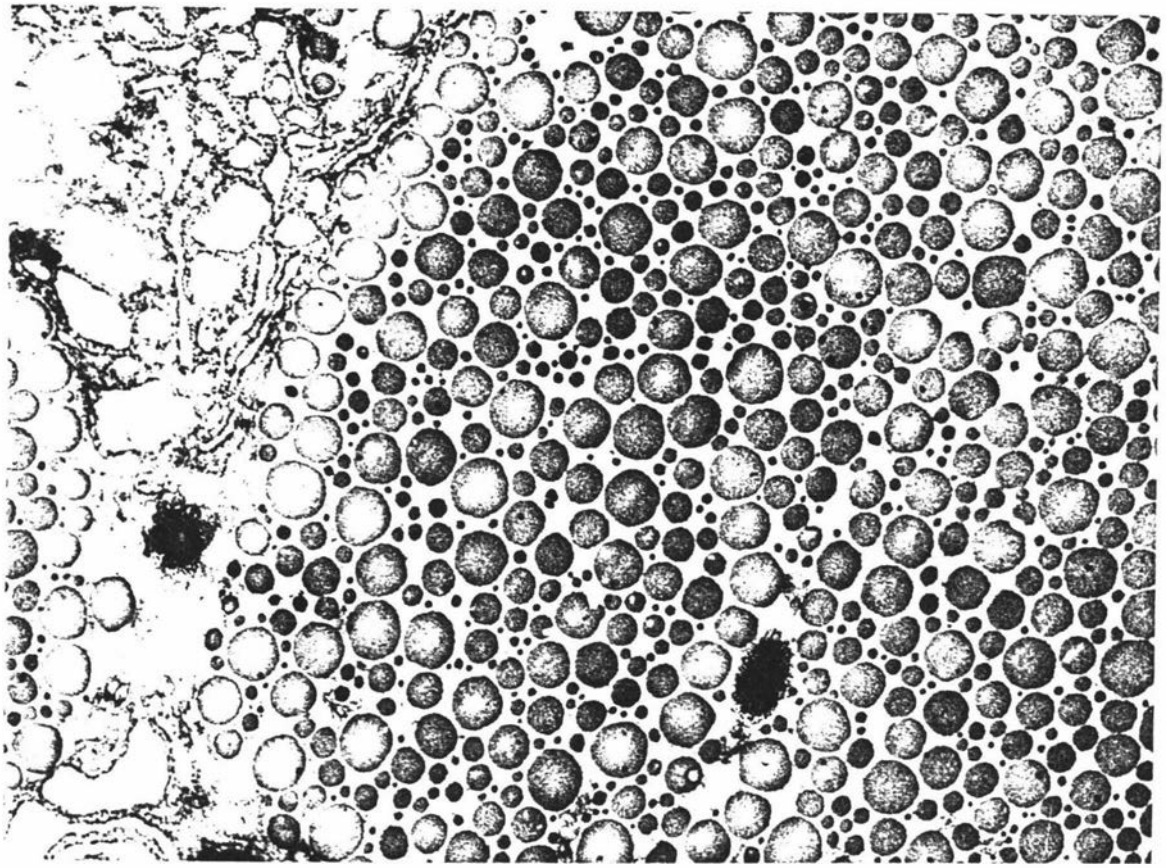

Figure 1.6 Electron micrograph showing the collagen fibrils in mature (adult) rat tail-tendon. The tissue now has a broad bimodal distribution of fibril diameters, the largest being ~500 nm. Two darkly-staining elastic fibres are also seen in this section; in this preparation the elastic fibrils have been enhanced by post-staining with 0.2% Orcein in 50% ethanol after Nakamura et al., 1977. Magnification: 16 000 X.

inflammatory and auto-immune responses. The fixed population of cells include undifferentiated mesenchymal cells, macrophages, adipocytes and fibroblasts. Of all of these cell types it is only those playing a fibroblastic role which are of importance for consideration in this thesis. Such cells, the fibroblasts, differentiate from mesenchymal cells and are the predominant cell type in the more dense connective tissues - tendon, cartilage and bone. Here the cells are known as fibrocytes (or tenocytes), chondrocytes and osteocytes respectively and have specialized functions peculiar to their parent tissue. In other rather unusual cases a fibroblastic role has been attributed to other cell types such as the corneal epithelial cells which are responsible for the synthesis of the collagenous component of the primary corneal stroma (see Hay and Revel, 1969).

1.3.2. The Fibrous Components

Classically, connective tissues have been described as having three types of fibrous component; the collagen fibres, reticular fibres and elastic fibres. The advent of electron microscopy has shown that those fibres known to light microscopists as "reticulin" (by their affinity for silver stains) are indeed collagenous in nature. It would appear that the difference in argyrophilia of reticular and collagenous fibres is not of chemical origin but has a physical basis which depends on the number, size and arrangement of collagen fibrils and their relationship to the glycosaminoglycan-rich matrix. The structure and chemistry of the collagen fibril has already been described and the grouping of large numbers of fibrils accounts for the collagen fibres seen in light microscopy. It should be stressed that many authors appear to use the term "fibre" rather

loosely, but in this thesis that D-periodic element seen by electron microscopy is termed a fibril whereas aggregations or parallel arrays of such fibrils are referred to as fibres. The term microfibril is reserved for that (hypothetical) element considered to be some fixed grouping of molecules being the smallest stable sub-fibrillar element and that of which all collagen fibrils are composed.

Elastic fibres are present in varying degrees in most connective tissues being particularly prominent in elastic cartilage and aorta and predominant in elastic ligaments such as ligamentum nuchae and ligamenta flava (see Table 1.1). Such fibres first appear in developing connective tissues as clusters of elastic microfibrils each 11 - 14 nm in diameter (Figure 1.7a). As the microfibrils increase in number an amorphous material, elastin, is centrally deposited in such a fashion (Figure 1.7b) that mature elastic fibres are seen to be composed of a massive amorphous elastin "core" surrounded by satellite microfibrillar elements (Figure 1.7c; Greenlee et al., 1966; Greenlee and Ross, 1967; Ross and Bornstein, 1969; Parry and Craig, 1978; Parry et al., 1978a). Elastin is synthesised on the ribosomes of fibroblasts, smooth muscle cells and other mesenchymal derivatives and is released as tropoelastin at the cell surface. Elastin, like collagen, has high glycine and proline contents but unlike collagen it is also rich in valine and contains two unusual amino acids, desmosine and isodesmosine which are involved in the crosslinking of elastin (Partridge et al., 1963). The advantages of having mixed elastic-collagenous tissues are discussed in Section 7.1.

TABLE 1.1

COMPONENTS OF CONNECTIVE TISSUES IN MATURE ANIMALS

Tissue	Percentage of Composition *			
	Collagen	Elastin	GAGs **	Water
Tendon/Ligament	30	1.5	0.03-0.3	65
Skin	30	0.2	0.03-0.35	60-72
Fibrocartilage	20	0.1-0.2	0.6	75
Elastic cartilage	16	5-7	3-4	70
Hyaline cartilage	5-18	< 0.1	5-11	75
Bone ***	5-20	-	0.4	30-50
Cornea	12-15	-	0.2-1.0	80
Aorta	5-15	7-15	0.2-2.5	70-75
Elastic ligaments	9	35	-	55
Wharton's jelly	12	-	0.3	88
Vitreous humour	0.25	-	0.02	99

* All expressed as percentages wet weight of tissue. Total percentage does not always add up to 100% due to lack of inclusion of cellular components and non-collageous proteins. No value quoted means that no quantitative data are available.

** Glycosaminoglycans

*** From the ranges of values cited in the literature it would appear that bone contains ~45% mineral.

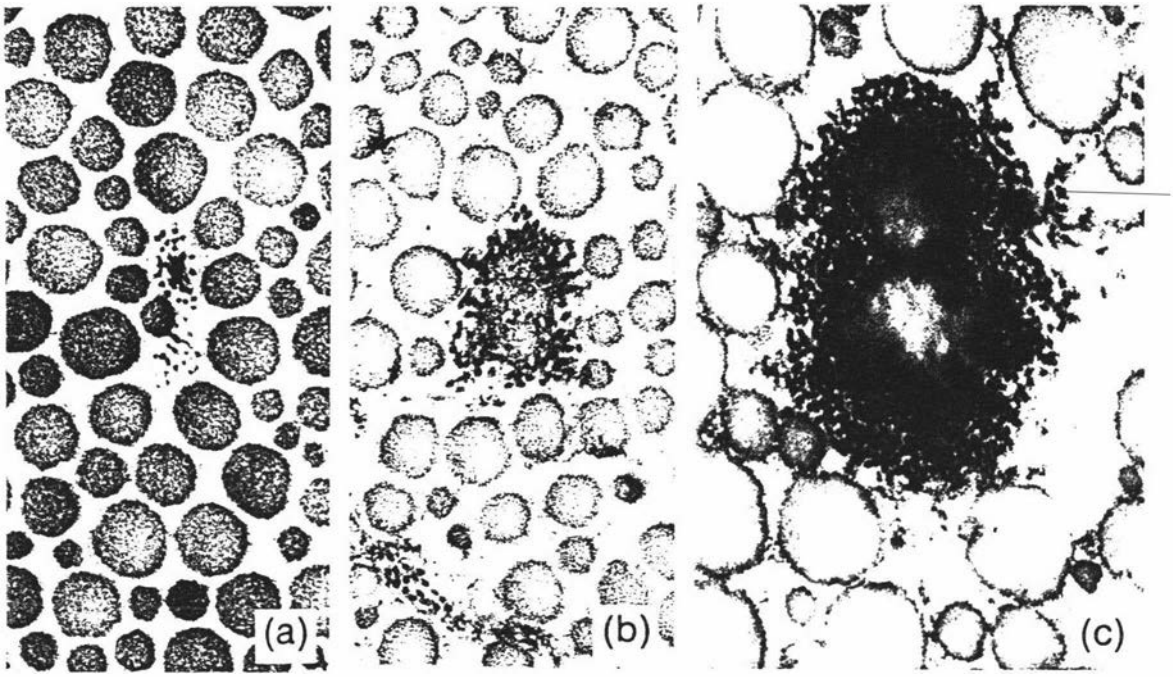

Figure 1.7 Electron micrographs showing transverse sections of elastic fibres. Magnification: 65 000 X.
(a) Immature elastic fibre first appears in a tissue as a cluster of elastic microfibrils ~14 nm in diameter.
(b) Maturing elastic fibre shows beginnings of deposition of an amorphous elastin core.
(c) Mature elastic fibre consists of a massive amorphous core of elastin surrounded by satellite microfibrils.

1.4 Scope and Aims of this Thesis

In Section 1.1 it was indicated that considerable information is now available concerning the molecular structure of collagen and the mode of molecular packing in the fibrils. However there is still little known about the manner in which fibrils grow and develop. In order to investigate growth patterns, tissues must be studied between the foetal and senescent stages of development. Generally, foetal tissues contain collagen fibrils of small diameter only - though some specialized tissues (e.g. the cornea) are known to contain small diameter fibrils throughout life. The comparative study of the vertebrate cornea (Chapter 3) was thus undertaken, not only to investigate a tissue containing small diameter ("foetal-like") fibrils but also to untangle the disparate results in the literature on the constancy of fibril diameter between differing species and that of differing diameter with increasing depth in the corneal stroma in any given species.

Prior to the work of Parry and Craig (Parry et al., 1978a, 1978b, 1980) little quantitative data were available on the diameter distributions of collagen fibrils in connective tissues. These workers began a systematic quantitative assay of fibril diameter distributions in tendons and this thesis is an extension of those studies. By measuring fibril diameters at differing stages of development (foetal - adult - senescence) the modes of fibril growth and fibril breakdown can be seen, and mechanisms of fibrillogenesis can be better understood. The results of these studies are presented in Chapter 4.

Just as biochemical pathways can often be elucidated when pathological processes are understood, so can the study of aberrant collagen forms lead to an understanding of possible fibrillogenetic mechanisms. A genetic disorder "dermatosparaxis" leading to malformed fibrils has been demonstrated by Helle and Ness (1972). Later research showed the presence of amino-terminal propeptides in procollagen molecules in dermatosparactic animals. It was ultimately demonstrated that this could be attributable to the low levels of procollagen amino-peptidase in these animals. In this thesis a variety of pathological or dysplastic tissues have been studied and the results are presented in Chapter 5.

Attempting to obtain quantitative electron microscope measurements from thin sections has many pitfalls. These include the failure of chemical fixation to preserve *in vivo* macromolecular assemblies, artefacts introduced by dehydration, embedding and sectioning, the stereographic interpretation of the recorded two-dimensional image and the methods of taking measurements from the micrographs. All of these difficulties lead to a spread of recorded diameters even for discrete populations of collagen fibrils. Preparative methods must then be chosen to minimize these artefacts and interpretive methods chosen which account for any residual artefacts. A discussion of these problems in relation to this thesis and the statistical methods employed to show the significance of the results are presented in Chapter 6.

As connective tissues have predominantly mechanical roles it is of importance to relate the sizes and dispositions of the constituent collagen fibrils (the tensile elements) with the mechanical attributes of the tissue. Earlier work (Parry *et al.*, 1978a) had listed eight

suppositions relating to such correlations and in Chapter 7 those new data relating to foetal, neonatal, mature and senescent tissues are discussed in terms of their contribution to the understanding of the growth patterns of connective tissues and their relationships to mechanical properties.

The controlling mechanisms and regulatory feedback mechanisms of collagen fibrillogenesis are still unknown, but *in vitro* and *in vivo* studies have implied a variety of physico-chemical controls. These include pH, ionic strength, the cell surface glycoprotein (fibronectin), composition and concentration of the glycosaminoglycans (GAGs) of the matrix, the degree of glycosylation of collagen molecules, the copolymerization of genetically distinct molecular species of collagen molecules, and the partial retention of the terminal propeptides. This thesis investigates the GAG composition and content in the matrix of a variety of connective tissues from which the collagen fibril diameter distributions have been measured, and in Chapter 8 an hypothesis is outlined which suggests a role for the glycosaminoglycans in the regulation of collagen fibrillogenesis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Instrumental Method

This thesis is largely a discussion of the implications of electron microscope measurements made on the collagenous components of connective tissues. It is therefore of importance to discuss the physical basis of the technique of electron microscopy so that its limitations can be understood and the results obtained interpreted in the most realistic manner. This section (2.1) outlines the theory of electron microscopy and draws on review articles by Siegel (1965), Hall (1966), Sjostrand (1967), Wischnitzer (1973) and Agar (1974).

The resolution of any optical system may be considered in terms of the intensity profiles of two near-neighbouring point sources limited by a circular aperture of diameter d. The normalized intensity transform I(R) of such an aperture is given by the expression

$$I(R) = \left[\frac{2J_1(x)}{x}\right]^2 \qquad (1)$$

where J_1 is a first order Bessel function of argument x (= πRd), R is equal to $2 \sin \alpha / \lambda$, λ is the wavelength of the information-seeking beam and α is the semi-angular aperture of the objective lens. Object points are said to be resolved as discrete in the image when the central maximum of the Airy disc of one image point is coincident with the first minimum of the Airy disc of the other. This "diffraction limit" of the system can be used to determine a value for the minimal separation of resolvable object points (d_o) and

is such that

$$d_o = \frac{0{\cdot}61\,\lambda}{n \sin \alpha} \qquad (2)$$

where n is the refractive index of the medium between the object and the objective lens.

In light optical sytems the design-dependent characteristics are maximized by using oil-immersed glass objectives with n ~1.5 and semi-angular apertures approaching 90° ($\sin\alpha$ tending to unity). The diffraction limit of the system then approaches 0.4λ and the ultimate resolution is thus dependent on the wavelength of the irradiating source (d_o ~200 nm for a monochromatic source of wavelength 500 nm).

Electron microscopy is used in preference to light microscopy in many biophysical investigations since it employs an incident (information-seeking) beam of much shorter wavelength than light and hence a greater inherent resolving power. Commercially available electron microscopes commonly use a tungsten filament as a thermionic electron source as part of an electron "gun" whose emission is controlled by a Wehnelt cylinder. The beam is produced by accelerating such electrons with anode-applied voltages (50 - 100 kV) in an evacuated column (typically ~10^{-5} torr or better; i.e. ~1 Pa). The wavelength of the electrons can be ascertained from the de Broglie equation

$$\lambda = \frac{h}{m v} \qquad (3)$$

where h is Planck's constant (6.626×10^{-34} J s), m is the mass of an electron (kg) and v is the magnitude of the velocity of the electron (m s^{-1}) after acceleration. Further, since the kinetic energy of an electron of charge e subjected to an accelerating voltage V is given

by the expression

$$eV = \tfrac{1}{2} m v^2 \qquad (4)$$

the following velocity-independent expression can be derived

$$\lambda = \frac{h}{(2 e m V)^{0.5}} \qquad (5)$$

(where $e = -1.6 \times 10^{-19}$ C, and $m \sim mo$ (rest mass of electron) $= 9.1 \times 10^{-31}$ kg). By substitution this expression reduces to

$$\lambda = \frac{1{\cdot}226 \times 10^{-9}}{V^{0.5}} \qquad (6)$$

Thus for an accelerating potential difference of 100 kV, a beam will be produced whose electrons have a wavelength of ~0.004 nm and it would seem possible that the resolving power of the instrument should tend towards this value. However, it will be seen from the following discussion that there are other factors which limit the achievable resolution to a considerably poorer figure.

Electron microscope lenses can be either electrostatic or electromagnetic but it is the latter which are almost exclusively used in commercial electron microscopes (see Figure 2.1) as they can be manufactured having smaller imaging defects. To focus electrons the magnetic field must be very strong, highly localised and coaxial with the column. Electrons travel in a helical path through these magnetic lenses but as this motion does not affect the focusing ability of the lens, the "envelope" of electrons emerging from the lens is analagous to the light-envelope emerging from a converging lens. The ray-diagrams for electron beams passing through magnetic lenses are thus similar to those of light beams passing through optical lenses (see Figure 2.2) and the lens formulae used in light-optics are equally applicable to electron-optics.

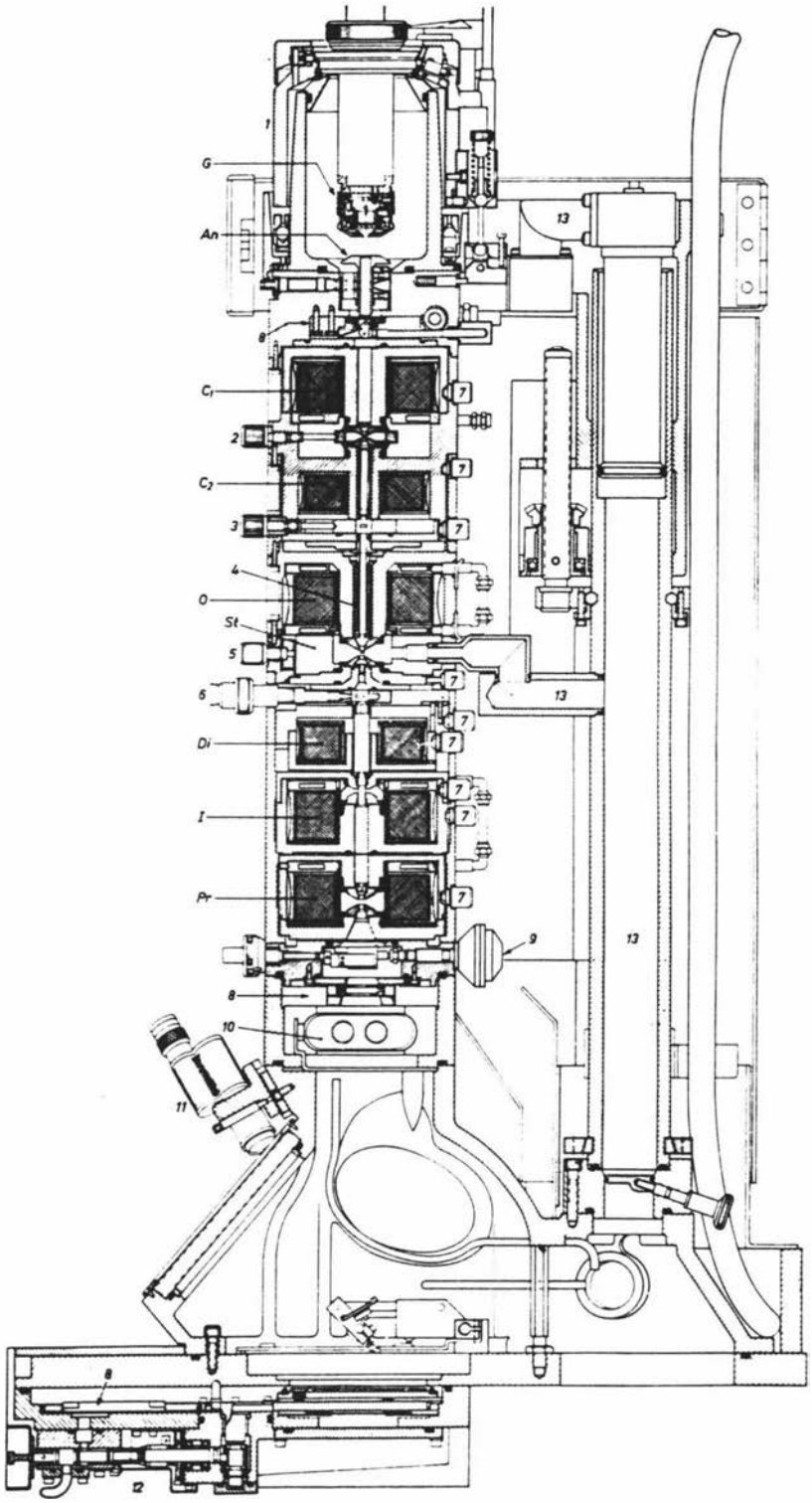

Figure 2.1 Detailed section of a Philips electron microscope column. G - gun; An - anode; C1 and C2 - condenser lenses; O - objective lens; St - specimen stage; Di - diffraction lens; I - intermediate lens; Pr - projector lens; 1 - gimbal ring; 2, 3, 5 and 6 - diaphragm alignment controls; 4 - supplementary objective lens coils for oblique illumination and focusing; 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. (Reproduced with permission of Philips N.Z. Ltd. from Philips Bulletin EM31.)

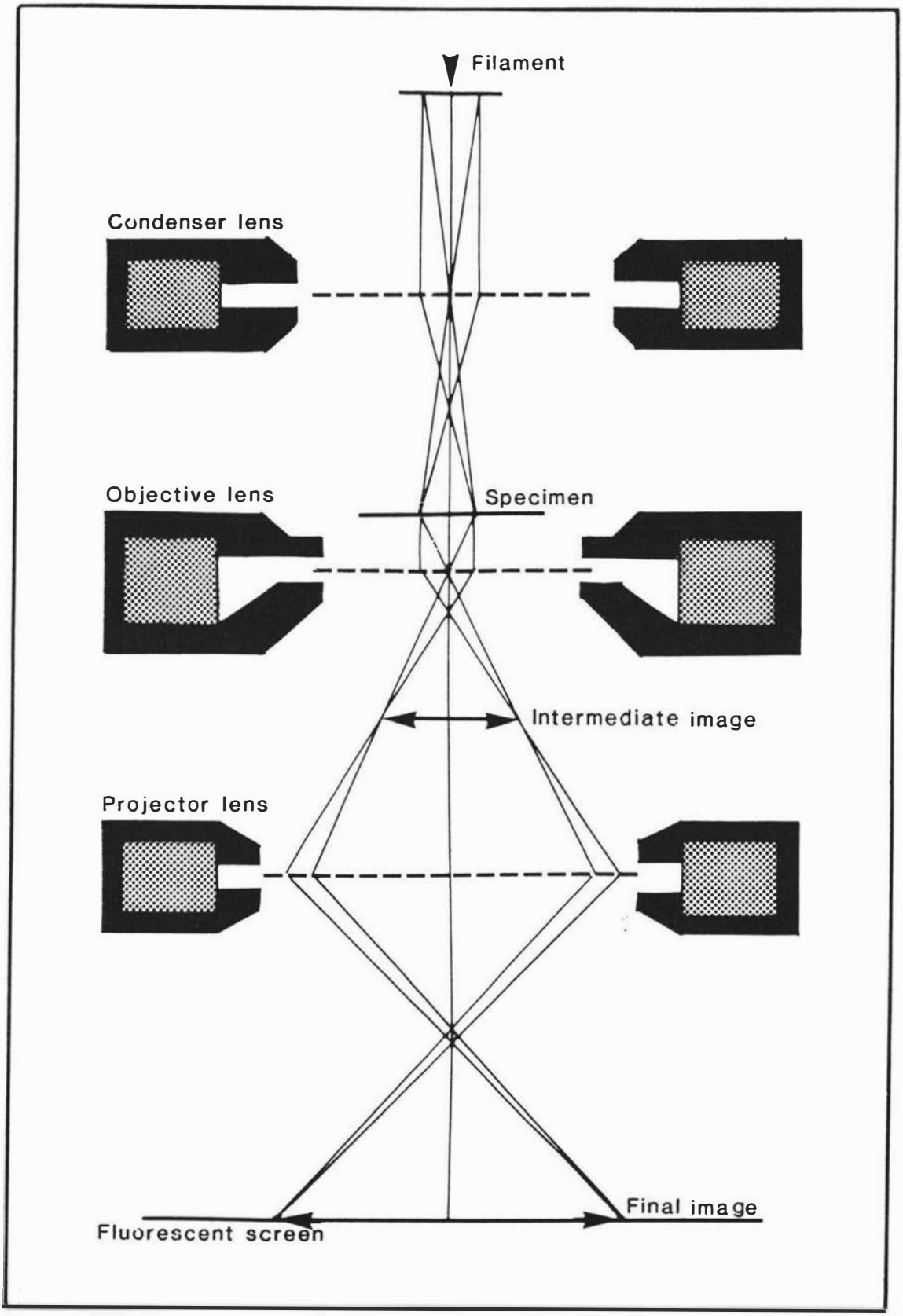

Figure 2.2 Simplified ray diagram for an electron microscope. The condenser lens forms part of the illuminating system and focuses the electron beam on to the specimen. The objective lens produces a magnified intermediate image which is magnified further by subsequent lenses (only one shown here) to form an image which can be viewed on a fluorescent screen.

The focal length (f) of such lenses can be calculated in terms of the relativistically corrected accelerating voltage (V_r), and the number of turns (N) and current (I) flowing in the lens. An expression for f can thus be derived as

$$f = K \frac{V_r}{(NI)^2} \qquad (7)$$

where K is the proportionality constant. The "relativistic voltage" as commonly defined by electron microscopists, is given by the expression

$$V_r = V \left[1 + \frac{eV}{2 m_o c^2} \right] \qquad (8)$$

where V is the accelerating potential (V) and c is the velocity of light (3×10^8 m s^{-1}). Thus numerically V_r is given by

$$V_r = V (1 + 0{\cdot}978 \times 10^{-6} V) \qquad (9)$$

Differentiation of Equation (7) leads to the expression

$$df = K \left[\frac{dV_r}{(NI)^2} - \frac{2V_r\, dI}{N^2 I^3} \right] \qquad (10)$$

Dividing Equation (10) by Equation (7) an expression may be derived relating the fractional change in focal length of a magnetic lens to both the accelerating voltage and the lens current:

$$\frac{df}{f} = \frac{dV_r}{V_r} - \frac{2dI}{I} \qquad (11)$$

Thus fluctuations in either accelerating voltage or lens current, if present in the objective lens, will affect resolution. This effect can be quantitated in terms of the so-called chromatic aberration coefficient (C_c), a parameter which allows computation of the minimum resolution (d_c) as controlled by the residual chromatic defects in the lens. This is given by the expression:

$$d_c = C_c \alpha \left[\frac{dV_r}{V_r} - \frac{2dI}{I} \right] \qquad (12)$$

In modern electron microscopes the chromatic aberration coefficient has a value similar to that of the focal length of the objective lens (~5 m). Thus if an instrument resolution of better than 1 nm is to be achieved high tension and objective lens supply voltage must have stabilities of the order of one part in a million.

Of a greater consequence to the designers of magnetic lenses, however, is the effect of spherical aberration. In general the best focus is attained as a "circle of least confusion" inside the paraxial focus on a plane perpendicular to the optical axis. The diameter (d_s) of this limiting disc is given by the expression

$$d_s = C_s \alpha^3 \qquad (13)$$

where C_s is the spherical aberration coefficient.

In light microscopy this problem is overcome by constructing compound lenses where the unwanted aberration of a converging lens is removed by a compensatory aberration introduced by a supplementary diverging lens of differing refractive index. As the refractive index in an evacuated electron-optical system cannot be manipulated such corrective lenses cannot be constructed and the defect can thereby only be minimised by reducing the angular aperture of the objective lens. There is a limit to how far this aperture may be reduced, however, as the diffraction limit requires that the angular aperture is large. In an electron microscope the refractive index of the immersing medium (the vacuum) is unity, $\sin\alpha = \alpha$ (as α is very small) and the expression for the diffraction limit (Equation 2) becomes

$$d_o = \frac{0{\cdot}61\,\lambda}{\alpha} \qquad (14)$$

Consequently there is an optimum aperture at which the two limits to resolution d_s (Equation 13) and d_o (Equation 14) are equal and for which the combined effects of these aberrations is minimal. By equating Equation 13 with Equation 14 it can be seen that this optimal angular aperture (α_{opt}) can be expressed as

$$\alpha_{opt} = \left[\frac{0{\cdot}61\,\lambda}{C_s}\right]^{0{\cdot}25} \qquad (15)$$

By substituting this value for the angular aperture in the diffraction limit Equation (14) it can be shown that

$$d_{min} = 0{\cdot}7\, C_s^{0{\cdot}25}\, \lambda^{0{\cdot}75} \qquad (16)$$

This expression defines the theoretical limitation (d_{min}) to the resolving power of the electron microscope. With electron lenses currently available the spherical aberration coefficient has been made as small as 10^{-4} nm and with accelerating voltages of 100 kV (λ = 0.0037 nm) a resolution (d_{min}) of ~0.2 nm can be achieved.

The depth of field (D_o) of a lens is the axial distance over which it may be focused without any perceptible change in the image sharpness. From a consideration of Figure 2.3 it can be seen that if o and o' are object points separated by the limiting resolution (d_{min}) of the lens and if parallel rays intersect the optical axis at y and y' respectively, then any points within the distance yy' can be made parfocal. The distance yy' (D_o) is thus the depth of field and it can be seen that

$$\frac{d_{min}}{2} = \frac{D_o}{2} \tan\alpha \qquad (17)$$

and that when α is small

$$D_o = d_{min}/\alpha \qquad (18)$$

Thus for an electron microscope having a resolution of 1 nm and an

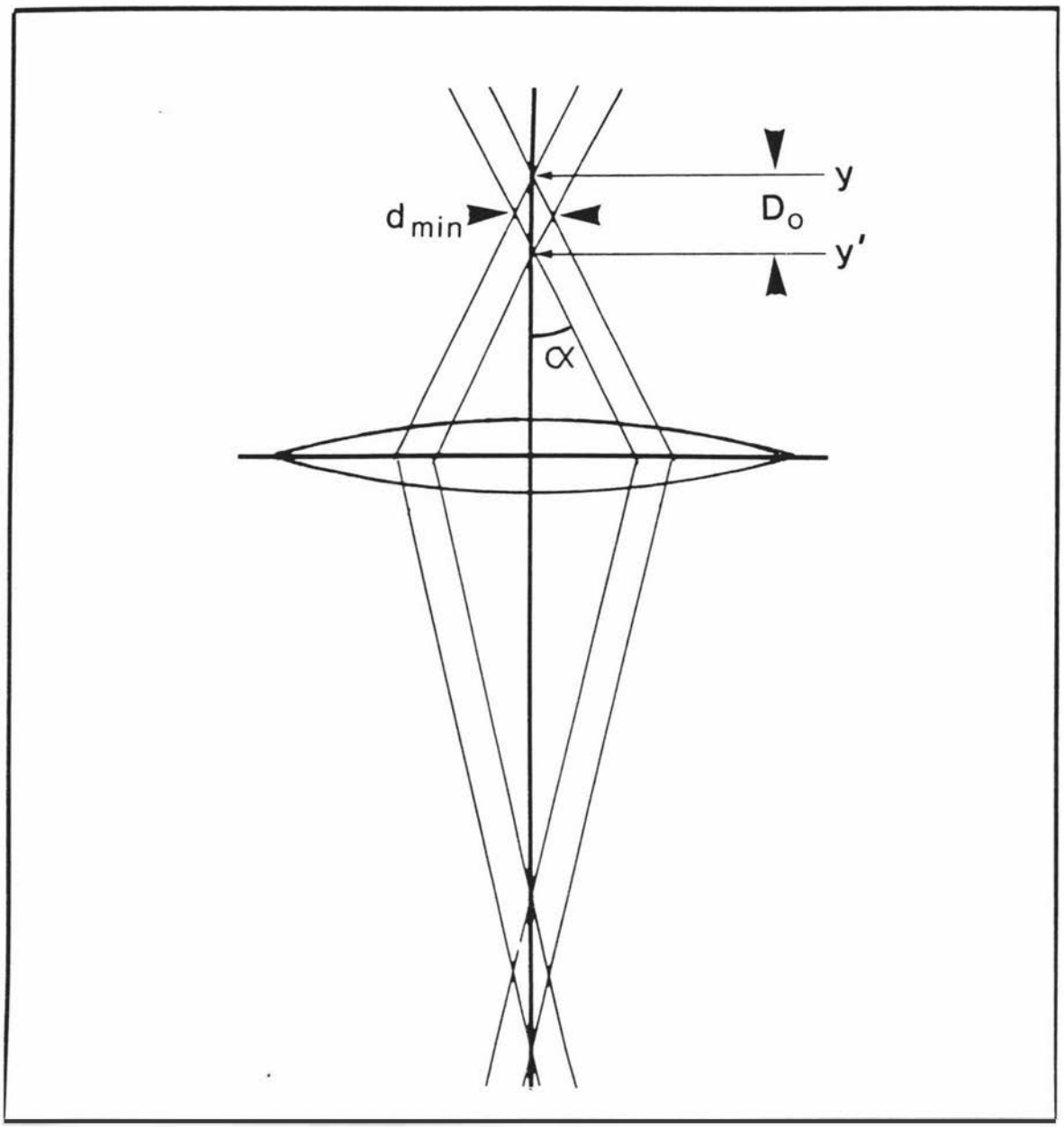

Figure 2.3 Schematic representation of the depth of field of an electromagnetic lens of semi-angular aperture α. When object points are separated by the resolution limit d_{min} of the lens then rays passing through these points intercept the optical axis at y and y' and the distance yy' can be defined as the depth of field (D_o) i.e. all points lying between y and y' will appear equally sharp in the final image.

objective lens semi-angular aperture of 5×10^{-3} radians, the depth of field will be ~200 nm. As this distance is more than twice the thickness of sections of biological specimens normally examined, such sections will appear equally in focus throughout. It should be stressed, however, that not all parts of the in-focus image will be equally resolved, as resolution will always be best at the section surface nearest the lens owing to subsequent scattering of electrons as the beam passes through the section.

The image formation is achieved by the attenuation of the beam by primary and secondary electron emission and by differential absorption and scattering of electrons from areas of the thin specimen having varying "electron opacities" or electron-scattering powers. Biological specimens either "whole-mounted" on grids or sectioned from epoxy-resin embedments, lie typically in the range of 1.5 to 100 nm thick. In sectioned biological materials it is common practice to achieve much of the beam attenuation by the use of heavy metal "stains", that is, contrast is achieved by the selective deposition of salts of osmium, uranium, lead, tungsten, silver, gold etc., to lipids, proteins, lipo-protein membranes, nucleic acids, nucleoproteins and other cellular constituents.

The information-containing beam is ultimately observed on a fluorescent screen or recorded directly on to a photographic film or plate. The transmission electron microscopes used to undertake this study were a Philips EM200 and Philips EM201C.

2.2 Collection of Specimens

Where possible fresh specimens were collected from animals

sacrificed for these experiments. Methods of killing experimental animals included intravenous or intraperitoneal injection of nembutal overdoses, chloroform or carbon dioxide asphyxiation, and decapitation. For the comparative corneal collagen studies, however, many specimens were taken from animals which had been formalin-fixed for teaching purposes or from eyes which had been formalin-fixed for transportation. The sources of all corneas and their state of preservation prior to fixation for electron microscopy are listed in Appendix 3. Those corneas described as being fresh were removed and placed into fixative within minutes of death with the exception of three bony fish (trout, butterfish, moki), the corneas of which were removed from eyes taken from 18 to 48 hour refrigerated heads. Specimens were obtained by excising the cornea and cutting small samples from the central region thus avoiding the corneo-scleral junction.

The non-corneal collagenous tissues investigated in this study were mostly from experimental animals available for sacrifice; chick, rat, guinea pig and sheep. The tissues taken for study were skin and metatarsal tendon (chick), skin, superficial flexor tendon, common digital extensor tendon and suspensory ligament (sheep), skin from both abdomen and foot-pad, bone from developing forelimb phalanx, central tendon of the diaphragm and forelimb digital flexor and extensor tendons (guinea pig), and tail-tendon, abdomen and tail-skin, forelimb and hindlimb flexor tendons, central tendon of the diaphragm and fibrocartilage from the annulus fibrosus of a lumbar intervertebral disc (rat). Many of these skin, tendon and ligament specimens were taken at a variety of ages as will be apparent from the

listings of results in Chapter 4.

Further, Achilles tendon specimens were taken from both adult dog and ox, and skin samples were also taken from lamprey, trout, human, greyhound and sheep, with the latter two specimens being controls for samples of dysplastic greyhound dermis and dermatosparactic sheep skin. These were all fresh specimens with the exception of the early developmental stages of lamprey and the foetal human skins which were formalin-fixed, and came from the same sources as the corneal specimens of the corresponding animals (see Appendix 3). The trout skin was taken from the head region of an adult fish. Tissues supplied already processed and epoxy-embedded for electron microscopy were lamprey notochord sheath and the umbilical cord of a normal neonate man. A freeze-dried specimen of the Cuvierian tubules of the sea cucumber (Holothuria forskali) was also made available for examination. The sources of these materials are listed in Appendix 4.

Pathological tissues and healing wounds were also investigated in this study. Biopsy samples of greyhound skin from an animal suffering from a dermal dysplasia were obtained from veterinarians at Massey University and processed for electron microscopy in the normal manner. Specimens of skin and tendon from a dermatosparactic lamb, and the palmar fascia of a man suffering from Dupuytren's contracture were obtained (with controls) already processed for electron microscopy. Superficial flexor tendons from the horse at a variety of time intervals (24 hours to 14 months) after in vivo intratendinous injection with bacterial collagenase were also supplied embedded, as were specimens of rat skin scars from experimental surgical incisions made either transversely or longitudinally to the direction of the

Langer's lines of the dermis. The sources of all of these materials are listed in Appendix 4.

2.3 Preparative Procedures

Routinely, specimens were fixed at 4° C in a modified Karnovsky fixative (Karnovsky, 1965) comprising 2% formaldehyde and 3% glutaraldehyde in 0.1M phosphate buffer (Na_2HPO_4, KH_2PO_4) at pH 7.4. The formaldehyde was prepared by the alkaline depolymerisation of paraformaldehyde. The duration of the primary fixation was usually 2 - 4 hours although some specimens were held in the fixative overnight. This was followed by three phosphate buffer washes (10 - 30 min, 4° C) and secondary fixation in 1% osmium tetroxide in the same buffer for 2 hours at 4° C. Further buffer washes (three of 10 - 30 min) preceded dehydration in a graded ethanol-water series (25%, 50%, 75%, 95% and 100% ethanol). The durations of these steps were 20 - 30 minutes and sometimes, for convenience, the specimens were held in 75% ethanol overnight. A second change of 100% ethanol preceded two ten minute rinses in propylene oxide and specimens were then held overnight in propylene-oxide-resin mixture (30:70) in uncapped glass vials on a motor-driven stirrer in a fume-hood. They were placed in fresh 100% resin for the duration of the next day (7 hours) then flat-embedded in further fresh resin in butyl-rubber moulds and polymerized at 60° C for 48 hours. The resins used were Araldite (Durcupan ACM, Fluka, Buchs, Switzerland) and Spurr's low-viscosity resin. Araldite was mixed to a standard formulation whilst Spurr's resin was prepared to a schedule intermediate between medium and hard (Spurr, 1969). Some tissues were supplied embedded in Epon 812 (see Appendix 4).

2.4 Sectioning and Staining Techniques

For electron microscopy all blocks were trimmed by hand and sectioned on glass or diamond knives using an LKB Ultratome. After cutting, sections were stretched with chloroform vapour, to relieve them of compressional artefacts. Sections were nominally of a pale gold interference colour (~80 nm thick) and were routinely picked up on 200 mesh copper grids having carbon-stabilized formvar support films. Formvar films were made by immersing an 80 x 30 mm piece of 7 mm polished plate glass into a 0.5% solution of formvar (polyvinyl formal formvar) in ethylene dichloride. This was drained to dryness, scored about its periphery with a thumb-nail and floated on a water surface which had been swept clean with a polyethylene bar. Grids laid on the floating film were picked up on blotting paper and oven-dried. Carbon was evaporated on the formvar-coated grids using spectrographic grade 3 mm graphite electrodes in a Kinney KDTG-3P vacuum evaporator. All sections from which quantitative measurements were to be made were thus supported, but some others were picked up unsupported on 400 mesh grids which had been pre-treated with a chloroform-Sellotape cement (prepared by dissolving the adhesive off 50 cm^2 of Sellotape with 100 g of chloroform).

Sections were stained by being immersed in saturated uranyl acetate in 50% ethanol in a staining dish, washed in three changes of 50% ethanol followed by three changes of water, then immersed in a droplet of lead citrate stain (Venable and Coggeshall, 1965) on Parafilm in a covered Petri dish, and finally washed copiously with running distilled water and air-dried. Staining times were routinely four minutes in each stain.

Other specimens were prepared as negatively stained "whole-mount" preparations. Aqueous suspensions of teased collagen fibrils were mixed with equal volumes of negative stain, droplets placed on coated grids, allowed to stand for 10 - 20 seconds then excess liquid removed by absorption at the edge of torn filter paper. The negative stains employed were 2% phosphotungstic acid neutralized to pH 7.0 with potassium hydroxide or 2% ammonium molybdate neutralized to pH 7.0 with ammonium hydroxide.

Some light microscopy was also undertaken using specimens which had been resin-embedded for electron microscopy. Sections of 2 μm thickness were cut on dry glass knives and flattened on to microscope slides by drying down at 60° C on a distilled water droplet. Such sections were stained for about 20 seconds at 45° C with 0.5% Toluidine blue in 0.1M (pH 7.2) phosphate buffer. Coverslips were mounted using objective immersion oil as this method has proven more satisfactory than the use of commercially available permanent mountants, all of which appear to produce daylight-enhanced stain-fading on storage.

2.5 Photomicrography and Mensuration Methods

When photographing electron microscope sections for subsequant quantitative analysis, an additional micrograph was taken of a 2 160 lines mm^{-1} diffraction cross-grating replica (Polaron 0736). Once an appropriate microscope magnification was selected it was left unchanged, and with the aid of a pre-pumped specimen air-lock the high tension was kept on, whilst micrographs of the section and grating were taken. This procedure eliminated any possible changes in magnification caused by fluctuations in high tension voltage or

changing currents in the electromagnetic lenses of the microscope. All transmission electron micrographs were recorded on 35 mm fine grain positive film (Kodak 5201) and developed in a high contrast developer (Kodak D19b). Measurement of the resulting developed emulsion showed it to have a grain size of <0.5 µm, a value well within the requirements of this work.

All collagen fibril diameter measurements were made from thin sections of transversely sectioned fibrils. Small fibrils such as those which constitute the cornea and embryonic or perinatal connective tissues were measured directly from the negatives using an eyepiece graticule in a Zeiss stereo-microscope at a magnification of 40 X. Four measurements (two pairs at right angles) were taken from the grating replica micrograph and the mean of these measurements was recorded as a magnification reference. Fibrils appearing elliptical in section were considered to have been cut obliquely and the minor diameter was measured. For sharp unimodal distributions of diameter a minimum of 100 measurements were recorded and for broad or multimodal distributions upwards of 1 000 measurements were commonly taken in order to ascertain the true form of the distribution. For larger collagen fibrils, diameters were measured directly from micrographs printed on Ilford resin coated paper at 67 000 X, a magnification determined by reference to the micrograph of the grating replica. An engraved metal metric-rule was then used to take fibril diameter measurements, to the nearest 0.5 mm directly from the prints.

Light microscope bright-field micrographs were taken using a compound research microscope (Zeiss GF) fitted with planachromatic objectives. Yellow or green filters were used for monochromatic photography and images were recorded on Ilford FP4 35 mm film.

2.6 Analytical Methods

The mean diameters (d) and mass-average diameters (d_m) of the collagen fibril diameter distributions measured were calculated from the following expressions

$$d = \frac{\sum n_i d_i}{\sum n_i} \qquad (19)$$

$$d_m = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \qquad (20)$$

where n_i is the number of fibrils whose diameters were measured as d_i. Diameter frequency distribution histograms were constructed by plotting n_i versus d_i and volume distribution histograms by plotting $n_i d_i^3 / \sum n_i d_i^2$ versus d_i.

Multimodal populations of small collagen fibrils (diameters 10 to 70 nm) were analysed using a computer program for non-linear least-squares decomposition of mixtures of populations and four possible models were considered. In all models it was assumed that the sub-populations were normal and in two of the models the mean diameters of the individual populations were not constrained whereas in the alternative models, the means were equally separated but not necessarily constrained to be an integral multiple of that separation from the origin. Further, the standard deviations of the means of the sub-populations were made equal in two of these models (one with constrained and one with unconstrained means) whilst the standard deviations were not constrained in the other two models.

Where quantitative electron microscope measurements were to be compared with low-angle X-ray diffraction data from concurrent collaborative studies, as in the case of chick metatarsal tendon, the

radial distribution function, g(r), was calculated from measurements made directly from electron micrographs printed at magnifications of 100 000 X. Micrographs were chosen which displayed extensive arrays of collagen fibrils and minimal cellular and other non-collagenous components. The occurrence of the latter features would modify the spatial relationships between fibrils and render the calculated radial distribution function less meaningful. The centres of all fibrils within a square of side 15 cm (or larger) were then marked on the micrographs and their Cartesian coordinates recorded for computer processing. The program employed considered each marked fibril centre as an origin and the frequency distribution of the distances from this origin to all other fibril centres within a radius ~10 X the nearest neighbour separation was computed and these results, which typically included ~5 X 10^4 independent values, were then summed. Difficulties arise in this method, however, when measurements are made from fibril centres lying close to the edge of the micrograph (less than 10 X nearest neighbour separation), as the true number of larger interfibrillar separations cannot be recorded here. A procedure which compensates for this deficiency in circular areas of micrographs has been outlined by Fraser et al., (1964). A different compensatory method devised by E.F.Eikenberry (Eikenberry et al., 1982a) was ultimately used in order to make full use of the data within a rectangular micrograph. This was achieved by weighting the contribution at each radius by the inverse of the fraction of the full circular circumference about that particular origin that lay within the micrograph. The normalised distribution was then seen to approach its expected asymptotic value of unity rather than falling off at larger radii as it does when the compensation for the edge depletion is not applied.

CHAPTER THREE

THE CORNEA

3.1 Introduction

The vertebrate cornea is the physical limit and the major light-collecting element of the eye, and its structure has evolved as a result of evolutionary pressure to function in these two distinctive ways. Being the physical limit of the anterior portion of the eye it must have the required structural integrity to maintain its shape and form a protective barrier between the underlying tissues and the surrounding environment. Further, in order to act as a light-collecting and a light-transmitting element in terrestrial and aquatic vertebrates, the cornea must not only be transparent but also have a refractive index greater than that of air or water. The collagenous nature of the cornea has simultaneously met both of these demands.

The cornea becomes continuous with the sclera at the limbus or sclero-corneal junction, which is marked by the termination of the vascular elements of the sclera and the internal and external scleral sulci. The anterior surface is bounded by a stratified squamous epithelium which in man is normally composed of five layers of cells with a total thickness of ~50 μm. The cells adhere to one another by many short interdigitating processes whose surfaces are rich with desmosomes. Below the corneal epithelium of many vertebrates is a region described by light microscopists as Bowman's membrane. By electron microscope observation this region is better described as Bowman's layer; it consists not of a membrane but a layer, of

disorientated collagen fibrils which is continuous with the underlying stroma.

The bulk of the cornea is composed of the stroma (substantia propria) which varies greatly in thickness between vertebrate species giving rise to so-called "thick" and "thin" corneas. The stroma is largely composed of lamellae of similarly directed small diameter collagen fibrils which are considered to have diameters that are relatively constant throughout life. The long axes of the fibrils lie parallel to the surface of the cornea and successive lamellae are rotated through ~90° about an axis perpendicular to the plane of the lamellae (Figure 3.1). The lamellae are kept tightly together by the interchange of fibrils from near-neighbouring lamellae, whose fibrils are disposed in a similar orientation. An exception to this arrangement has been described in the cornea of the skate and several other cartilaginous fish (Weber, 1878; Smelser, 1962; Payrau et al., 1964, 1965). These authors described "sutural fibres" which lie perpendicular to the corneal epithelium, and traverse the stroma from Bowman's layer to Descemet's membrane (Figure 3.2). The excised elasmobranch cornea, unlike other vertebrate corneas, neither swells nor becomes opaque when placed in distilled water and these properties have been attributed to the existence of the sutural fibres (see Goldman and Benedek, 1967).

Early in the development of the cornea the collagen of the presumptive stroma is produced by the epithelium. Although it was once considered that Type II collagen was peculiar to cartilaginous tissues it has been shown in the last decade that it is also produced by the embryonic epithelia of the notochord (Linsenmayer et al., 1973), neural retina (Newsome et al., 1976) and cornea (Linsenmayer et

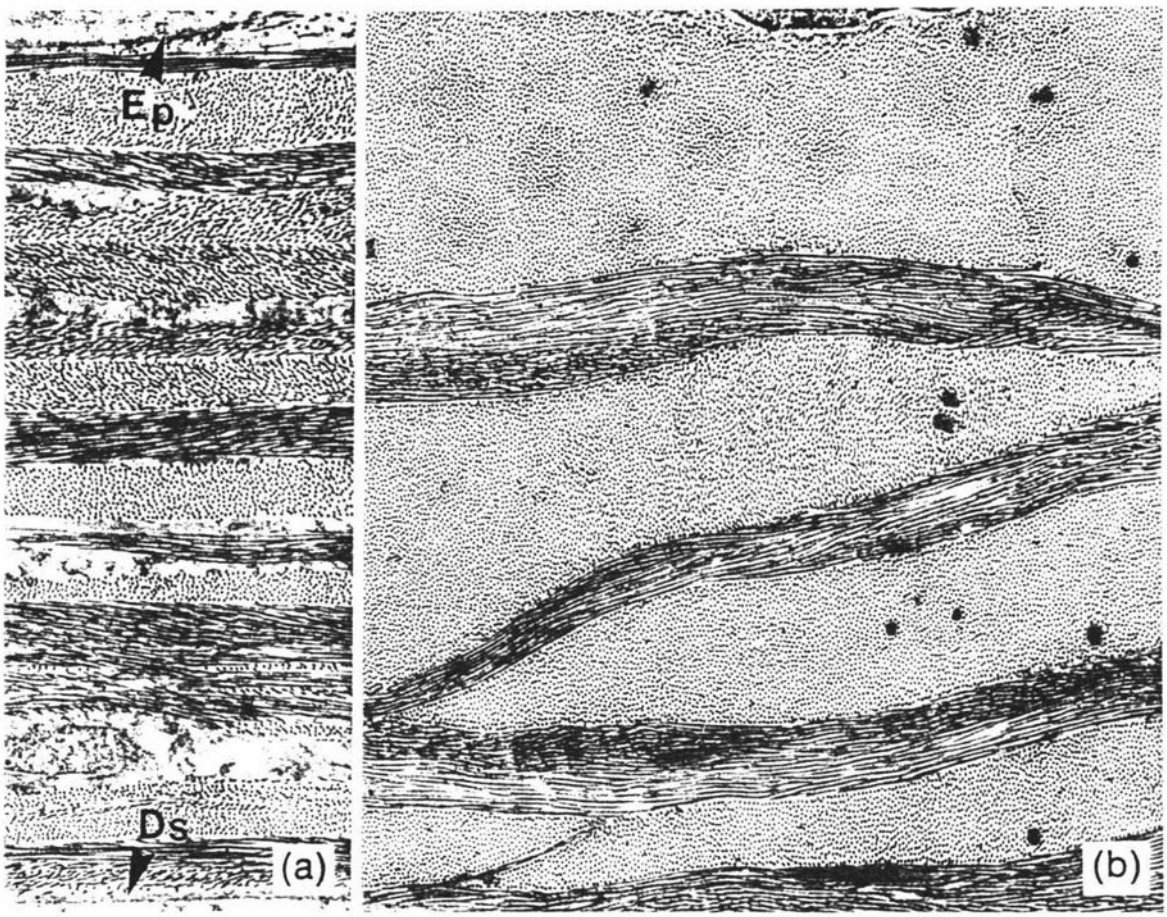

Figure 3.1 Electron micrographs of the corneal stromata of the snake and magpie. Magnification: 12 000 X.
(a) Section through the stroma of the thin cornea of the snake showing the anterior, epithelial surface (Ep), and the posterior surface limited by Descemet's membrane (Ds). The thickness of the lamellae range between 0.3 and 0.8 μm, and the mean diameter of the collagen fibrils is ~23 nm.
(b) Section through a central portion of the thick cornea of the magpie. The thickness of the lamellae range from between 0.5 and 3 μm and the mean diameter of the collagen fibrils is ~25 nm.

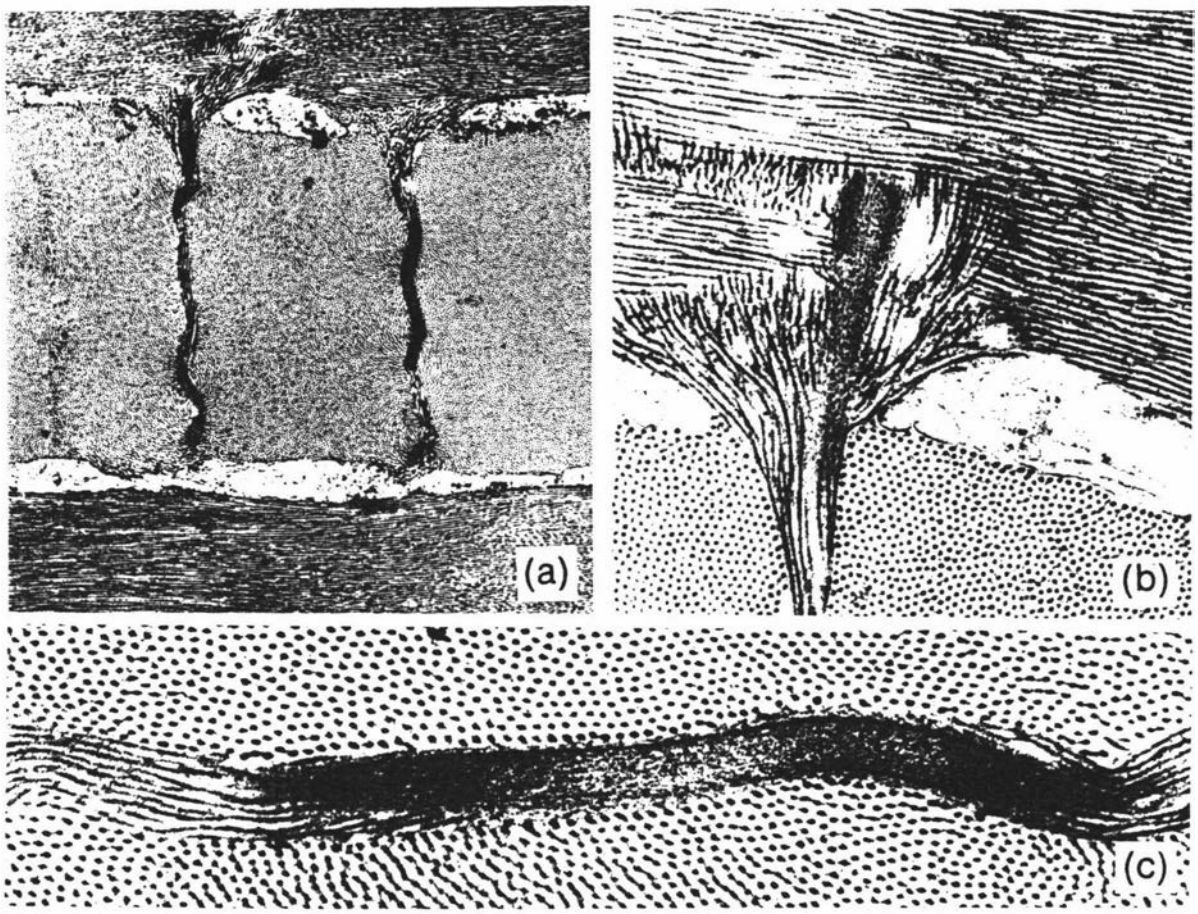

Figure 3.2 Electron micrographs of the collagenous sutural fibres in the corneal stromata of an elasmobranch (cartilaginous fish; dogfish).
(a) Bundles of collagen fibrils, in the form of a sutural fibre, traversing a stromal lamella and lying perpendicular to the fibrils of this and the neighbouring lamellae. Magnification: 8 000 X.
(b) Sutural fibres appear to arise from the fibrils of a lamella and turn through 90° to traverse the adjacent lamella. Magnification: 24 000 X.
(c) Detail of sutural fibre in mid-lamella - where individual collagen fibrils of the sutural fibre appear to lose their identity. Magnification: 28 000 X.

al., 1977). Although Type I collagen is still the predominant molecular species of collagen synthesised by the developing corneal epithelium it has been demonstrated in the four-day-old embryonic chick eye that Types I and II collagens are deposited between the epithelium and the lens capsule in the primary acellular stroma (von der Mark et al., 1982). Further it has been shown that, at this stage of development, Types I and II collagen are codistributed in the same fibrils (Hendrix et al., 1982). These authors speculate that the self-assembly of the highly regular structure of the cornea may be controlled by the participation of Type II collagen in Type I collagen fibrils.

In a later stage of development fibroblasts from the mesenchyme of the embryonic head migrate into the primary stroma and initiate the development of the secondary stroma by the deposition of a new matrix which is later to contain Types I and V collagens in the ratio of 4:1 (Poschl and von der Mark, 1980). Some days after birth the Type II collagen has completely disappeared from the stroma of the chick cornea although it is known to persist as a minor species in Descemet's membrane (von der Mark et al., 1977).

A point of interest which should be noted from the above discussion is the absence of Type III collagen in the development of the cornea, as it is Type III collagen which plays a predominant role in the development of most Type I containing fibrous tissues. It has been suggested by von der Mark et al., (1982) that "the coincidence of Type III collagen-absence with the transparent region of the corneal stroma and the beginning of the non-transparency in the limbus suggests that the highly regular Type I/V collagen network in the transparent region cannot form in the presence of Type III collagen."

Between the fibrils of the lamellae is a hydrated proteoglycan gel which, in the adult cornea, is largely composed of chondroitin sulphate and corneal keratan sulphate (see for example Anseth, 1961; Breen et al., 1972; Mathews, 1965; Gregory et al., 1982). The increased biosynthesis of corneal keratan sulphate in the human takes place at a late stage of embryonic development (Smelser and Ozanics, 1957; Breen et al., 1972). It is believed to occur at the time at which there is an atrophy of the hyaloid artery, an increase in the corneal thickness and an opening of the eyelids (Barber, 1955). It has been suggested by Breen et al., (1972) that these changes may indeed "affect the nutrient supply of the cornea and stimulate the production of keratan sulphate." The cells of the stroma are long slender fibrocytes (keratocytes) which lie between the lamellae, and a smaller number of wandering lymphoid cells which migrate from the vessels of the limbus.

Posterior to the corneal stroma is Descemet's membrane which is limited by a typical squamous endothelium. Structurally this is a very thick membrane (5 - 10 μm in man) and although having an homogeneous appearance by light microscopy is shown by electron microscopy to contain a filamentous network (Jakus, 1956, 1961; Hay and Revel, 1969). This network is attributable to the collagenous components which are currently considered diverse. Kefalides and Denduchis (1969) have demonstrated the presence of Type IV ("basement-membrane") collagen, whilst more recently it has been shown to contain Type I (Davison and Cannon, 1977) and Type II (von der Mark et al., 1977) collagens. It has been indicated by tissue culture (Perlman and Baum, 1974) and electron microscope studies (Hay and Revel, 1969; Perlman et al., 1974) that the membrane is elaborated by the underlying endothelium. The developmental inter-relationships

between the components of the cornea (epithelium, stroma, Descemet's membrane and endothelium) have been described in a comprehensive review on corneal morphogenesis (Hay, 1980).

A survey of the literature reveals a wide range of values quoted for the mean diameter of the collagen fibrils in cornea. At the lower end of the scale Cox et al., (1970) have reported that the collagen fibrils in the cornea of the glass catfish have diameters of about 10 nm whereas Klein et al., (1981) have suggested that in rabbit cornea the fibrils have a mean diameter as large as 78 nm. Other values lying between these extremes include those for human cornea (25 - 33 nm, Schwarz, 1953; 20 - 23 nm, Maurice, 1957; 24 - 28 nm, Jakus 1961; 32.5 nm, Cox et al., 1970; 22.5 - 36.5 nm, Borcherding et al., 1975; 24.2 - 32.3 nm, Trelstad et al., 1977), rabbit cornea (24 - 35 nm, Smith and Frame, 1969; 16.4 - 23.2 nm, Cox et al., 1970; 20 - 45 Cintron et al., 1978), chick cornea (25 nm, Hay, 1973), rat cornea (25 - 30 nm, Jakus, 1954), monkey cornea (33 - 35 nm, Ozanics et al., 1976) and pig cornea (45 nm, Ruggeri et al., 1979).

Further it has been stated (Jakus, 1961) that larger diameter collagen fibrils are found with increasing depth in the human corneal stroma; ~19 nm in the anterior stroma (Bowman's layer), ~24 nm centrally, and ~34 nm deep within the stroma towards Descemet's membrane. Despite the fact that these values have been quoted many times (for example Maurice, 1969; Smelser and Ozanics, 1972; Borcherding et al., 1975), no attempts have been made to confirm this point. Contrarily, Goldman and Benedek (1967) claim that the collagen fibrils in the dogfish cornea decrease in diameter with increasing depth in the stroma (~31 nm in the anterior stroma and ~27 nm in the posterior stroma). Finally it has been shown for rabbit cornea (Cox

et al., 1970) that collagen fibril diameters have mean values of 18.6, 19.8 and 17.8 nm in the anterior, central and posterior stroma respectively. Since these measurements differ by only 2 nm and have standard deviations of ~3 nm it follows that there is clearly no significant change in the collagen fibril diameters with increasing depth in the corneal stroma of the rabbit.

If the corneal collagen fibril diameters quoted in this and other works are to have significance for comparative purposes, it is necessary to establish that the values obtained do not vary with location in the stroma. This study is concerned with determining those features of the collagen fibrils in the corneal stromal lamellae which are invariant in different vertebrate genera and in finding how the distribution of collagen fibril diameters changes with age. Further, the diameter distribution of collagen fibrils in the stroma will be determined as a function of the distance from the anterior surface.

Corneas were prepared for light and electron microscopy by the methods described in Chapter 2. The corneas of 29 vertebrate genera were studied in their adult form, and the corneas of four of the animals included in this group (frog, rat, guinea pig and man) were studied as a function of age. The neonatal corneas of a further three mammals (capuchin monkey, squirrel monkey and hippopotamus) were obtained from animals which had died at birth or close to birth. Finally, the possible variation of collagen fibril diameter distribution with increasing distance from the anterior surface of the cornea was determined by cutting thin sections across the total thickness of the cornea and measuring the distributions at six equi-spaced locations between the most anterior portion (underlying

the corneal epithelium) and the most posterior portion (overlying Descemet's membrane). This was done for six of the vertebrate genera; snake, magpie, chick, rat, guinea pig and man.

3.2 Results and Discussion

Light microscope observations have revealed that the thickness of the cornea varies markedly between the vertebrate species studied; stromal thickness ~560 μm in man and ~10 μm in the snake (Craig and Parry, 1981a). The extremely thin cornea of the snake may be explained in part by the presence of a chitinous "exocornea". The thickness of this exocornea was ~20 μm in the specimen studied, hence it is the chitin and not the corneal epithelium which forms the most anterior protective layer in this animal. Similarly, depending on the animal and its age, the thickness of the lamellae constituting the stroma may also vary greatly (approximate range 0.1 - 3.0 μm, Figure 3.1). Each lamella contains an array of similarly orientated collagen fibrils whose diameters have been measured in transverse section. The mean values and standard deviations of the collagen fibril diameter distributions of the 29 vertebrates used in the comparative study are listed in Table 3.1. It can be seen that none of the distributions have a standard deviation greater than 2.3 nm and thereby can be considered populations of uniform diameter collagen fibrils (Parry and Craig, 1979, 1981b).

Further, it can be seen that the mean diameters of the corneal collagen fibrils in the four bony fish and in the adult sea-lion are all close to 17.5 nm, while the collagen fibrils of the corneal

TABLE 3.1

MEAN DIAMETERS OF POPULATIONS OF COLLAGEN FIBRILS FROM THE CORNEAL STROMAL LAMELLAE OF THE ADULT VERTEBRATES STUDIED

Class	Common Name	Genus and Species	Mean Diameter ± std.devn. (nm)
Chondrichthyes			
	Dogfish	*Koinga lebruni*	26.2 ± 1.4
	Elephant fish	*Callorhynchus milii*	26.4 ± 1.3
	Stingray	*Bathytoshia brevicaudata*	25.3 ± 1.3
Osteichthyes			
	Goldfish	*Cyprinus carpio*	16.7 ± 2.3
	Butterfish	*Coridodax pullus*	17.7 ± 0.9
	Moki	*Latridopsis ciliaris*	16.8 ± 1.1
	Brown trout	*Salmo trutta*	18.1 ± 1.0
Amphibia			
	Salamander	*Necturis sp.*	24.5 ± 1.7
	Toad	*Bufo marinus*	23.5 ± 1.5
	Frog	*Hyla aurea*	23.9 ± 1.6
Reptilia			
	Turtle	*Pseudemys scripta*	25.1 ± 1.9
	Snake	*Thamnophis sp.*	23.6 ± 1.8
	Tuatara	*Sphenodon punctatus*	27.6 ± 1.5
Aves			
	Kiwi	*Apteryx australis*	26.1 ± 1.8
	Magpie	*Gynnorhina hypoleuca*	25.0 ± 1.3
	Chicken	*Gallus domesticus*	23.8 ± 1.8
	Pigeon	*Columba livia*	24.5 ± 1.7
	Thrush	*Turdus philomelos*	26.2 ± 1.5
Mammalia			
	Sea-lion	*Zalophus californianus*	18.7 ± 1.4
	Opossum	*Trichosurus vulpecula*	27.6 ± 1.8
	Rabbit	*Oryctolagus cuniculus*	26.2 ± 1.3
	Rock wallaby	*Petrogale penicillata*	25.7 ± 1.2
	Wallaroo	*Macropus robustus*	23.8 ± 1.6
	Diana monkey	*Cercopithecus diana*	25.5 ± 1.8
	Blackbuck	*Antilope cervicapra*	26.9 ± 1.3
	Rat	*Rattus norvegicus*	23.5 ± 1.7
	Human	*Homo sapiens*	23.8 ± 1.5
	Guinea pig	*Carvia porcellus*	26.1 ± 1.7
	Ox	*Bos taurus*	27.3 ± 2.2

stromata of all the other vertebrates have mean diameters ~25 nm (Figures 3.3 a-b, 3.4 a-e). With the exception of the sea-lion it would thus appear that the bony fish are unique with respect to the diameters of their corneal collagen fibrils. It should be emphasised that the vertebrates chosen for study embrace six classes of living vertebrates, and that the corneal collagen fibril diameters appear invariant over five of these. The diameters of the collagen fibrils in the cornea of the sea-lion would therefore appear anomalous and it is not clear whether this value reflects the relative immaturity of the specimen (1-year-old) or whether the sea-lion represents another group of mammals (say marine) whose collagen fibrils attain a diameter of only ~17.5 nm at adult life. Conversely the specimen may have suffered adversely in its preparation for electron microscopy.

The diameters of corneal collagen fibrils which have been published in the literature are often quoted as single values (e.g. human cornea; 20 nm, Jakus, 1961) or as a range of values (e.g. human cornea; 22.3 - 36.6 nm, Borcherding et al., 1975). These diameters are listed in Table 3.2 as either the single value or the mid-point of the range of values quoted, so that they may be compared with the diameters of collagen fibrils measured in this work from similar animal species. The only corneal collagen fibril diameter published for a bony fish is 10 nm (glass catfish; Cox et al., 1970), and although this value is much lower than the mean value of 17.5 nm suggested by this work it does indeed confirm that the corneal collagen fibrils from bony fish are very small. The values of Jakus (1954) and Hay (1973) showing 25 nm diameter fibrils in both rat and chick, are in excellent agreement with these results, but the mid-range value of 34 nm (Ozanics et al., 1976) for the diameter of

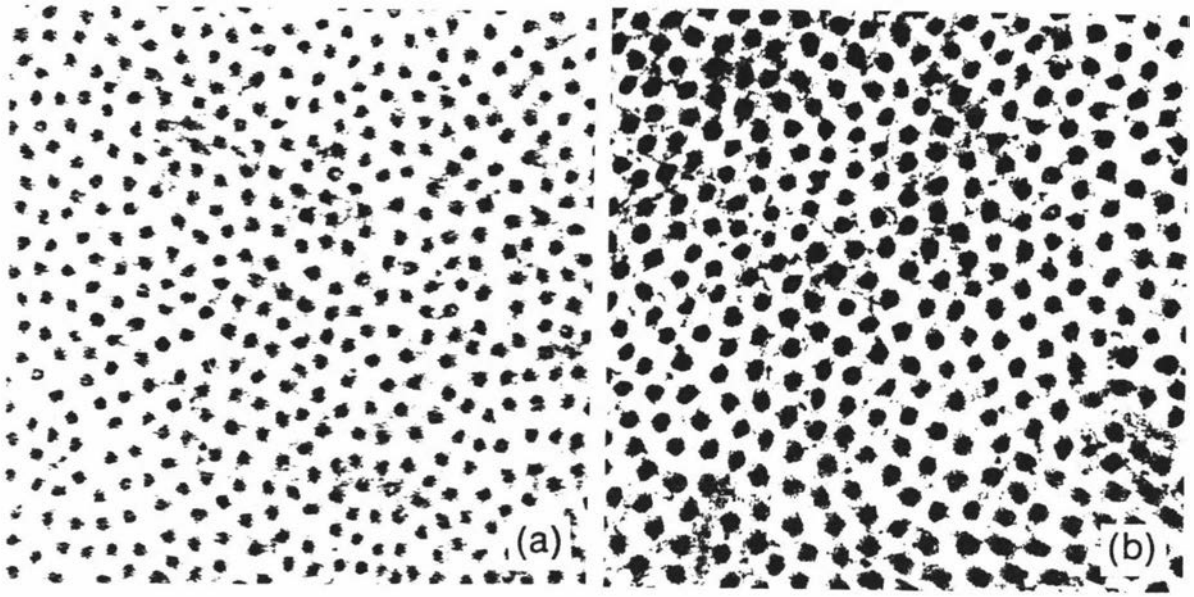

Figure 3.3 Transverse sections of the collagen fibrils from the corneal stromata of fish. Magnification: 82 000 X.
(a) Population of collagen fibrils from the corneal stroma of a bony fish (goldfish); the mean diameter of the fibrils is ~17 nm.
(b) Population of collagen fibrils from the corneal stroma of a cartilaginous fish (stingray); the mean diameter of the fibrils is ~25 nm.

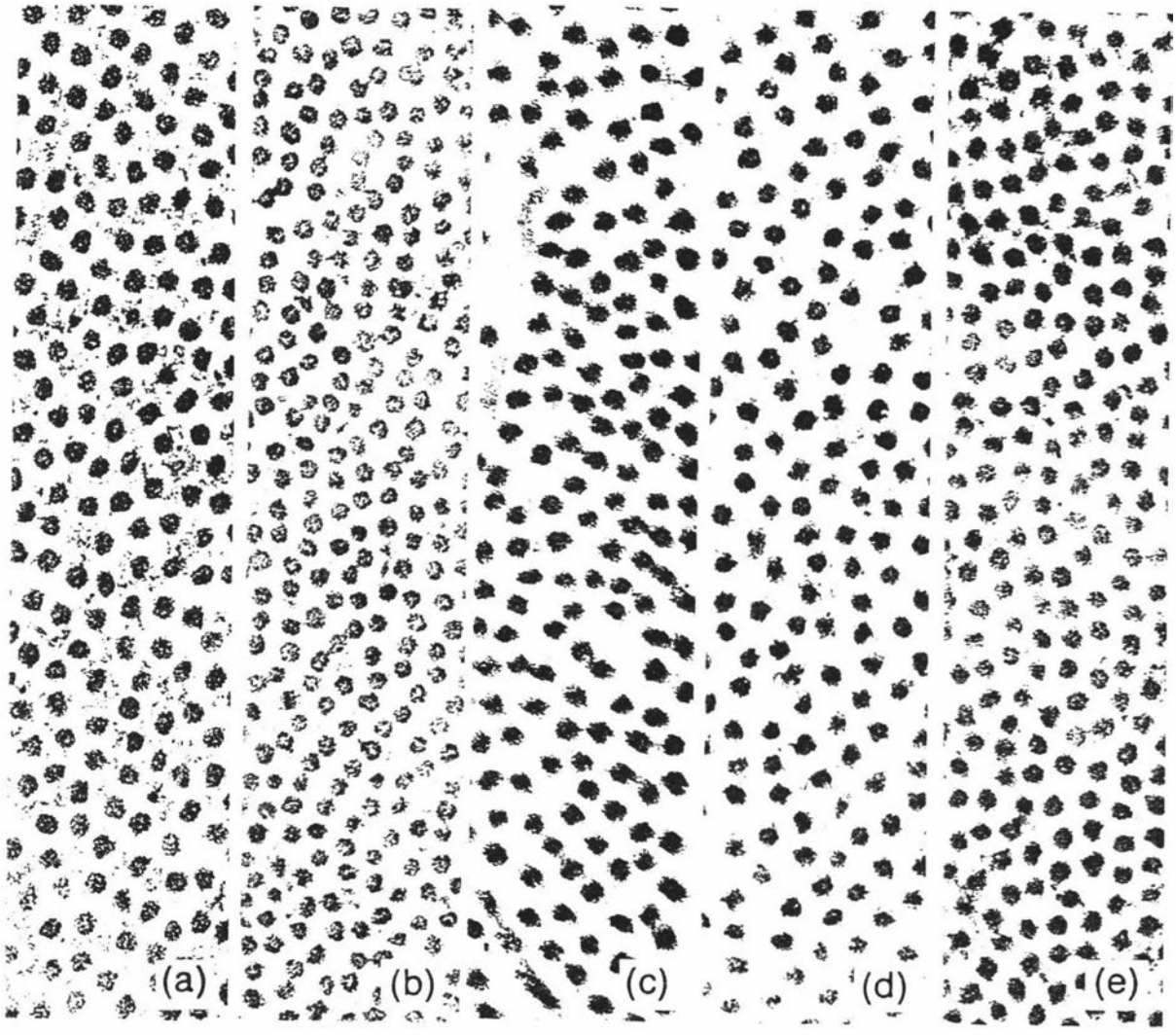

Figure 3.4 Electron micrographs of populations of collagen fibrils from the corneal stromata of (a) dogfish (chondrichthyes), (b) salamander (amphibia), (c) snake (reptilia), magpie (aves), and rabbit (mammalia). All collagen fibrils have diameters ~25 nm. Magnification: 92 000 X.

TABLE 3.2

MEAN COLLAGEN FIBRIL DIAMETERS OF THE CORNEAL STROMA AS CITED IN VARIOUS WORKS

Animal	Mean Diameter or mid-point of range (nm)	Range of Diameters (nm) (where known)	Method of Determin-ation	Reference
Glass catfish	10.0	-	S	Cox et al., 1970
Bony fish	17.3	14.1 - 20.5	S	This work
Chick	23.8	21.2 - 27.4	S	This work
Chick	25.0	-	S	Hay, 1973
Rat	23.5	20.1 - 26.9	S	This work
Rat	27.5	25.0 - 30.0	S	Jakus, 1954
Monkey	25.5	21.9 - 29.1	S	This work
Monkey	34.0	33.0 - 35.0	S	Ozanics et al., 1976
Man	21.5	20.0 - 23.0	S	Jakus (see Maurice, 1957)
Man	23.8	20.8 - 26.8	S	This work
Man	25.0	23.0 - 27.0	S	Schwarz, 1966
Man	26.0	24.0 - 28.0	S	Jakus, 1961
Man	29.0	25.0 - 33.0	D	Schwarz, 1953
Man	29.5	22.3 - 36.6	S	Borcherding et al.,1975
Man	29.6	24.2 - 32.3	S	Trelstad et al., 1977
Man	32.5	-	S	Cox et al., 1970
Man	32.5	30.0 - 35.0	S	Francois et al., 1954
Man	34.0	32.0 - 36.0	S	Hogan et al., 1971
Man	37.4	33.0 - 41.6	S	Kayes & Holmberg, 1960
Pig	45.0	-	F	Ruggeri et al., 1979
Rabbit	20.0	16.4 - 23.2	S	Cox et al., 1970
Rabbit	26.2	23.6 - 28.8	S	This work
Rabbit	29.5	24.0 - 35.0	S	Smith & Frame, 1969
Rabbit	30.0	20.0 - 45.0	S	Cintron et al., 1978
Rabbit	32.5	30.0 - 35.0	S	Francois et al., 1954
Rabbit 3 w	52.0	22.0 - 82.0	F	Klein et al., 1981
Rabbit 5 mo	78.0	44.0 -112.0	F	Klein et al., 1981

D = Measurements from micrographs of sonically dispersed fibrils.
S = Measurements from micrographs of thin-sectioned material.
F = Measurements from micrographs of freeze-fracture replicas.

corneal collagen fibrils in the monkey is 8 - 9 nm higher than that recorded here. Although diameters of thin-sectioned material from both rabbit and man have been reported many times the values quoted have varied significantly from one observer to another. The mean values given for fibril diameters in man range from 21.5 to 37.4 nm in the works cited in Table 3.2 and are spread about a value of ~28 nm. This is ~4 nm larger than that measured in this work. Similarly the mean diameters of fibrils in the rabbit corneal stroma when determined from thin section measurements by other workers are, with one exception, reported to lie between 29.5 to 32.5 nm. This again is significantly larger than the 25 nm diameter expected of the corneal collagen fibrils of vertebrates which had been measured in this work as 26 nm. These high values may be accounted for by the difficulties in making such measurements (see Chapter 6).

Further, those published values of collagen fibril diameter obtained from measurement of freeze-fracture replicas of corneal stromata are all higher than those observed by thin sectioning techniques. The value of 45 nm quoted by Ruggeri *et al.*, (1979) for pig corneal collagen has taken into account the ~2.5 nm replica thickness claimed to be produced by the technique employed, and all subsequent measurements rest on the assumption that this replica thickness is correct. It must also be argued, however, that freezing as a method of specimen preparation might be expected to lead to fibrils having a greater (and closer to *in vivo*) diameter than those processed for electron microscopy by fixation, dehydration and resin embedding. Nonetheless, it is surprising that these techniques should lead to such widely differing estimates of fibril diameter. Even more

surprising are the diameters of 52 nm and 78 nm determined for corneal collagen fibrils of three-week and five-month rabbits (Klein et al., 1981). These values are not only extremely high but, without gross differences in replica thickness and measuring technique between the two samples, they also imply a 26 nm growth between these two ages in the rabbit. Detailed examination of this paper (Klein et al., 1981), however, reveals that no allowance was made for replica thickness and although it was stated that the 67 nm D-period was chosen as an internal magnification reference, the published micrographs with their scale-markings do not bear this out. It would thus appear that any further consideration of these results must be undertaken with caution.

Such a post-natal growth of corneal collagen fibrils is not suggested by the results of this study. The mean diameters of such fibrils from four vertebrates at a variety of ages are listed in Table 3.3. It can be seen that in the three foetal mammalian specimens studied (18 d foetal rat, 14 w foetal and 24 w foetal man) the collagen fibrils have diameters ~17.5 nm, whereas all other measurements indicate fibril diameters ~25 nm from birth to adult in the guinea pig, birth to senescence in the rat and maturity to senescence in man. It could be expected that 25 nm fibrils would also be found in the cornea of man at some time close to birth, but specimens at these ages were not available. The corneal collagen fibril diameters of three other mammals which were studied perinatally are also listed in Table 3.3. Although the 1.5 d hippopotamus had a mean fibril diameter ~25 nm expected of a mammal, it was found that the five-day hippopotamus and newborn capuchin and squirrel monkeys all had corneal collagen fibrils which measured ~20 nm. Such

TABLE 3.3

MEAN DIAMETERS OF POPULATIONS OF COLLAGEN FIBRILS FROM CORNEAL STROMAL LAMELLAE IN DEVELOPING FROG, RAT, GUINEA PIG, MAN AND SOME NEONATAL MAMMALS.

Species	Age (F = foetal)	Mean Diameter ± std. devn. (nm)
Frog		
(*Hyla aurea*)	Tadpole *	24.2 ± 1.8
	Adult	23.9 ± 1.6
Rat		
(*Rattus norvegicus*)	18 d F	18.2 ± 1.1
	0 w	24.0 ± 1.0
	8 w	23.3 ± 2.1
	16 w	23.5 ± 1.6
	2 y	23.7 ± 1.5
Guinea Pig		
(*Carvia porcellus*)	0 d	25.1 ± 1.9
	7 d	26.0 ± 1.5
	17 d	26.8 ± 1.6
	66 d	26.6 ± 1.5
	Adult	26.2 ± 2.1
Man		
(*Homo sapiens*)	14 w F	17.2 ± 1.0
	24 w F	16.7 ± 1.7
	18 y	24.1 ± 1.6
	73 y	23.1 ± 1.3
	75 y	23.8 ± 1.4
Hippopotamus		
(*Hippopotamus amphibius*)	1.5 d	24.2 ± 1.2
	5 d	19.4 ± 1.0
Capuchin Monkey		
(*Cebus appela*)	0 d	20.9 ± 1.5
Squirrel Monkey		
(*Saimiri sciureus*)	0 d	20.5 ± 1.6

* Age unknown but hindlimbs had emerged.

measurements are considered to be too large to be included in the bony fish and foetal mammal grouping of ~17.5 nm and not large enough to be considered ~25 nm fibrils.

The data relating to the depth study of the corneal stromata are given in Table 3.4. This shows that the diameters of the collagen fibrils of the cornea are invariant throughout the stroma with the exception, in some species, of some fibrils immediately adjacent to Descemet's membrane. The fibrils throughout the stroma are of uniform diameter (~25 nm) in snake, guinea pig and man, but the most posterior fibrils in the rat and chick stromata had fibrils whose diameters were measured at 17.4 nm and 18.2 nm respectively, and which can be considered ~17.5 nm diameter fibrils similar to those recorded for bony fish and foetal mammals. Further, the most posterior fibrils in the magpie cornea were measured at 19.7 nm and, like the stromal fibrils of some neonate mammals, must also be considered intermediate between ~17.5 nm and ~25 nm fibrils. It must thus be concluded that, with minor exception, the collagen fibrils of the vertebrate corneal stroma are of uniform diameters and do not vary with their position in the stroma. This is in agreement with the data of Cox et al., (1970) but is contrary to the oft-cited results of Jakus (1961) - that the collagen fibrils increase in diameter with increasing depth in the corneal stroma of man; or of Goldman and Benedek (1967) - that the collagen fibrils decrease in diameter with increasing depth in the corneal stroma of the dogfish.

In this present work, 84 collagen fibril diameter distributions have been measured from the 44 vertebrate corneas studied, and of these, but four can be considered anomalous. They do not fit the 17.5 / 25 nm pattern which has otherwise been established. These data are

TABLE 3.4

VARIATION OF MEAN COLLAGEN FIBRIL DIAMETER WITH DEPTH BELOW THE ANTERIOR SURFACE OF CORNEAL STROMA

Level in Stroma	Mean collagen fibril diameter ± std. devn. (nm)					
	Snake	Magpie	Chick	Rat	Guinea Pig	Human
1	23.3±1.8	25.9±1.6	24.7±1.6	24.5±2.1	28.4±1.8	22.2±1.3
2	22.1±1.6	25.8±1.4	24.1±1.8	25.7±1.8	27.4±1.3	22.0±1.6
3	22.8±1.8	22.5±1.6	23.7±2.2	24.7±1.6	27.7±1.8	22.1±1.4
4	23.7±1.6	24.5±1.7	23.6±1.7	22.1±2.3	28.2±1.7	22.4±1.3
5	22.8±1.6	24.8±1.6	22.7±1.7	23.6±2.4	27.7±1.6	22.1±1.5
6	21.9±1.7	19.7±1.3	18.2±1.6	17.4±1.4	26.3±1.3	22.4±1.6
Mean of 1-5	22.9±1.8	25.3±1.7	23.8±1.9	24.1±2.4	27.9±1.7	22.2±1.4

listed in Table 3.5 and consist of the distributions measured from the corneas of the neonate hippopotamus, capuchin and squirrel monkeys and the distribution obtained from the most posterior region of the magpie cornea. It is not known whether these fibril diameters are the result of ill-managed preparative procedures for electron microscopy or whether they represent short-lived transient forms of fibrils of the established preferred diameters.

Early theories explaining the transparency of the cornea required that the stromal collagen fibrils be disposed in a regular hexagonal lattice within the lamellae (Maurice, 1957), but later theorists claimed that no such regular lattice existed (Goldman and Benedek, 1967; Smith and Frame, 1969; Farrell and Hart, 1969). Smith (1969) postulated that the corneal transparency was due to the near equivalence of the refractive indices of the collagen fibrils and the surrounding matrix, while others (Hart and Farrell, 1969; Benedek, 1971; Twersky, 1975) claim that the phenomenon must be accounted for by light scattering from cylinders (collagen fibrils) which are of uniform diameter and separated from each other by distances smaller than the wavelength of light. More recent workers (e.g. Sayers et al., 1982) favour theories of short range order between fibrils, though the mechanism of transparency is still not fully understood. The results of the ultrastructural studies of this work would favour any theoretical considerations which necessitated a constancy of collagen fibril diameter.

The results obtained from the work undertaken for this thesis may thus be summarized:

a) collagen fibrils of the adult vertebrate corneal stroma have preferred sizes of ~17.5 nm in the bony fish and ~25 nm in the other

TABLE 3.5

COLLAGEN FIBRILS OF THE VERTEBRATE CORNEAL STROMA HAVING DIAMETERS WHICH ARE NOT SIMPLE MULTIPLES OF ~8 nm

Animal	Age	Fibril Diameter ± std. devn. (nm)
Hippopotamus	5 d	19.4 ± 1.0
Capuchin monkey	0 d	20.9 ± 1.5
Squirrel monkey	0 d	20.5 ± 1.6
Magpie *	Adult	19.7 ± 1.3

* Fibrils of most posterior portion (layer 6) adjacent to Descemet's membrane.

vertebrate classes,

b) the fibrils are not initially formed at their definitive sizes, but do indeed grow during pre-natal development.

c) the fibrils are invariant in diameter throughout the depth of the mature stroma, and

d) the fibrils remain at a fixed diameter throughout adult life.

Although good comparative anatomy can be undertaken by conventional electron microscopy, it is important to realise that the specimens examined in this work are all dehydrated and resin-embedded and it is likely therefore that the collagen fibril diameters reported are smaller than the true in vivo values. Evidence is reported in Chapter 6 that small collagen fibrils in a variety of tissues (cornea, notochord, chick metatarsal tendon) have diameters that measure from 10 - 30% less by electron microscopy than when measured in a hydrated state by a non-destructive method (X-ray diffraction). The in vivo value of the diameters of collagen fibrils from the corneal stroma could thus be as great as 25 nm in bony fish and 36 nm in other vertebrate classes.

CHAPTER FOUR

EXPERIMENTAL OBSERVATIONS ON THE GROWTH AND DEVELOPMENT OF COLLAGEN FIBRILS

4.1 Introduction

Prior to the work of Parry et al., (Parry et al., 1978a, 1978b, 1980; Parry and Craig, 1977, 1978, 1979; Craig and Parry, 1981a, 1981b) little quantitative data was available on the form of the collagen fibril diameter distribution in connective tissues as a function of age. The few diameter measurements which had been reported in the literature were often both unreliable and incomplete; mean or ranges of diameter were quoted from which the form of the distribution could not be derived, the age of the tissue was often unspecified, insufficient measurements were recorded to render the data significant, and inadequate precautions were taken to establish the true magnification of the electron microscope. It was thus considered necessary to establish reliable data, as a function of age, for the size distributions of collagen fibrils in diverse connective tissues. Such data will not only lead to a better understanding of the mechanisms of fibrillogenesis but should form the basis of an interpretation of the mechanical attributes of a connective tissue in terms of its constituent collagen fibrils.

Tendons and ligaments are dense regular connective tissues; the constituent collagen fibrils, with their long axes lying parallel to one another, are close packed in an hydrated matrix which is rich in proteoglycans and contains fine elastic networks. Almost exclusively, the cellular elements in tendon are the elongate fibrocytes. Such

cells, which are responsible for the synthesis of collagen, proteoglycans and elastin, are arranged parallel to the bundles of collagen fibrils and have long slender cytoplasmic processes ramifying throughout the tissue. Fibrocytic processes, collagen fibrils and elastic fibres can be seen in the transverse section of rat-tail tendon shown in Figures 1.5 and 1.6. Such axially orientated collagenous tissues, being flexible and having high tensile strength, are well suited for sustaining tensile force.

Skin forms the physical limit between an animal and its environment and in most vertebrates its outermost layer is composed of a keratinizing stratified squamous epithelium. The basal (germinal) layer of this epidermis is commonly undulating and is separated from the underlying dermis by a basement membrane. The most superficial part of the dermis is thrown into folds which interdigitate with the undulations of the epidermis. The collagen fibrils of this, the papillary layer of the dermis, are usually smaller in diameter than those deeper in the dermis and are less well orientated though many of the fibrils within the papillary core lie at right angles to the epidermal surface. It has been suggested (Jarrett, 1973) that the epidermal cells rather than the fibrocytes may produce low molecular weight glycosaminoglycans in the papillary layer of the dermis, and that this region may act as a relatively fluid viscous layer which facilitates cohesion between the epidermis and the superficial dermis. The remainder of the dermis, the reticular layer, forms the greater part of the skin and consists largely of elongate fibrocytes, orientated interweaving bundles of collagen fibrils and the associated hydrated proteoglycan matrix.

The work undertaken in this thesis is thus an extension of that of Parry et al., (Parry and Craig, 1977, 1978, 1979; Parry et al., 1978a, 1978b, 1980; Craig and Parry, 1981a, 1981b) and consists of age-related studies of the skins of lamprey (a cyclostome), trout (a bony fish), frog (an amphibian), chick (a bird) and rat, guinea pig, sheep, greyhound and man (mammals) and the tendons and ligaments from the chick, guinea pig, rat and sheep. The collagenous features of a small selection of other vertebrate connective tissues will also be reported.

4.2 Tendons and Ligaments

4.2.1 Avian Metatarsal Tendon.

An electron microscope study of developing chick metatarsal tendons was first undertaken by Jackson (1956) who showed that the mean diameters of the collagen fibrils comprising the metatarsal tendons of 8, 11, 11-13, 14, 16 and 20 day foetal chickens were 8, 12, 17, 25, 31 and 40 nm respectively. Confirmation that these diameters do indeed reflect a possible 8 nm "microfibril" in the collagen fibril (see Fraser et al., 1979a; Parry and Craig, 1979, Parry et al., 1980 and Chapter 6) indicated that this tissue should be further investigated by both electron microscopy (this thesis, Eikenberry et al., 1982a) and low-angle x-ray diffraction (Eikenberry et al., 1982b). In addition these studies will allow important correlations to be made between the physical parameters determined by each of these techniques (see Chapter 6).

The collagen fibril diameter distribution data measured from chick metatarsal tendons are listed in Table 4.1. Those distributions which appeared to be composed of discrete populations (15 d F,

TABLE 4.1

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRILS IN CHICK METATARSAL TENDONS

Age (1,2)	Mean Diameter ± Std.devn. (nm)	Mass-average Diam. (nm)
11 d F*	33.1 ± 2.9	33.6
12 d F*	38.9 ± 1.9	39.1
13 d F*	44.9 ± 3.4	45.4
14 d F(i)*	39.1 ± 3.0	39.5
14 d F(ii)*	32.3 ± 1.8	32.5
15 d F**	37.9 ± 5.4	39.5
17 d F(i)*	41.2 ± 3.2	41.7
17 d F(ii)**	40.2 ± 5.0	41.4
18 d F**	50.4 ± 10.8	54.5
0 d	45.6 ± 11.1	50.5
Adult	132.9 ± 91.1	235.9

(1) F refers to foetal.

(2) Designations (i) and (ii) represent distributions obtained from different specimens from different areas of the same specimen.

* Standard deviations < 3.5nm. Populations are sharp and unimodal.

** Populations multimodal and can be resolved into sub-populations (see Table 4.2)

17 d F(ii) and 18 d F: d F specifies "days foetal") were analysed using a computer program for non-linear least squares decomposition of mixtures of populations with the assumptions that the populations were normal, and had equal standard deviations. From such analyses of the overall distributions, the mean values of the constituent populations and the proportion of the data falling within each population were derived and these are listed in Table 4.2. A low resolution micrograph of 18 day foetal chick metatarsal tendon is illustrated in Figure 4.1.

Further, the radial distribution functions derived from the spatial arrangement of the collagen fibrils were calculated (see Chapter 2) from micrographs of those chick metatarsal tendon specimens which displayed sharp unimodal distributions of fibril diameters (Figure 4.2a), and for the 18 day foetal specimen (Figure 4.2b). The modal separations of fibrils ascertained from such functions are then compared with the mean separations determined by X-ray diffraction studies (see Chapter 6) and, for the sharp unimodal distributions, the mean surface separation of fibrils is calculated as the difference between the modal separation and measured mean diameter; these data are listed in Table 4.3.

The foetal specimens examined in this study had all been fixed and embedded for electron microscopy by E.F. Eikenberry (Rutgers Medical School, Piscataway, New Jersey) and three of these (13 d F, 15 d F and 17 d F(ii)) were the same specimens used for X-ray diffraction studies. Subsequent electron microscopy of one of these specimens (15 d F) showed that it had suffered bacterial contamination, presumably whilst being held in buffer during the diffraction studies. Details of the fibril packing in this tissue are illustrated in Figure

TABLE 4.2

RESOLUTION OF MULTIMODAL DISTRIBUTIONS OF COLLAGEN FIBRILS IN CHICK METATARSAL TENDONS.

Age	Population mean diam. ± std.devn. (nm)	% total fibrils in population	Separation of means of population (nm)
15 d F	33.4 ± 2.0	45	
	40.8 ± 2.0	43	
	48.2 ± 2.0	12	7.4
17 d F*	34.3 ± 1.7	21	
	40.6 ± 1.7	55	
	46.9 ± 1.7	24	6.3
18 d F	36.6 ± 2.5	11	
	45.0 ± 2.5	31	
	53.4 ± 2.5	32	
	61.9 ± 2.5	22	
	70.3 ± 2.5	4	8.4

* Corresponds to 17 d F(ii) of Table 4.1.

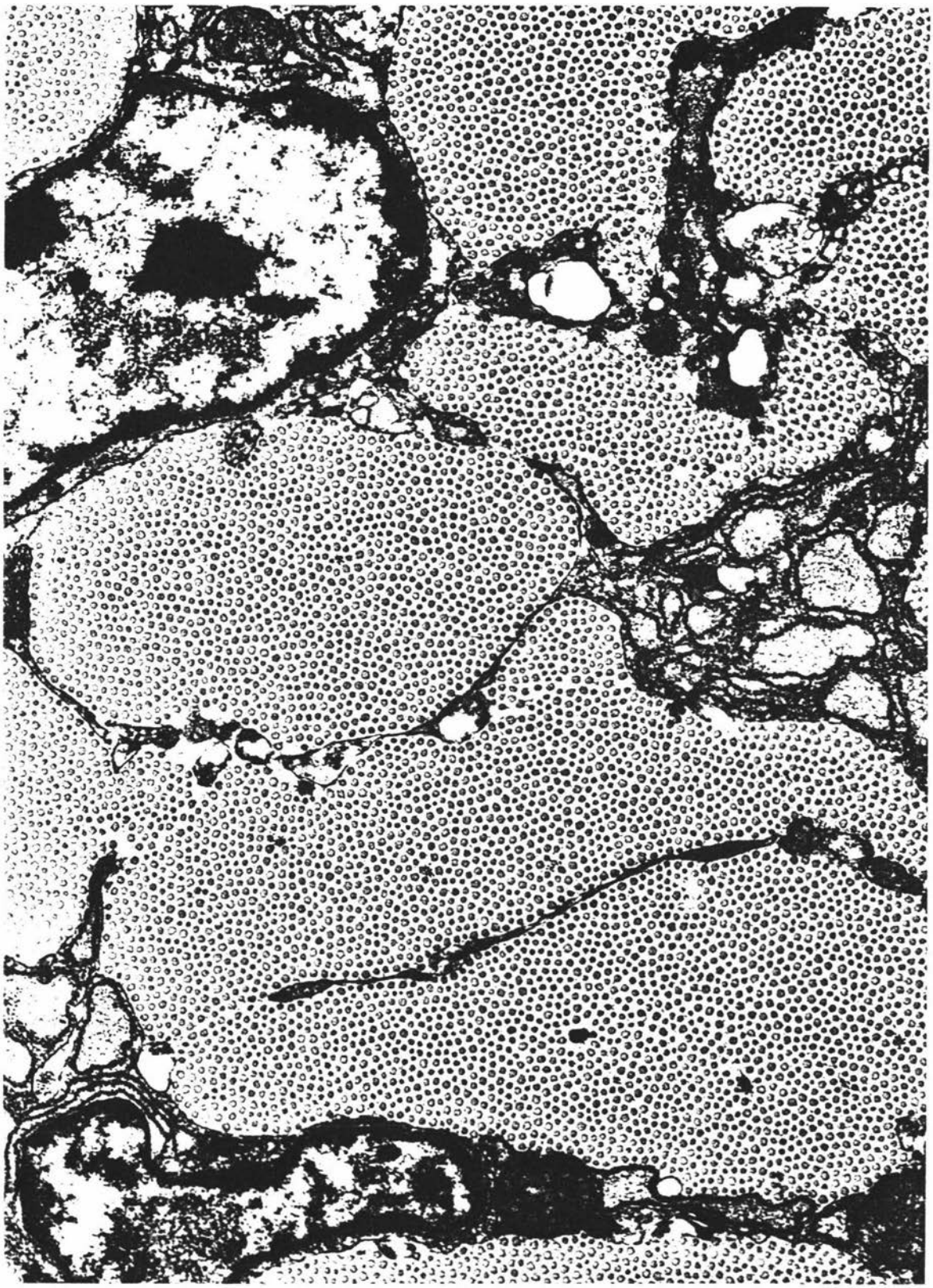

Figure 4.1 Electron micrograph of 18 day foetal chick metatarsal tendon showing cross sections of immature collagen fibrils organized into fibres which are delineated by the cytoplasmic processes of surrounding tenocytes. Magnification: 21 000 X.

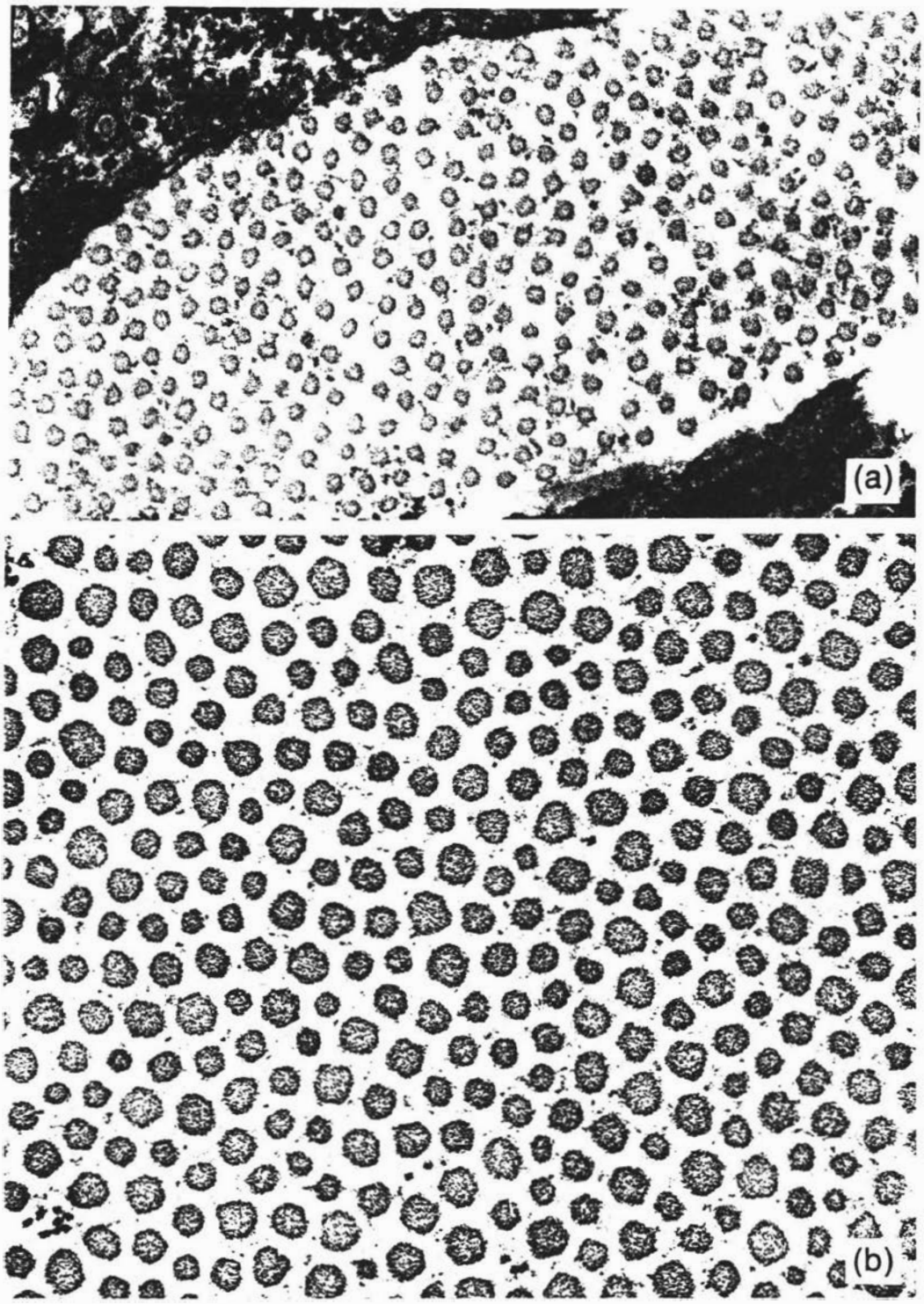

Figure 4.2 Electron micrographs of collagen fibrils in developing chick metatarsal tendons. Magnification: 65 000 X.
(a) 14 day foetal tendon displaying a population of uniform diameter collagen fibrils (mean diameter ~40 nm).
(b) 18 day foetal tendon displaying a heterogeneous population of collagen fibril diameters.

TABLE 4.3

MODAL CENTRE-TO-CENTRE AND MEAN SURFACE SEPARATIONS OF COLLAGEN FIBRILS IN FOETAL METATARSAL TENDONS

Age	Mean Diam. (nm)	Modal Sepn. (nm) (1)	Mean Surface Sepn. (nm) (2)	X-ray modal Sepn. (nm) (3)
11 d F	33.1	65	31.9	
12 d F	38.9	75	36.1	
13 d F	44.9	79	34.1	87
14 d F*	39.1	74	33.9	
15 d F	37.9	— (4)	---- (5)	96
17 d F**	41.2	75	32.8	
18 d F	50.4	82	---- (5)	
19 d F	----	--	----	87

(1) Calculated from radial distribution function.

(2) Calculated as the difference between the modal separation and the mean diameter.

(3) Data from Eikenberry et al., 1982b.

(4) Modal separation could not be calculated because of bacterial contamination (see Section 4.2.1).

(5) Cannot be meaningfully calculated as a difference for a broad multimodal distribution (see note 2).

* Corresponds to 14 d F(i) in Table 4.1.

** Corresponds to 17 d F(i) in Table 4.1.

4.3. These micrographs show the invasion of bacteria into the tissue and the resultant disorganization of packing leading to fibrils falling into configurations of close array.

Although such contamination must be considered an undesirable phenomenon, the resultant observations may indeed be providing some useful information. The collagen fibril diameter distribution appears to fit the pattern of growth established, in that the fibrils are larger than those present in the 13 day foetal specimen and are almost identical to one of the 17 day foetal specimens. It could thus be argued that the growth substrate for the bacterium is the tendon matrix rather than the collagen fibrils and that the digestion of this matrix has allowed the collagen fibrils to collapse into the close arrays observed. As the fibrils are never seen in such arrays in tendons in their normal physiological state, the observations on the bacterially contaminated tendon would indicate the importance of the matrix (presumably the proteoglycans) in maintaining some minimum surface to surface separation of the fibrils *in vivo*.

4.2.2 Mammalian Tendons and Ligaments

Earlier studies on foetal, newborn and adult rat and horse tissues (Parry and Craig 1977, 1978; Parry *et al.*, 1978a, 1978b, 1980) have suggested that, during the gestation period, modes of development of connective tissues vary significantly between mammalian species. Most mammals, including rat and man, have offspring which are very immature at birth and such newborn mammals require a considerable degree of parental care or nursing. Such a mode of development is referred to here as "altricious". In contrast the horse, an ungulate, is capable of locomotion within a short time of

Figure 4.3 Electron micrographs (a - d) showing the disorganization of the collagen fibrils within the bacterially contaminated matrix of the 15 day foetal chick metatarsal tendon. It would appear that the bacteria have digested the supportive matrix and allowed the collagen fibrils to fall into "close-arrays" as indicated here by the lines marked on the micrographs. Magnification: 100 000 X.

birth and it has been shown at this age to have tendinous tissues which are well developed in terms of collagen fibrillar contents (Parry et al., 1978b). Such a high degree of development in utero of the mammalian offspring gives rise to a neonate referred to here as "precocious".

Such locomotory precocity is found in the ungulates (Perissodactyla, Artiodactyla), sub-ungulates (Proboscidea, Sirenia, Hyracoidea), marine mammals (Cetacea, Pinnipedia), and the caviomorph rodents (guinea pig), whilst the mammals of the other placental orders give birth to their young in varying altricial states (see Table 4.4). There are undoubtedly some exceptions to this general classification. For example a few genera of families from other orders of placentals (mainly all Rodentia), which are listed as altricious, may tend towards being precocious. The two developing precocious mammals chosen for this study were the sheep and the guinea pig and the developing altricious mammal studied was the rat.

Three tissues were chosen for study in the sheep and these were the common digital extensor tendon, superficial digital flexor tendon and suspensory ligament. These were taken from the forelimbs of a selection of pre-natal and post-natal animals ranging in age from 60 day foetal to five weeks after birth. The gestation period of the sheep is nominally 145 days. In the 60 day foetal flexor tendon two separate specimens were studied, whilst in the 72 day foetal extensor tendon, 72 day foetal flexor tendon and 70 day foetal ligament two different areas of the same specimen were studied; in each of these cases two distinctive collagen fibril diameter distributions were recorded. All of the data obtained from this age-related study of sheep tendons and ligaments are listed in Table 4.5 and the forms of

TABLE 4.4

PRECOCIOUS-ALTRICIOUS CLASSIFICATION OF NEONATE PLACENTAL MAMMALS *

"GROUP" Order ** (sub-order)	Examples of order	Neonate state ***
UNGULATES		
Perissodactyla (16)	Horse, Tapir, Rhinoceros	P
Artiodactyla (171)	Sheep, Antelope, Hippopotamus	P
SUB-UNGULATE		
Proboscidea (2)	Elephant	P
Sirenia (5)	Dugong, Sea Cow, Manatee	P
Hyracoidea (11)	Hyrax	P
MARINE-MAMMALS		
Cetacea (84)	Whale, Dolphin, Porpoise	P
Pinnipedia (30)	Seal, Walrus, Sea-lion	P
OTHER PLACENTALS		
Rodentia (1690)		
(Caviamorpha)	Guinea pig, Paca, Agouti	P
(Other sub-orders)	Rat, Squirrel, Beaver	A
Insectivora (406)	Hedgehog, Mole, Shrew	A
Dermoptera (2)	Flying lemur	A
Chiroptera (853)	Bats	A
Edentata (31)	Anteater, Armadillo, Sloth	A
Pholidota (8)	Pangolin	A
Tubulidentata (1)	Aardvark	A
Lagomorpha (63)	Rabbit, Hare	A
Carnivora (254)	Cat, Dog, Bear	A
Primates (166)	Lemur, Monkey, Man	A

* Table compiled for this thesis from data obtained from Walker, (1975) and other sources.

** Figures in brackets indicate number of living species (Vaughan, 1978).

*** P, Precocious; A, Altricious.

TABLE 4.5

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS OF TENDONS AND LIGAMENTS IN THE DEVELOPING SHEEP

Age (1) (F, foetal)	Mean Diameter ± std.devn. (nm)	Mass-average diam. (nm)
(a) Forelimb common digital extensor tendon		
60 d F*	39.5 ± 2.7	39.9
72 d F(i)*	40.3 ± 2.3	40.6
72 d F(ii)**	44.0 ± 4.4	43.0
120 d F	71.2 ± 29.8	93.4
5 w	80.7 ± 28.4	99.1
(b) Forelimb superficial digital flexor tendon		
60 d F**	29.5 ± 3.8	30.5
72 d F(i)**	38.7 ± 4.2	39.6
72 d F(ii)*	40.4 ± 2.2	40.6
120 d F	74.4 ± 21.1	85.3
5 w	95.0 ± 34.2	116.7
(c) Forelimb suspensory ligament		
70 d F(i)*	24.8 ± 1.3	25.0
70 d F(ii)**	29.4 ± 3.9	30.4
72 d F*	40.5 ± 3.5	41.0
120 d F	69.7 ± 22.1	82.2
5 w	98.7 ± 30.1	114.8

(1) Designations (i) and (ii) represent distributions obtained from different areas of the same specimen.

* Sharp unimodal distribution; standard deviation < 3.5nm.

** Bimodal distribution, can be resolved into component populations (see Table 4.6)

the distributions of the collagen fibril diameters from some of these tissues are shown in Figures 4.4 - 4.6, as both frequency and volume distributions. Four of the distributions were relatively sharp (standard deviations 3.8 - 4.4 nm) but clearly contained two populations of fibrils; these were further analysed to resolve their constituent populations. These data are listed in Table 4.6 along with those populations listed in Table 4.5 which were unimodal and sharp (standard deviation < 3.5 nm).

The tendinous tissues studied in the guinea pig were the forelimb digital flexor and extensor tendons and the central tendon of the diaphragm. The ages of the animals studied were 0, 7, 17 and 66 day and adult (~1 year). The mean and mass-average diameters of the collagen fibrils in these tissues are listed in Table 4.7 and the forms of the distributions are shown in Figures 4.7 - 4.9, as both frequency and volume distributions. All such distributions were broad at birth and none could be resolved into constituent populations.

A considerable amount of data relating to the diameter distributions of collagen fibrils from tendinous tissues in post-natal, mature and ageing rats has already been published (e.g. rat tail-tendon, Parry and Craig 1977, 1978; tibial collateral ligament, flexor digitorum longus and Achilles tendons, Parry et al., 1980). These studies reflected the degree of immaturity of the tissues in this altricious mammal two days after birth, and this present study is an extension of the earlier work in that it examines a forelimb (flexor digitorum longus) and a hindlimb flexor tendon in their late pre-natal and early post-natal development. The gestation period of the rat is 21 days and the ages chosen for study were 18 day foetal, 0, 2 and 5 days.

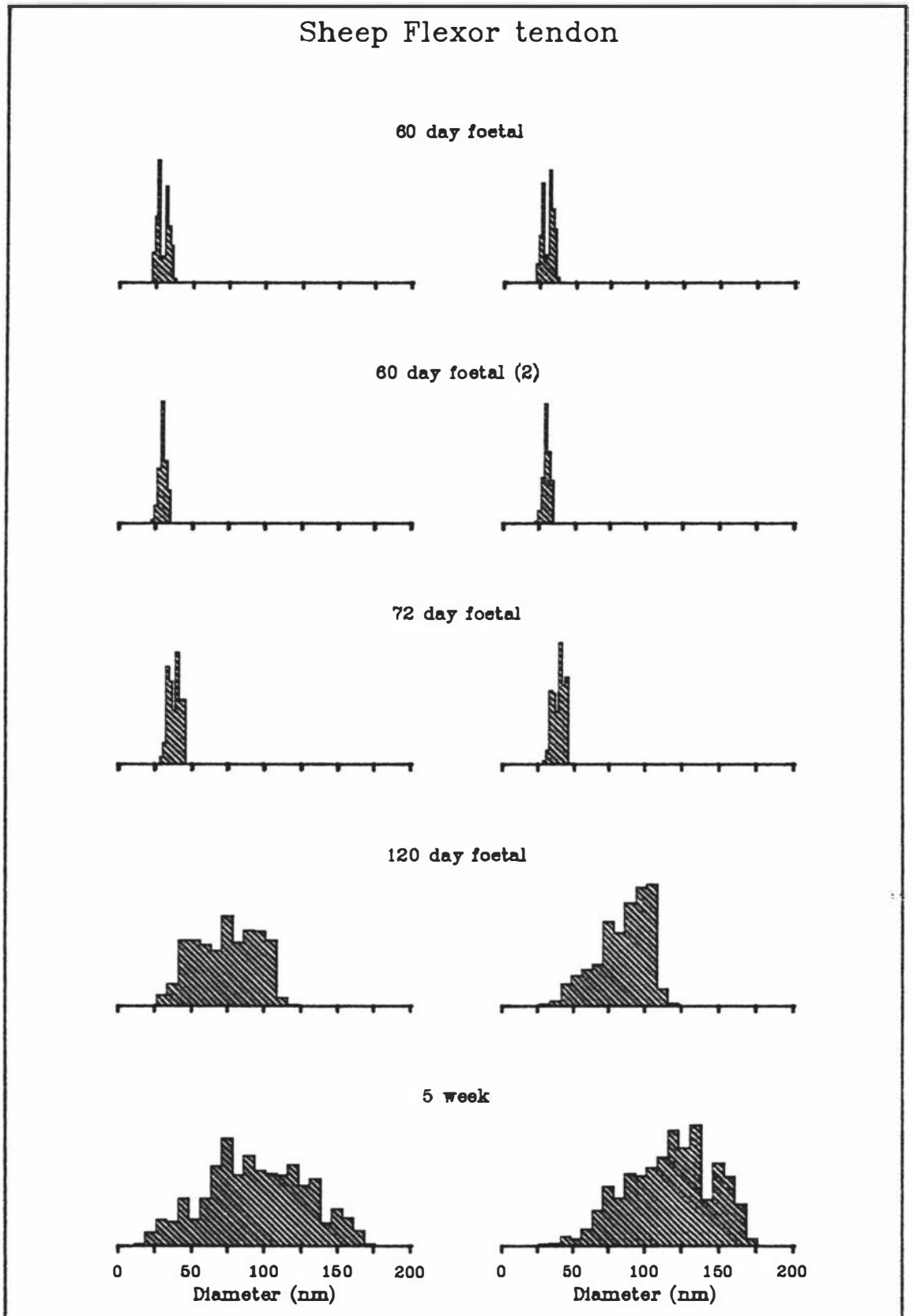

Figure 4.4 Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a sheep flexor tendon.

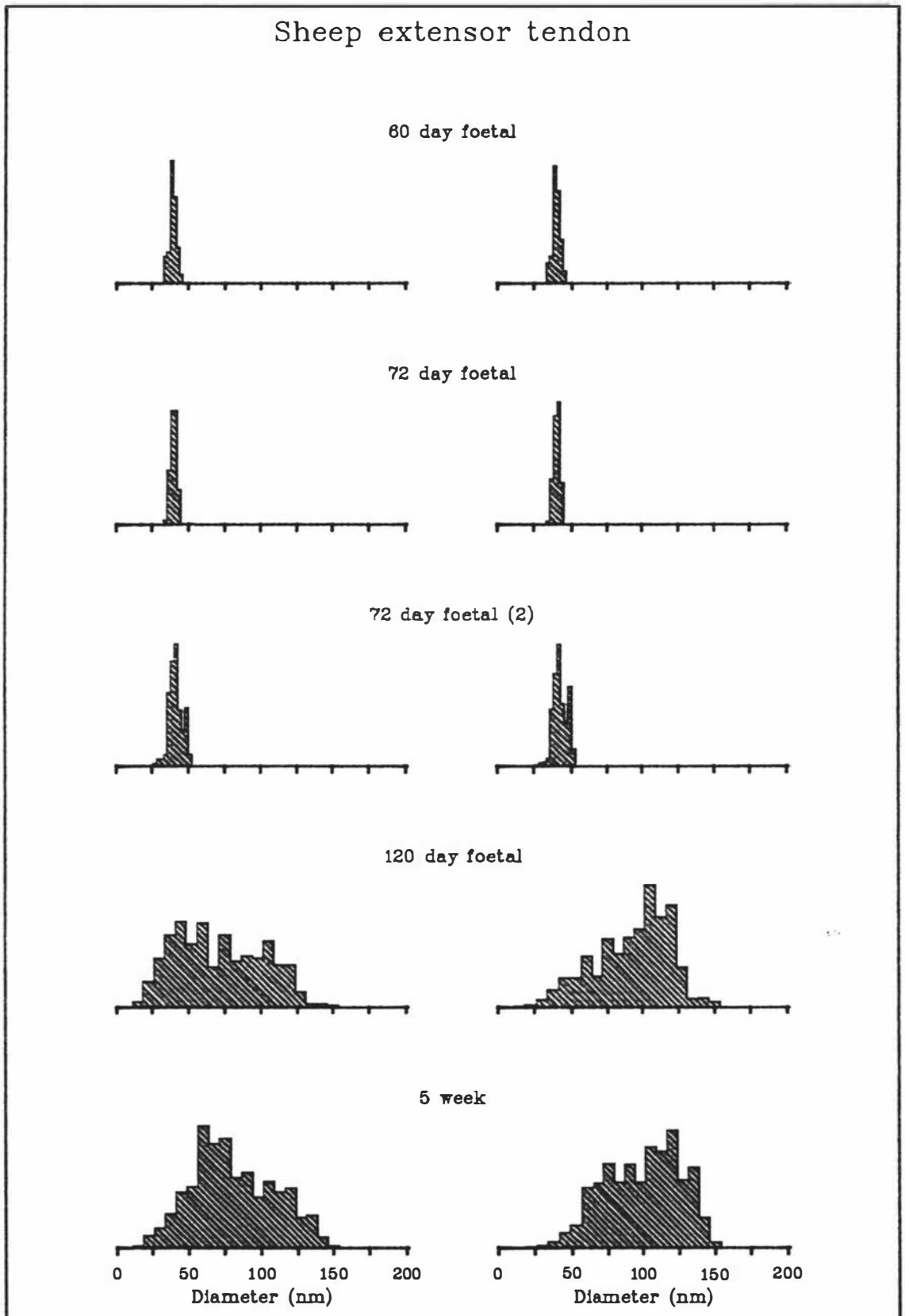

Figure 4.5 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a sheep extensor tendon.**

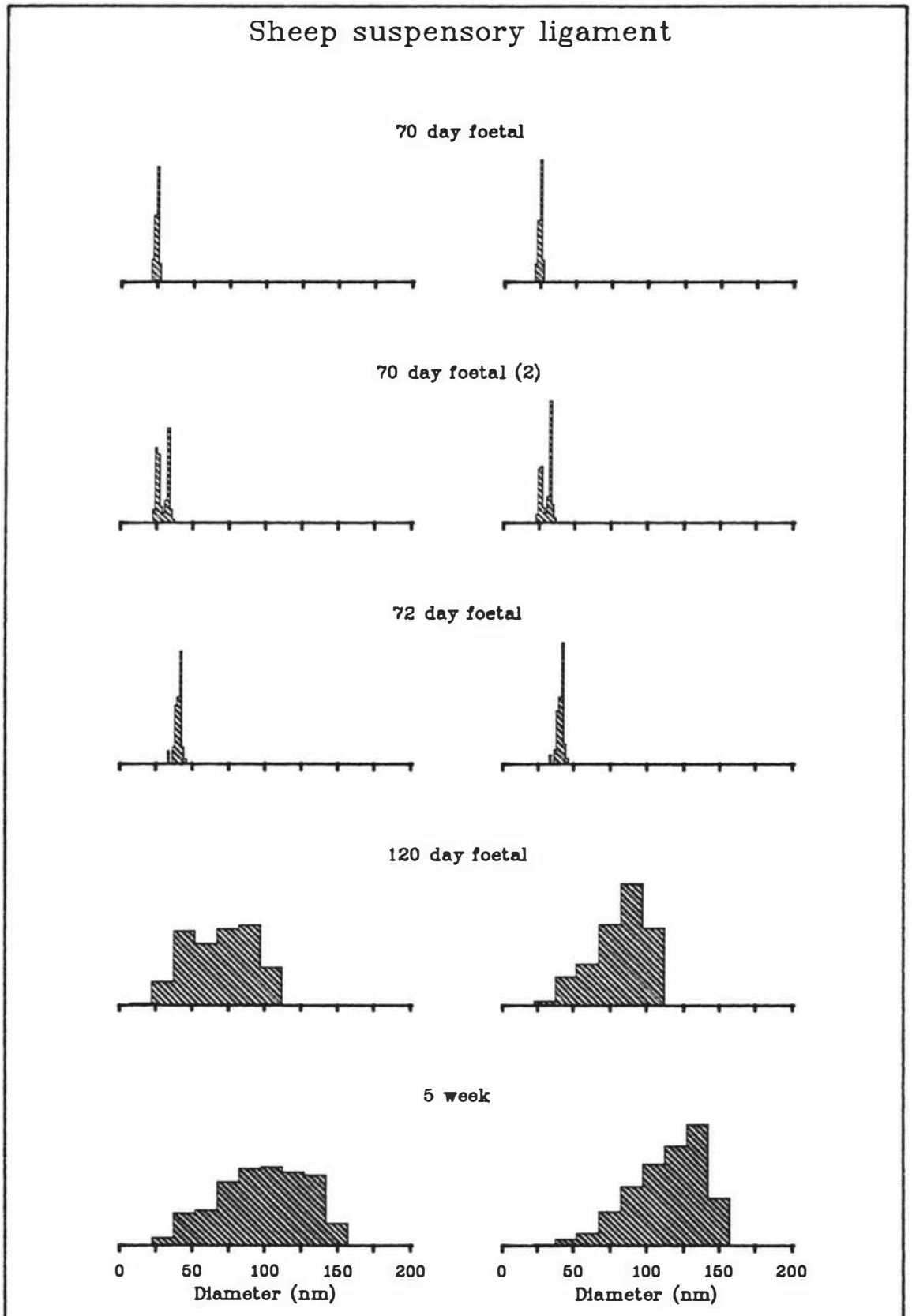

Figure 4.6 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a sheep suspensory ligament.**

TABLE 4.6

RESOLUTION OF SUB-POPULATIONS OF COLLAGEN FIBRIL DIAMETERS IN SHEEP TENDONS AND LIGAMENTS; THEIR COMPARISON WITH THE SHARP UNIMODAL DISTRIBUTIONS OBSERVED.

Age (1) (F, foetal)	Mean Diam. ± std.devn. (nm)	Nominal Fibril Diam.* (nm)	% Total fibrils in (sub)population
(a) Forelimb common digital extensor tendon			
60 d F	39.5 ± 2.7	40	100
72 d F(i)	40.3 ± 2.3	40	100
72 d F(ii)	40.6 ± 2.7	40	75
	48.9 ± 2.7	48	25
(b) Forelimb superficial digital flexor tendon			
60 d F	26.5 ± 1.5	24	55
	32.9 ± 1.5	32	45
72 d F(i)	34.8 ± 2.3	32	48
	41.9 ± 2.3	40	52
72 d F(ii)	40.4 ± 2.2	40	100
(c) Forelimb suspensory ligament			
70 d F(i)	24.8 ± 1.3	24	100
70 d F(ii)	26.0 ± 0.9	24	52
	33.6 ± 0.9	32	48
72 d F	40.5 ± 3.5	40	100

(1) Designations (i) and (ii) represent distributions obtained from different areas of the same specimen.

* Fibril diameter expressed as multiple of 8 nm closest to the observed value.

TABLE 4.7

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS OF TENDONS FROM NEONATE TO ADULT GUINEA PIGS.

Age	Mean Diam. ± std.devn. (nm)	Mass-average diam. (nm)
(a) Digital extensor tendon		
0 d	72.5 ± 26.3	88.9
7 d	81.4 ± 31.3	102.5
17 d	82.8 ± 34.1	106.7
66 d	128.9 ± 53.5	164.4
Adult	165.6 ± 81.1	224.1
(b) Digital flexor tendon		
0 d	87.6 ± 24.8	99.7
7 d	84.5 ± 37.8	115.0
17 d	68.0 ± 29.9	89.7
66 d	63.8 ± 34.4	100.6
Adult	73.4 ± 45.3	121.6
(c) Central tendon of diaphragm		
0 d	50.0 ± 12.1	55.3
7 d	47.4 ± 12.7	53.6
17 d	59.4 ± 21.6	73.4
66 d	73.0 ± 27.8	90.9
Adult	86.6 ± 50.5	134.9

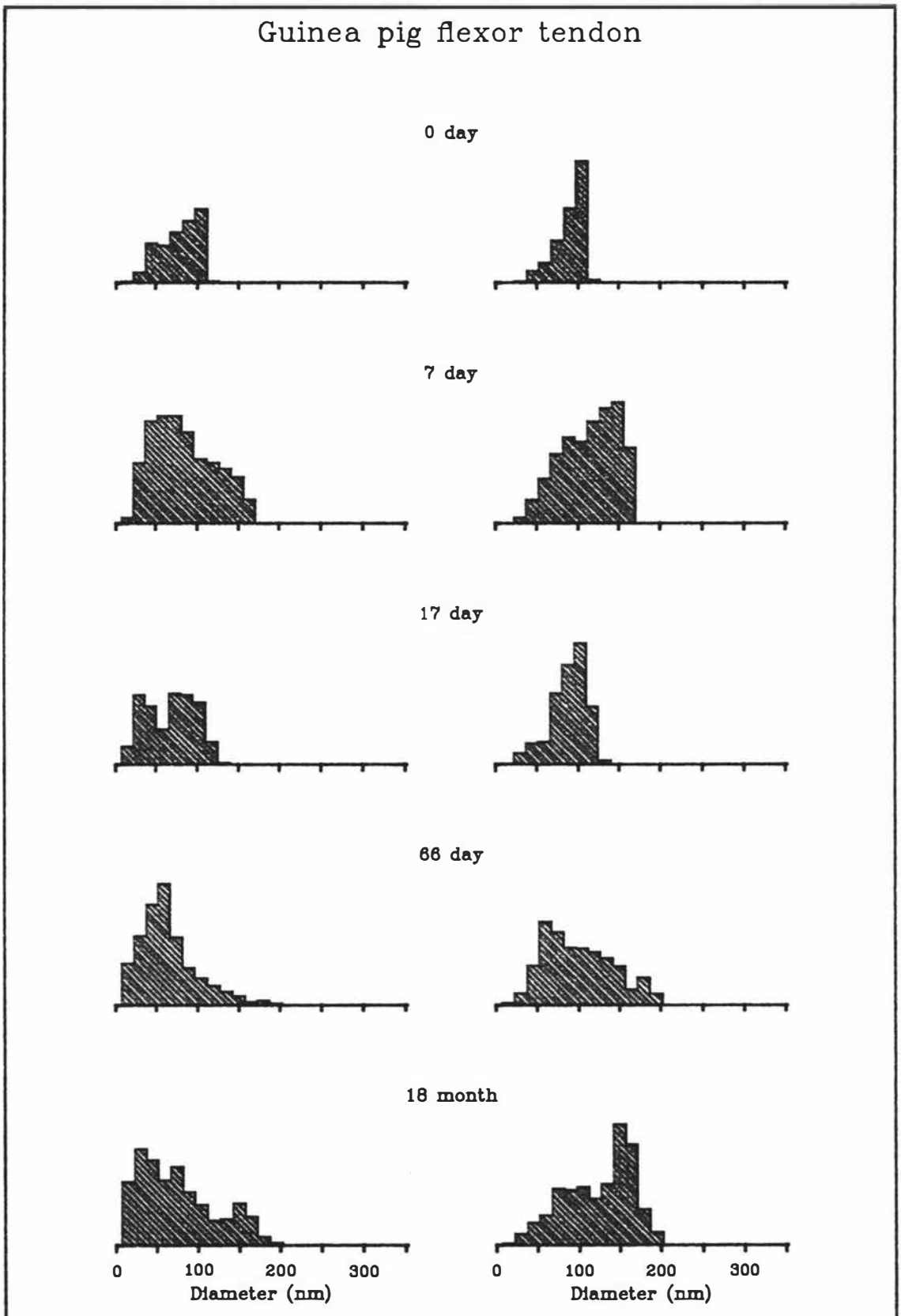

Figure 4.7 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a guinea pig flexor tendon.**

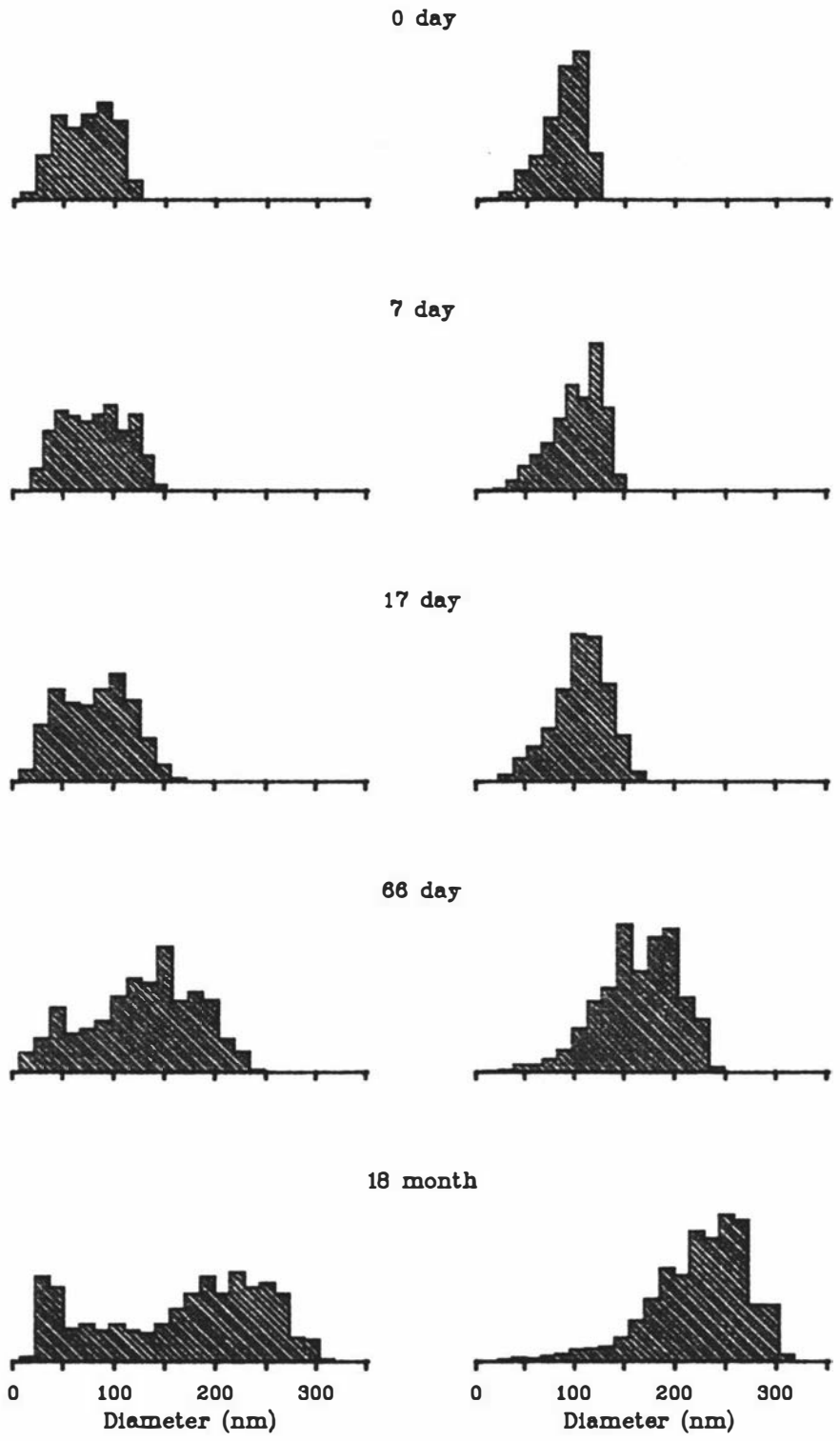

Figure 4.8 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a guinea pig extensor tendon.**

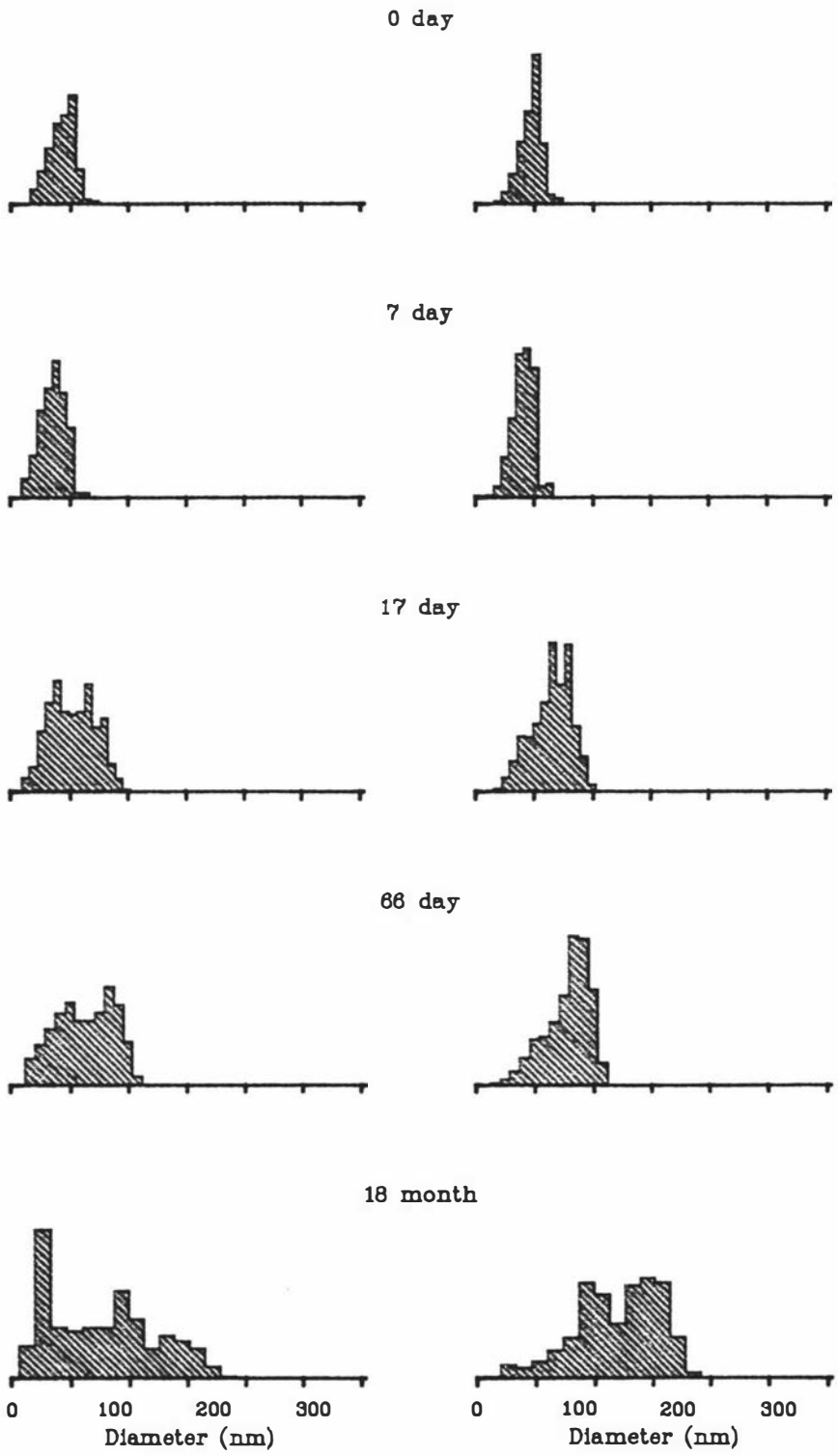

Figure 4.9 Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a guinea pig diaphragmatic tendon.

The data obtained from this study are listed in Table 4.8 along with the data of Parry et al., (1980) for the 5 day, 8 week and 16 week forelimb flexor tendons. Three of the distributions were relatively sharp (standard deviations ~5-6 nm) but contained two populations, and these were also analysed to resolve their constituent populations. These data are listed in Table 4.9 along with those populations from Table 4.8 which were seen to be unimodal and sharp (standard deviations < 3.5 nm). The forms of the distributions of the collagen fibril diameters are shown in Figures 4.10 - 4.11.

4.3 Skins

A large number of skin samples from a diversity of animals have been studied. Guinea pig and rat were chosen for study as examples of precocious and altricious mammals which are available for sacrifice for experiment. Two types of skin were examined in each of these animals. From the guinea pig, samples were taken from the dorsal and ventral abdominal skin and from the pressure-bearing skin of the foot-pad, whilst from the rat, samples were taken of abdominal skin and of the "tensile-skin" of the tail. Skin samples from the sheep, a second precocious mammal, were made available at known stages of foetal development by research colleagues who were sacrificing the animals for lipid metabolism experiments. This allowed the important pre-natal stage of development of collagen fibrils in a precocious mammal to be studied. The second altricious mammal studied was man. Formalin fixed foetal specimens and neonate, mature and senescent specimens from biopsy/autopsy were made available, allowing the collagen fibril diameter distribution to be determined at these ages. Normal adult greyhound skin was taken (biopsy) as a control for the

TABLE 4.8

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS OF FLEXOR TENDONS IN FOETAL TO MATURE RATS

Age (1) (F, foetal)	Mean diam. ± std.devn. (nm)	Mass-average diam. (nm)
(a) Hindlimb flexor tendon.		
18 d F*	17.9 ± 1.3	18.1
0 d*	31.1 ± 1.5	31.2
2 d**	34.1 ± 4.9	35.5
5 d*	34.9 ± 2.0	35.1
(b) Forelimb flexor tendon.		
18 d F*	24.3 ± 2.2	24.7
0 d*	30.6 ± 1.6	30.7
2 d**	32.3 ± 6.1	34.5
5 d(i)**	26.7 ± 5.6	28.9
5 d(ii)	44.3 ± 4.9	45.3
8 w	125.4 ± 63.5 (2)	175.2
16 w	132.9 ± 74.8 (2)	204.1
(c) Achilles tendon		
5 d	47.6 ± 4.4	47.5

(1) Designations (i) and (ii) represent distributions obtained from different areas of the same specimen.

(2) Data from Parry *et al.*, 1980.

* Sharp unimodal distribution; standard deviation <3.5nm.

** Bimodal distribution, can be resolved into component populations (see Table 4.9)

TABLE 4.9

RESOLUTION OF BIMODAL DISTRIBUTIONS OF COLLAGEN FIBRIL DIAMETERS IN RAT TENDONS AND THEIR COMPARISON TO THE SHARP UNIMODAL DISTRIBUTIONS OBSERVED

Age (F, foetal)	Mean diam. ± std.devn. (nm)	Nominal Fibril Diam.* (nm)	% total fibrils in (sub)population
(a) Hindlimb flexor tendon			
18 d F	17.9 ± 1.3	16	100
0 d	31.1 ± 1.5	32	100
2 d	28.2 ± 1.5	24	21
	35.1 ± 1.5	32	66
	43.0 ± 1.5	40	13
5 d	33.5 ± 2.7	32	100
b) Forelimb flexor tendon			
18 d F	24.3 ± 2.2	24	100
0 d	30.6 ± 1.6	32	100
2 d	26.4 ± 2.5	24	39
	34.1 ± 2.5	32	41
	40.6 ± 2.5	40	20
5 d	22.9 ± 3.3	24	55
	32.5 ± 3.3	32	45

* Fibril diameter expressed as multiple of 8 nm closest to the observed value.

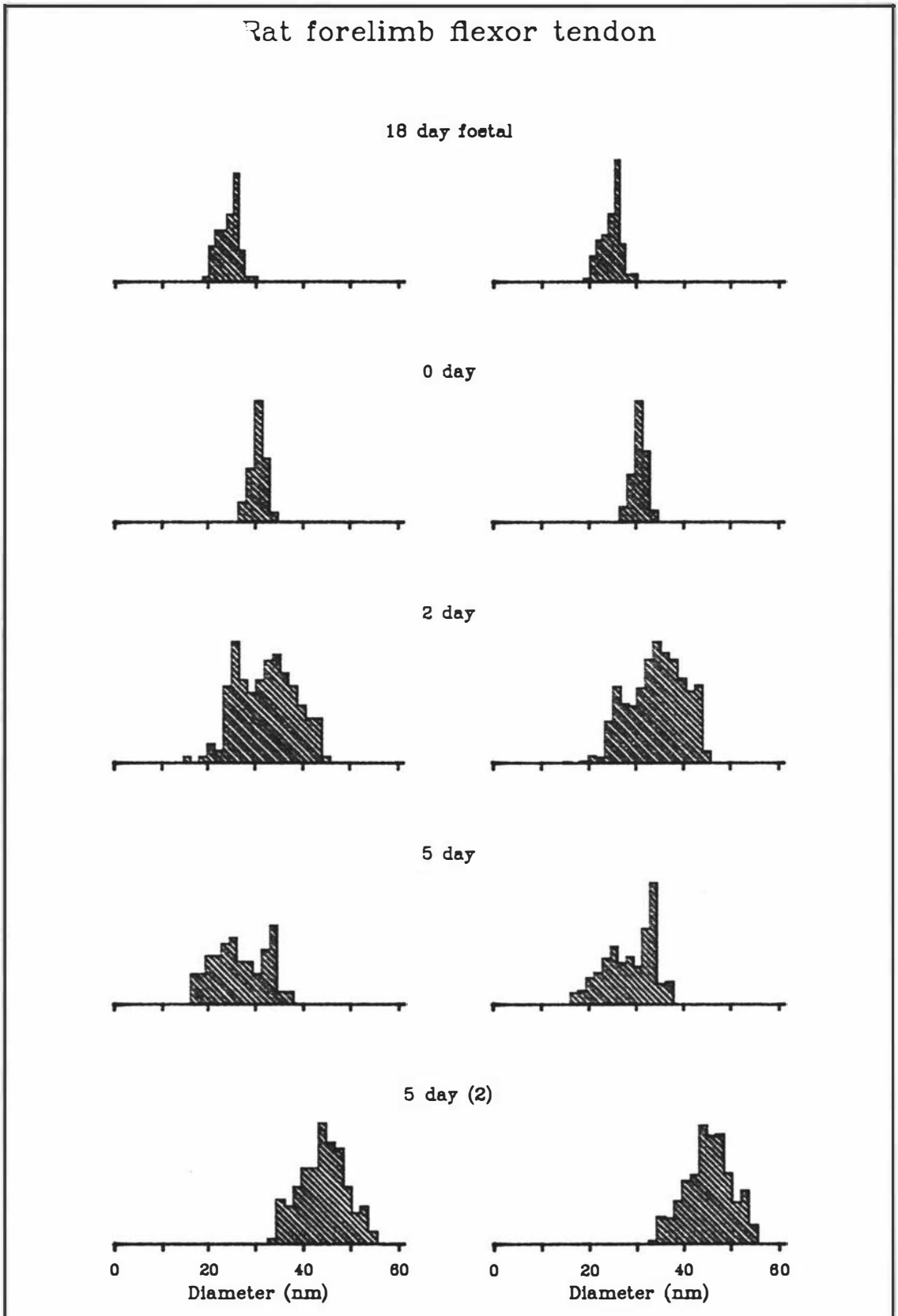

Figure 4.10 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a rat forelimb flexor tendon.**

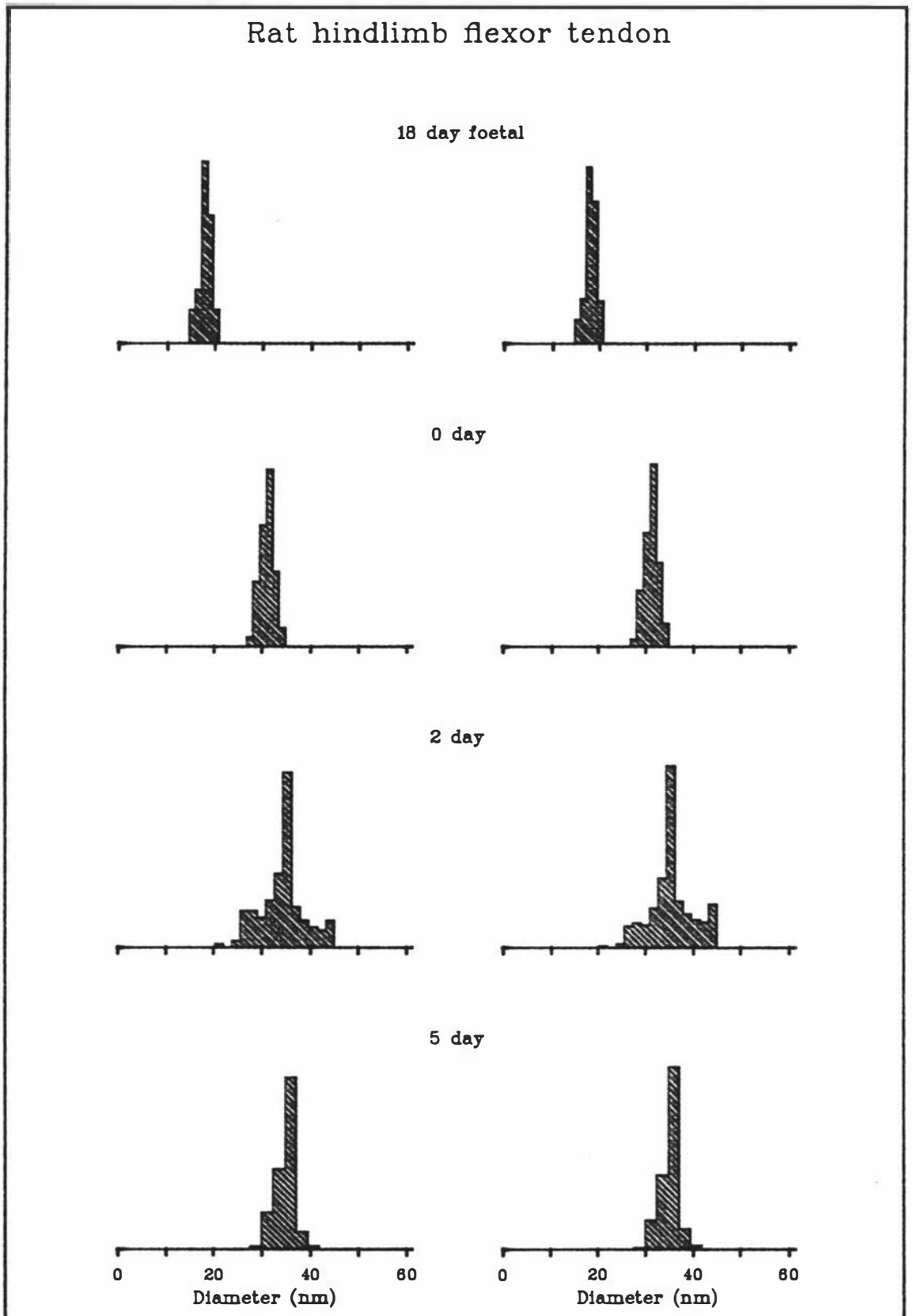

Figure 4.11 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from various stages of development in a rat hindlimb flexor tendon.**

dysplastic greyhound skin studied (Chapter 5) and chicken skin samples at birth and adult were taken along with metatarsal tendons necessary for the completion of the chicken tendon study.

Wainwright et al., (1978) have described the gross histology of shark skin, showing that it differs from mammalian skin and suggesting that the structure is probably peculiar to both the cartilaginous and bony fish, and for this reason the skin of adult trout, and lamprey at three stages of development (ammocoete, macrophthalmia and adult), were examined in this present work. A summary of all collagen fibril data relating to the skins studied are listed in Table 4.10.

4.4 Other Tissues

During the course of these studies a variety of tissues have been examined other than those already outlined in this and the preceding chapters. These are the notochord sheath of the lamprey, tadpole skin, the central tendon of the diaphragm and fibrocartilage of the lumbar annulus fibrosus of the rat, bone of a developing forelimb phalanx of the guinea pig, Achilles tendons of both dog and ox, and Whartons jelly from the umbilical cord of man. For completeness of inclusion of all results the measurements made from these tissues are listed in Table 4.11 along with the mean and mass-average diameters of their constituent collagen fibrils. These data will be made use of in the relevant sections of Chapters 6 to 8.

TABLE 4.10

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS IN SKIN

Source (1)	Age (2) (F, foetal)	Mean Diam. ± std.devn. (nm)	Mass-av. diam. (nm)
Lamprey	Macrophthalmia	44.0 ± 2.0	44.2
	Adult	51.9 ± 3.4*	52.4
Trout	Adult 1	69.3 ± 43.8	131.1
	Adult 2	135.5 ± 66.4	195.0
Frog	1.2 g tadpole	32.1 ± 2.0**	--
		38.8 ± 2.0**	--
Chicken	0 d	48.4 ± 6.4	50.1
	Adult	91.8 ± 9.6	93.7
Rat	18 d F	25.8 ± 2.1*	26.1
(ventral-abdominal)	0 d	32.0 ± 3.4*	32.8
	2 d	33.2 ± 1.3	33.3
	2 d	39.5 ± 1.8	39.7
	5 d	31.9 ± 2.2	32.2
	5 d	38.6 ± 1.8	38.7
	6 d	43.1 ± 3.4	43.6
	5 w	95.1 ± 18.4	101.3
	8 w	112.0 ± 21.1	119.7
	12 w	105.2 ± 18.7	112.0
	16 w	124.6 ± 21.1	131.8
	20 mo	92.5 ± 14.7	97.4
Rat	2 d	30.0 ± 3.8	30.9
(dorsal-abdominal)	1 mo	59.9 ± 10.8	63.4
	3 mo	117.0 ± 26.0	128.9
	5 mo	103.1 ± 23.0	113.9
Rat	2 d	31.0 ± 4.3	32.2
(tail)	1 mo	85.0 ± 28.5	102.5
	3 mo	100.4 ± 22.4	110.5
	5 mo	109.5 ± 49.6	153.4
	1 y	112.2 ± 51.8	154.8
Sheep	60 d F(i)	24.2 ± 1.5*	24.4
	60 d F(ii)	31.8 ± 2.0*	32.1
	72 d F	32.1 ± 2.1*	32.3
	120 d F	84.1 ± 12.2	87.5
	0 d	82.7 ± 15.0	87.6
	Adult	62.6 ± 9.2	72.5
Guinea pig	0 d	75.6 ± 15.5	81.4
(ventral-abdominal)	7 d	88.7 ± 12.2	91.9
	17 d	75.3 ± 15.7	81.7
	66 d	88.5 ± 14.0	92.6
	Adult	72.1 ± 9.6	74.5
Guinea pig	5 d	56.6 ± 10.5	60.3
(dorsal-abdominal)	11 w	109.7 ± 14.8	113.7

continued next page

Table 4.10 continued

Guinea pig (foot-pad)	0 d	70.5 ± 9.8	73.1
	66 d	55.3 ± 14.5	62.0
	Adult	56.5 ± 13.3	62.9
Greyhound	Adult	85.8 ± 16.1	91.6
Human	14 w F	26.5 ± 1.6*	26.7
	24 w F	56.5 ± 8.3	58.6
	5 d	67.4 ± 10.0	70.1
	20 y	91.7 ± 13.3	95.4
	70 y	76.8 ± 9.6	79.3

(1) All samples are of body-skin unless stated otherwise.

(2) Designations (i) and (ii) represent distributions obtained from different areas of the same specimen.

* Sharp unimodal distribution; standard deviation < 3.5nm.

** Resolved into two populations and mass-average diameters were not computed.

TABLE 4.11

MEAN AND MASS AVERAGE DIAMETERS OF COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS RECORDED FOR SOME MISCELLANEOUS TISSUES.

Source	Tissue	Age	Mean Diam. ± std.devn. (nm)	Mass-average Diam. (nm)
Lamprey	Notochord sheath	Adult	15.2 ± 1.5	15.5
Tadpole	Fin	1.2 g	35.7 ± 3.2	36.3
Rat	Diaphragm (1)	0 d	40.4 ± 7.4	43.1
		4 d	38.4 ± 8.0	41.4
	Fibrocartilage (2)	8 w	36.2 ± 21.2	61.2
Guinea pig	Bone (3)	0 d	~80 *	
		7 d	~80 *	
Greyhound	Achilles tendon	Adult	156.5 ± 77.2	219.1
Ox	Achilles tendon	Adult	111.8 ± 74.9	197.9
Dog	Sesamoid region (4)	Adult	35.4 ± 16.1	48.4
	A-region (4)	Adult	109.6 ± 52.4	151.8
Man	Whartons Jelly	0 d	38.9 ± 4.9	40.1

(1) Central tendon of the diaphragm; (2) Annulus fibrosus (lumbar);
(3) Developing forelimb phalanx; (4) Flexor digitorum sublimis tendon.

* Not enough measurements taken to make the standard deviation representative of the range of fibril diameters or to determine the form of the distribution.

CHAPTER FIVE

COLLAGEN FIBRIL ASSEMBLY DISORDERS

5.1 Introduction

A large number of hereditable and acquired diseases either arise from or lead to defects in the mechanism of collagen fibrillogenesis. The likely aetiology of some of these disorders have been reviewed (Uitto and Lichtenstein, 1976; Minor, 1978; Light and Bailey, 1979; Bailey and Etherington, 1980; Francis and Duksin, 1983) and range widely from cistron-translation in Marfan Syndrome and Ehlers-Danlos Syndrome Type IV (EDS-IV), the presence of aldehyde blocking agents in homocystinuria, low levels or activities of enzymes such as lysyl hydroxylase in EDS-VI and Osteogenesis imperfecta, and lysyl oxidase in EDS-V, Menkes Kinky Hair Syndrome and lathyrism, and unusual metabolic regulation in the Werner Syndrome and Progeria. Other disorders include the usually rare and mostly hereditable diseases such as Cutis laxa, scurvy, rickets, diabetes, dermatosparaxis, osteopetrosis and rheumatoid arthritis (Light and Bailey, 1979). Although no morphological changes have been reported in the collagen fibrils in some of these disorders, others present a ready diagnosis through either fibrillar malformations or a changed collagen fibril diameter distribution.

The most studied of these disorders is possibly the Ehlers-Danlos Syndrome (EDS) which has seven distinctive forms, involving different defects in the metabolism of collagen in the skin, skeletal fibrous tissues, gastro-intestinal tract, cardiovascular system, placenta and eye in man. Three forms of the syndrome (EDS-I, EDS-II and EDS-III)

have an autosomal dominant pattern of inheritance, three (EDS-IV, EDS-VI and EDS-VII) have an autosomal recessive pattern of inheritance, whilst EDS-V has an X-linked mode of inheritance. All of these disorders are now assumed to be due to defects in the metabolism of collagen and since the primary function of collagen is to provide the appropriate mechanical attributes for the tissue it is not surprising that sufferers of this syndrome present clinical symptoms characterized by both hyperextensibility and increased fragility of one or more tissues.

A most extreme degree of collagen fibrillar malformation is displayed in the hereditable disorder known as dermatosparaxis, which has been shown to occur in Belgian and Texan cattle (O'Hara et al., 1970; Simar and Betz, 1971), Norwegian sheep (Helle and Ness, 1972; Fjolstad and Helle, 1974) and a Himalayan cat (Holbrook et al., 1980). Using electron microscopy the fibrils have been described as twisted sheets or ribbons which have a stellate or pinwheel appearance in transverse section (O'Hara et al., 1970; Fjolstad and Helle, 1974). It has been claimed that the thickness of such ribbons and the predominant four-fold symmetry of the fibrils may reflect the size and packing-symmetry of underlying sub-fibrillar structure (Fraser et al., 1979a).

Dermatosparaxis is caused by an abnormally low level (or activity) of procollagen amino-peptidase in the tissue and a subsequent lack of cleavage of the amino-terminal propeptide. The persistence of the terminal peptide gives rise to a molecular form known as pN-collagen. The presence of pN-collagen in tissues leads to a cross-linking deficiency which results in the gross disorganization of the fibrils. An hypothesis proposed by Delvoye et al., (1983)

would suggest that the activity of the procollagen peptidase has been affected indirectly by a defective intracellular processing of the oligosaccharide side-chain in the amino-terminal region of the procollagen molecule. Other connective tissue dysplasias show similarly malformed fibrils though usually to a lesser extent than seen for the dermatosparactic tissues. These include human emphysematous lung (Belton et al., 1977), "tight-skin" mutant mouse dermis (Menton and Hess, 1980), dermal lesions of humans suffering from Buschke-Ollendorff syndrome (Uitto et al., 1981) and the dermis of man in EDS-I (Vogel et al., 1979).

Other dysplasias of connective tissue are characterised by distributions of collagen fibril diameters which are markedly changed from normal. Examples of such changed distributions have been shown in the cornea of man suffering from leucoma (Schwarz, 1957), in corneal stromal dystrophy (Witschel et al.,1978), in tendon from a horse suffering from a disorder known as "contracted tendon" (Parry et al., 1978b) and in skin of man afflicted with EDS-I, II, IV and V (Black et al., 1980; Holbrook and Byers, 1981).

The pathological tissues studied in this thesis were skin and tendon of a dermatosparactic lamb, the skin of a greyhound having a dysplastic dermis and the palmar fascia from a man afflicted with Dupuytren's contracture. Wounding responses were studied in rat skin and the effects of in vivo intratendinous injection of bacterial collagenase were examined in the horse. Also studied were the collagen fibril diameter distributions of the "normal" (native) collagen fibrils from the Cuvierian tubules of a sea cucumber. The results of these studies are discussed in terms of the resulting fibrillar malformations or changing fibril diameter distributions.

5.2 Fibrillar Malformations

5.2.1 Hereditable disorders

In this work skin samples from a dermatosparactic lamb showed varying degrees of fibrillar malformation throughout the dermis. In those regions where the degree of malformation of the fibrils was least severe the fibrils still have irregular outlines in section and neighbouring fibrils often appear to be fused (Figure 5.1a). In these regions, however, an estimate of the range of fibril diameters can be made (20 - 130 nm); such a range of diameters is comparable to that found in normal newborn lamb skin (82.7 $\pm$ 15.0 nm; Figure 5.1b). Other areas of the dermis displayed gross fibrillar disorganization and no estimate of the fibril diameter could meaningfully be made (Figure 5.1c). Forelimb flexor tendon from a newborn dermatosparactic lamb was also studied by electron microscopy. A general survey of this tissue showed that, for the most part, the collagen fibrils were circular in section and had mean diameters of ~80 nm. This value is similar to that obtained previously by Craig and Parry (1981b) for neonate sheep flexor tendon. However, small regions of tendon can be found in which the fibril diameters were less than normal and where many of the fibrils were irregular in outline (Figure 5.2).

These observations indicated that the structure of the collagen fibrils in this tendon were largely normal and that the defects in fibrillogenesis were primarily confined to the skin. These results confirm the conclusions of Cassidy *et al.*, (1980) derived from biochemical and X-ray analyses on these tissues. In particular they noted that dermatosparactic skin contained about 70% pN-collagen, whereas in the dermatosparactic tendon only about 15% pN-collagen was present. These values parallel, in a qualitative manner, the degree

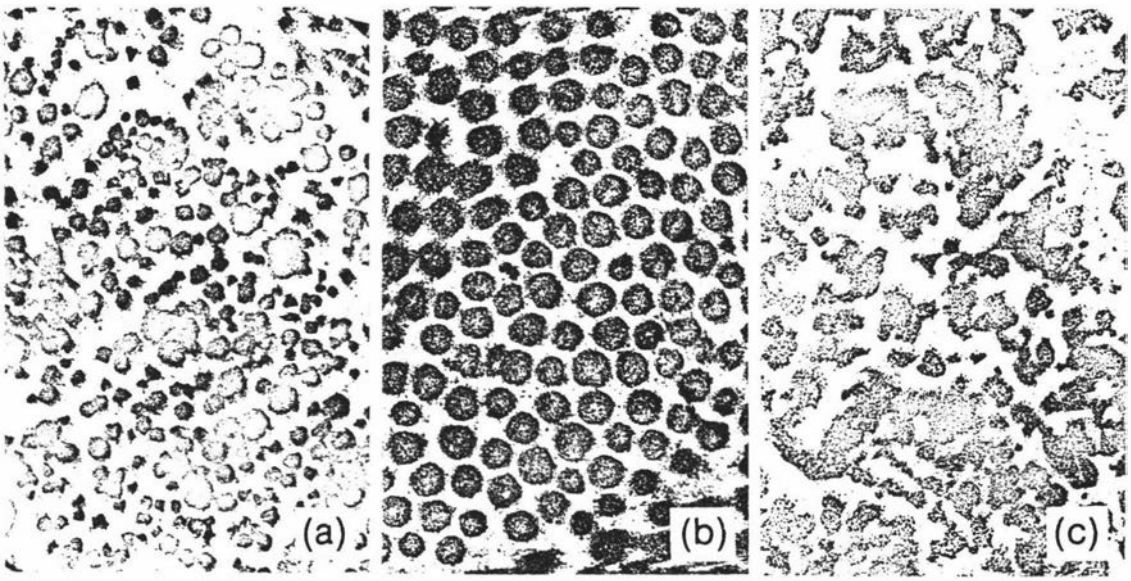

Figure 5.1 Electron micrographs of transverse sections of collagen fibrils from the skin of newborn lambs. Magnification: 43 000 X.
(a) Dermis of a dermatosparactic lamb showing fibrils which are irregular in outline and have a wide range of diameters.
(b) Dermis of a normal newborn lamb showing collagen fibrils which are circular in outline (mean diameter 83 nm; mass-average diameter 88 nm).
(c) Area in the dermis of the dermatosparactic lamb displaying a gross disorganization of its constituent collagen fibrils.

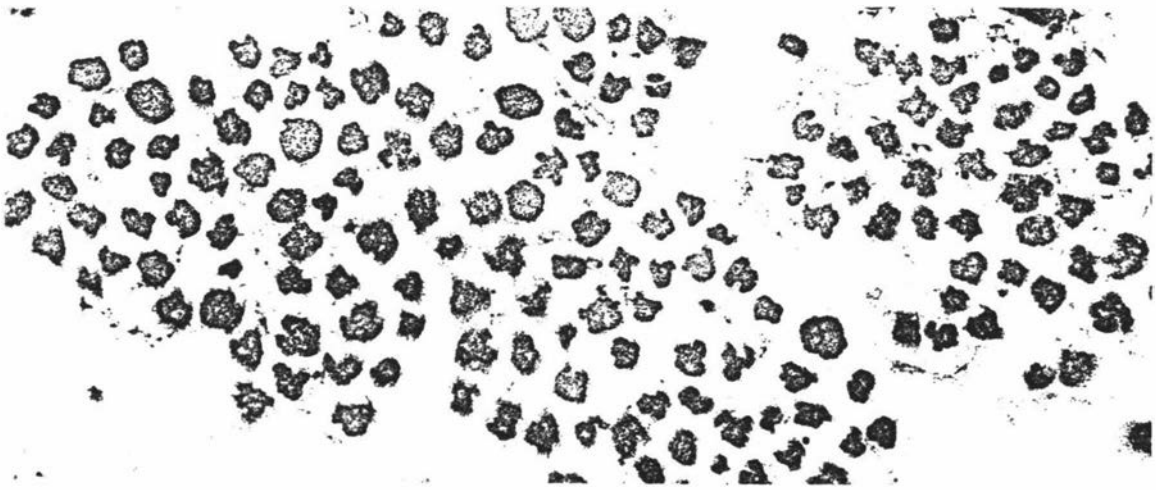

Figure 5.2 Electron micrograph of transverse sections of collagen fibrils from a tendon from a newborn dermatosparactic lamb. Although most of the fibrils in this tissue appeared normal, a small number appeared irregular in section. Magnification: 65 000 X.

of disorganization of the collagen fibrils observed in this electron microscope study.

The second dermal dysplasia investigated was that from a greyhound. The animal (a 10-month-old bitch) presented clinical symptoms of soft, thin, highly extensible skin which had become grossly scarred due to multiple lacerations arising from minor trauma. Healing of wounds, after suturing, was slow and the scars produced were thin and papyraceous. Similar conditions in other species are known to be inherited and, in canine breeds, an autosomal dominant pattern of inheritance has been confirmed (Hegreberg et.al., 1970). The mode of inheritance, however, was not ascertained in the animal studied. None of the dog's close relations or litter-mates had skin problems and although the animal was kept until of breeding age she failed to respond to any breeding attempts.

The extensibility of the skin had earlier been assessed by Cahill et. al., (1980) in terms of an extensibility index (Patterson and Minor, 1977); this index is defined as 100 times the ratio of the vertical height to which the lumbosacral skin can be lifted, to the body length. The body length is measured from the base of the tail to the occipital crest. The extensibility index of the skin of the affected bitch was 18.5, a value significantly greater than the mean value of 7.8 determined from four control animals of similar age. The skin fragility was also assessed as a tensile strength measurement (Cahill et al., 1980) and, at a strain rate of 0.7% per second, the ultimate tensile strength of the skin of the affected bitch was 1.5 MPa compared to the value of 10.6 MPa for a control animal.

The collagen fibrils in the deep dermis of a control greyhound skin (Figure 5.3a) were circular in cross-section and had a fairly sharp unimodal distribution of diameters ranging from 45 to 170 nm (mean diameter 100 ± 24 nm)*. In contrast, in the affected animal, the collagen fibril diameter ranged between 15 and 130 nm (mean diameter 80 ± 22 nm) and were thus ~20% smaller than those of the control. Generally, the dermal collagen of the affected animal showed little abnormality in collagen fibril structure, although areas deep within the dermis could be found where small numbers of fibrils showed fragmentation or assembly defects (Figure 5.3b). In the most superficial (papillary) layer of the dermis the greatest disorganisation of fibrils was seen; here, in many places, the fibrils were disorientated and irregular in cross-section, and when seen in longitudinal section appeared to be fragmenting into smaller elements (Figure 5.3c). This is in contrast to the findings of O'Hara et al., (1970) who described an increase in collagen fibril disorganisation with increasing depth from the dermal surface in a collagenous tissue dysplasia of the calf.

Throughout the dermis, and particularly in its more superficial aspects, there was seen to be an increase in lysosomal activity in the fibrocytes. This activity was reflected by the diverse morphology of the lysosomal complement as illustrated in Figure 5.4. These lysosomes are characterized by inclusions which, although of unknown origin, may be described as "multilamellar" (Figure 5.4b), "fibrinoid" (Figure 5.4c) or "electron-dense" (Figure 5.4d). Such grossly bizarre lysosomal architecture may well reflect some underlying biochemical defect in the degradation of macromolecular components of the matrix. Lysosomal disorders, known to be inherited, include the

* This figure differs from that quoted for greyhound skin in Table 4.10. The latter figure (mean diam. 85.1 ± 16.1 nm; mass-av. diam. 92 nm) was obtained from measurements made in the mid-dermal region.

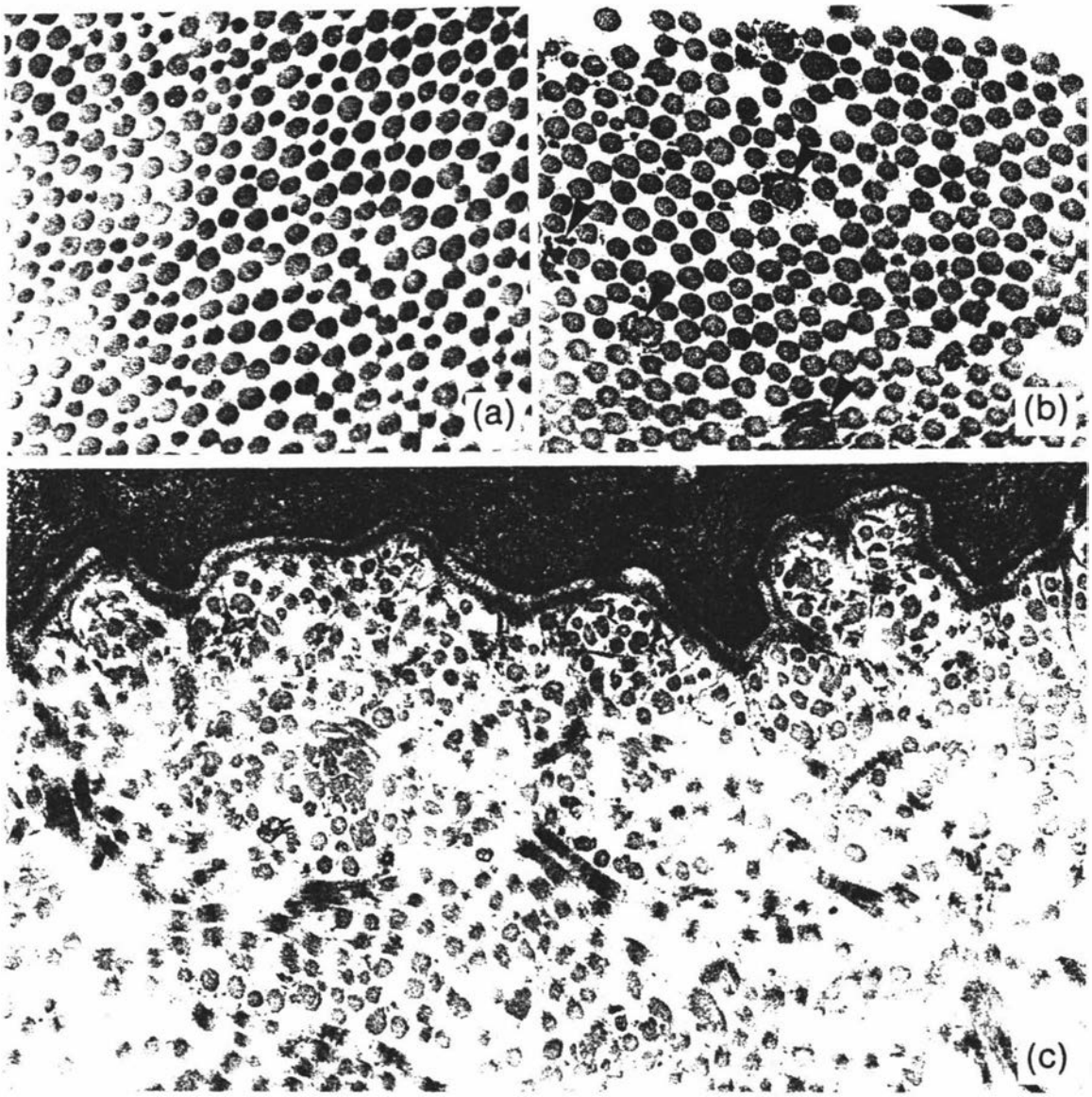

Figure 5.3 Electron micrographs of transverse sections of collagen fibrils from greyhound skin. Magnification: 34 000 X.
(a) Normal greyhound skin showing normal distribution of collagen fibril diameters (mean diameter 100 nm).
(b) Collagen fibrils with irregular profiles (arrowed) found deep within the dermis in a population of fibrils which otherwise appears relatively normal (mean diameter 80 nm).
(c) Collagen fibrils from the superficial (papillary) layer of the dermis showing many fibrils of bizarre architecture.

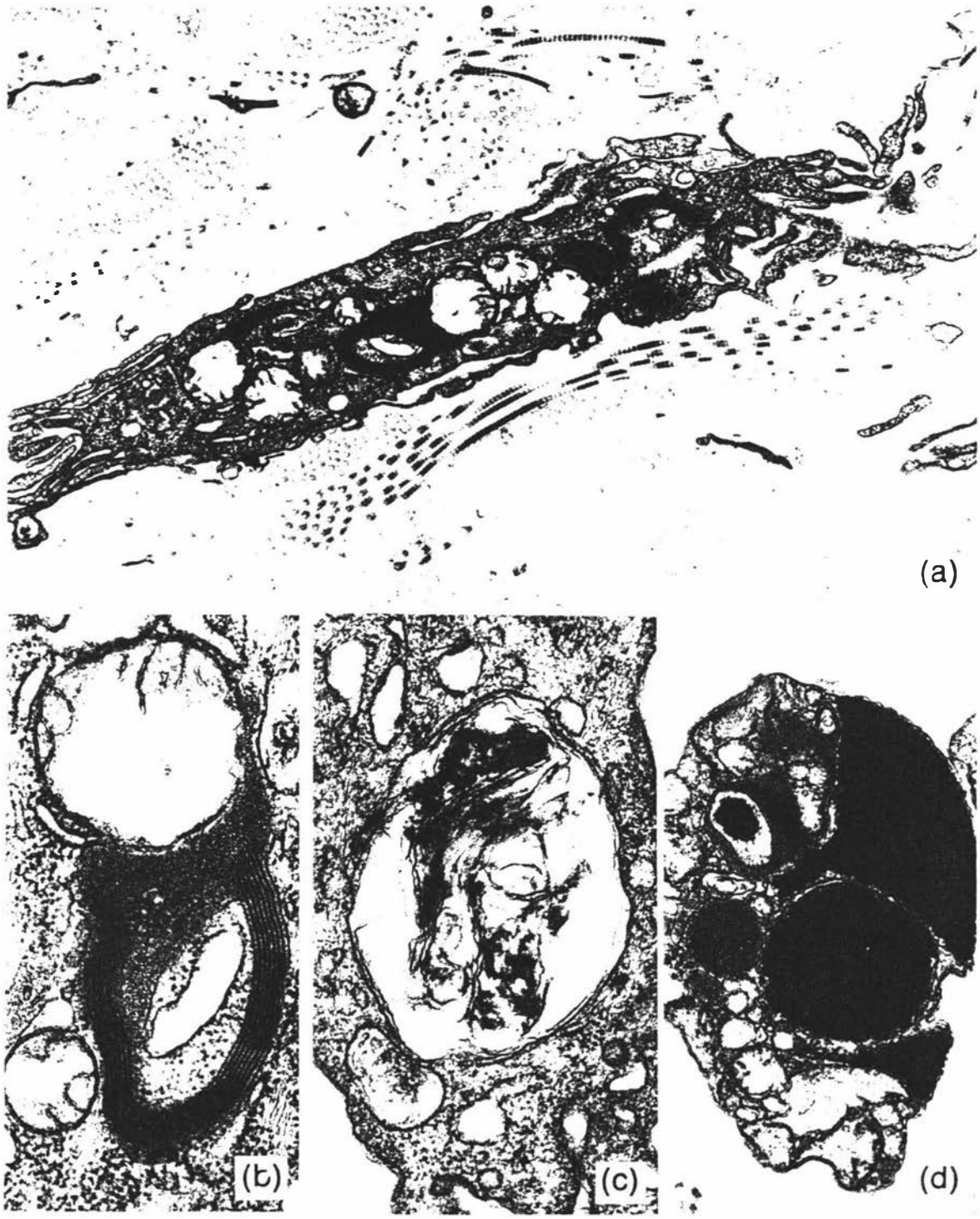

Figure 5.4 Electron micrographs of fibrocytes, containing abnormal lysosomal inclusions, in the superficial layer of the dysplastic greyhound dermis.
(a) Survey micrograph. Magnification: 12 000 X.
(b -d) Details of abnormal fibrocytic lysosomal inclusions: (b) lamellar, (c) fibrinoid and (d) "electron dense". Magnification: 38 000 X.

mucopolysaccharidoses and disorders of glycoprotein metabolism (for review see Neufeld et al., 1975). The mucopolysaccharidoses adversely affect metabolism of the sulphated glycosaminoglycans - molecules which may play an important part in the regulation of collagen fibrillogenesis (see Chapter 8).

5.2.2 Naturally occurring malformations

A non-pathological but striking example of collagen fibrillar malformation has been shown to occur in the sea cucumber, Holothuria forskali. Holothuria and some other genera of sea cucumbers have external appendages of their alimentary tracts, known as Cuvierian tubules; such organs consist of a secreted fibrillar protein system in tubular sacs which may be rapidly everted. The animal can thus eject a sticky, highly extensible fibrous network used both in food capture and defence against predators (Nichols, 1967). It has been shown by X-ray diffraction, electron microscopy and amino acid composition (Watson and Silvester, 1959) that collagen is the major protein constituent of such tubules. In the native tissue, layers of collagen fibrils are arranged in mutually rotated layers reminiscent of liquid crystals in a cholesteric phase (Dlugosz et al., 1979). When the tubules are ruptured, the fibrils are ejected and become axially and irreversibly aligned into a fibre which is highly extensible but which has little tensile strength. Bailey et al., (1982) have shown that, although the axial head-to-tail crosslinks between collagen molecules were present in this tissue, the "stable" crosslinks binding molecules together transversely were absent. It is thus not surprising that the fibrils formed are lacking in lateral stability. Further, these authors have suggested that the

irrecoverable extension of collagen fibres from this source could be explained by subfibrillar elements sliding past one another without causing fibril rupture.

In this work transverse sections were taken of Cuvierian tubule collagen fixed and embedded in either the native state or after extending the tubules by several orders of magnitude. The fibrils from an unstretched Cuvierian tubule are shown in Figure 5.5a. Most have irregular outlines but remain D-periodic in longitudinal section. Fibril "diameter" was estimated from both longitudinal and transverse section and a value of about 60 - 70 nm was obtained. In the highly extended ("stretched") material the fibril outline was even more irregular (Figure 5.5b). However there is no apparent change in the transverse dimensions of the fibrils and from longitudinal sections it can be seen that the D-periodic structure is still present.

As there is little change in fibril diameter before and after tubule extension it would seem unlikely that the sliding microfilament model proposed by Bailey et al., (1982) is correct in detail. Such a model would predict that fibrillar elongation with concomitant reduction in diameter would occur, assuming the process to be an isovolumetric one. These results rather support the observations of Gathercole and Keller (1975) that the elongation is accounted for by fibre (fibril bundle) slippage. The results, however, do not differentiate between sliding fibre or sliding fibril models.

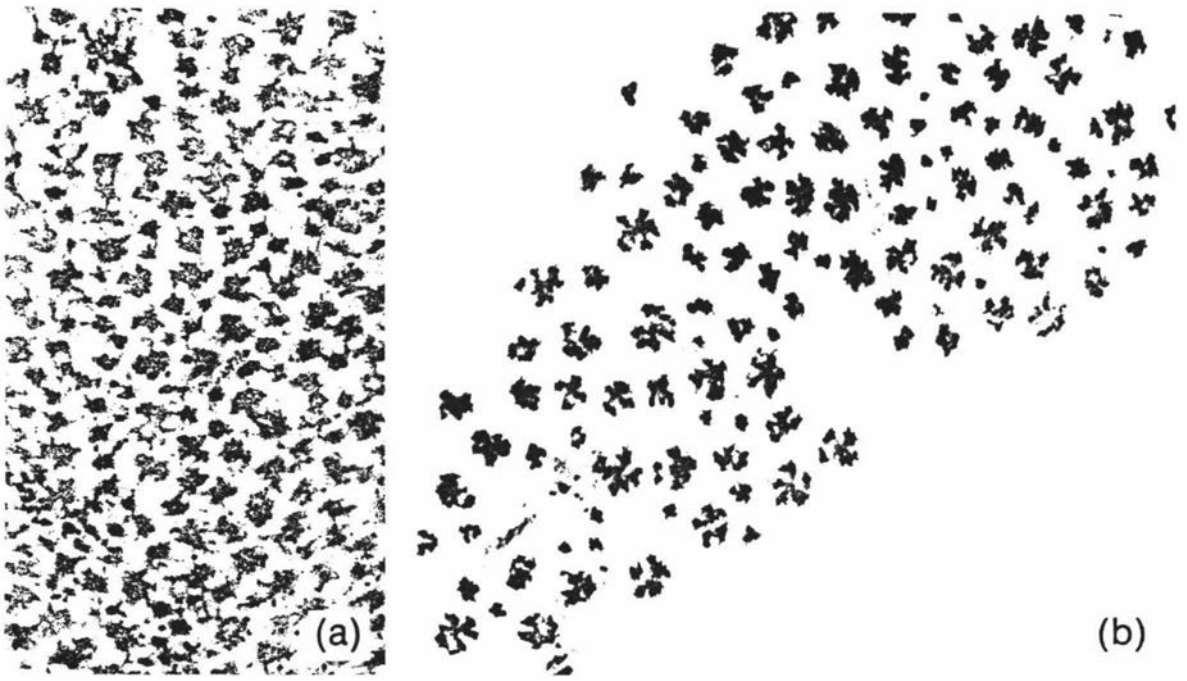

Figure 5.5 Electron micrographs of transverse sections of collagen fibrils from the (a) unstretched and (b) stretched Cuvierian tubules of the sea cucumber Holothuria forskali. Magnification: 66 000 X.

5.3 Changing Fibril Diameter Distributions

5.3.1 Induced disorders

Wounding provides a simple experimental method whereby connective tissue organization may be altered locally. A study of the subsequent repair mechanisms may provide an insight into both fibrillogenesis and the remodelling processes which are involved in scar formation. The mechanism of wound healing is beyond the scope of this thesis but for clarity some comments must be made here. Immediately after wounding, coagulation is effected by the interaction of enucleate platelets with thrombin and collagen. Once coagulation is complete there is a migration into the wound of polymorphonuclear neutrophils (granulocytes) and blood monocytes. The latter differentiate into the macrophages which are responsible for the debridement of the wound. By the second day fibrocytes begin to enter the wound and reach their maximum numbers after about seven days, at which time capillary regeneration is also beginning to take place. The fibrocytes are responsible for the synthesis of collagen, GAGs and, in the later stages of scar formation, some elastin (Williams, 1970). One of the first products of secretion of the wound-invading fibrocytes is hyaluronic acid (Bentley, 1969; Toole and Gross, 1971; Mathews, 1975). It is believed that high levels of this GAG facilitate the migration of cells and the organization of the extracellular components. In the later stages of remodelling the majority of the fibrocytes and capillaries disappear. A review on the processes of connective tissue formation in wound repair has been presented by Ross (1980).

In order to study aspects of wound healing longitudinal and transverse surgical incisions were made by M.H.Flint in the dorsal body skin of three-month-old rats. The incisions described as longitudinal and transverse were made parallel and perpendicular respectively to both the vertebral column and the Langer's lines. These wounds were left unsutured for two months after which time specimens were prepared for electron microscopy and sent to the author for further analysis. Collagen fibril diameter distributions were then measured for both of the healing wounds and the control. The longitudinal wound, which was coincident with Langer's lines, had healed well; in contrast the transverse wound appeared less mature at a similar time. The collagen fibril diameter distributions for each of these specimens (along with a control) are shown as both frequency and volume plots in Figure 5.6.

The number distributions for the collagen fibrils of the specimens taken from the transverse and longitudinal wounds differ significantly, not only from one another but most particularly from that of the normal rat dermis (mean diameter 117 $\pm$ 26 nm, mass-average diameter 129 nm at three months; mean diameter 103 $\pm$ 23 nm, mass-average diameter 114 nm at five months). The transverse wound has a right-skewed distribution (mean diameter 120 $\pm$ 32 nm, mass-average diameter 139 nm) which contains about 3% of large diameter fibrils that contribute significantly to the total mass. The longitudinal wound (mean diameter 137 $\pm$ 35 nm, mass-average diameter 156 nm) also contains about 3% of collagen fibrils whose diameters are markedly greater than those of any in the control.

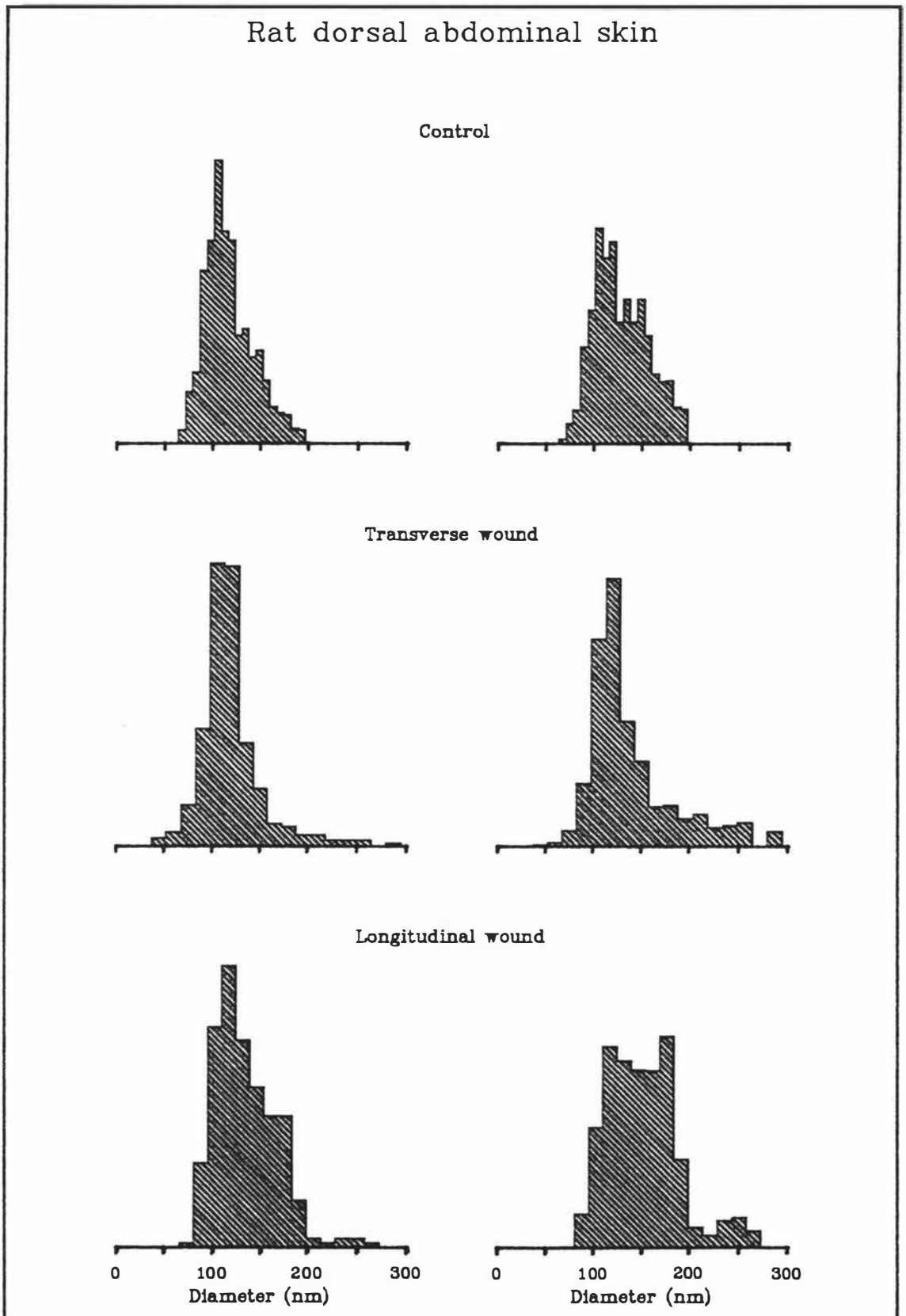

Figure 5.6 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from normal rat ventral abdominal skin and from skin eight weeks after the healing of "transverse" and "longitudinal" surgical incisions (see Section 5.3.1).**

It has been pointed out by Bentley (1980) that very little is known about the GAGs of healing wounds, mainly because it is difficult to obtain from a wound a sufficient amount of material for the GAG analyses to be undertaken. However, M.H.Flint (private communication) has analysed the GAGs in the longitudinal and transverse wounds previously described. His conclusions were that the GAG content changed significantly with the age of the wound but was always highly elevated relative to that of normal skin. Whilst hyaluronic acid and chondroitin sulphate reached their highest levels at about four weeks (longitudinal) and about seven weeks (transverse) after wounding the dermatan sulphate content showed the largest proportionate increase of any GAG and reached its highest level at a later stage of healing (about ten weeks after wounding).

The concomitant elevation in the proportions of hyaluronic acid and chondroitin sulphate and the subsequent elevation in the proportion of dermatan sulphate in healing wounds is consistent with the presence of large diameter fibrils, and is in accordance with the GAG-control mechanism for regulation of fibril diameters described in detail in Chapter 8. It is also worth noting that a higher proportion of Type III collagen is usually associated with scar tissue and that the degree of the fibril organization is less apparent (Bailey, 1975; Epstein and Munderloh, 1975). These factors are likely to influence the mechanical properties of the scar.

A second induced disorder which is reflected by a changed collagen fibril diameter distribution has been produced *in vivo* by biochemical intervention. Samples of forelimb superficial flexor tendons, 24 hour, 1, 4 and 8 week, 10, 11 and 14 month after intratendinous injection of bacterial collagenase were supplied by

I.F.Williams (Dept. of Pathology, School of Veterinary Sciences, University of Bristol). The protocol for bacterial collagenase treatment and the subsequent clinical and post-mortem observations are detailed in Appendix 5. The controls for these experiments were taken from the contralateral tendons of the affected animals and the collagen fibril diameter distributions from these tissues were plotted as both number and volume distributions in Figure 5.7. The form of the distributions obtained from the horse four weeks after bacterial collagenase treatment is characteristic of a young animal (see Parry et al., 1978b). The frequency and volume distributions of the collagen fibrils measured in the bacterial collagenase treated tendons are shown in Figure 5.8. The forelimb flexor of a normal horse has a collagen fibril diameter distribution which is both broad and bimodal. The two peaks in the distribution have mean diameters of about 35 and 165 nm and contain about 90 and 10% respectively of the number of fibrils in the distribution (see Parry et al., 1978b; Figure 5.9a). Within a day of collagenase treatment the small diameter fibrils had completely disappeared and the largest diameter fibrils had apparently been partially degraded to fibrils of intermediate size. The affected part of the tendon then contained a unimodal distribution of fibril diameters (mean diameter 73.1 $\pm$ 10.7 nm, mass-average diameter 76.7 nm; Figure 5.9b) which, in higher resolution micrographs, have been resolved into a discrete number of populations (Tables 5.1, 5.2) each with a mean diameter close to a multiple of ~8 nm. Specimens from four and eight weeks and also 10 and 14 months after bacterial collagenase treatment were similar to one another with sharp distributions of small diameter collagen fibrils (mean diameters ~50 nm, standard deviations ~7 nm; Figure 5.9c) though, once again, careful measurement of the highest quality micrographs revealed distributions containing several populations of fibril size with means close to a multiple of ~8 nm. (Tables 5.1, 5.2). The fibrils were of

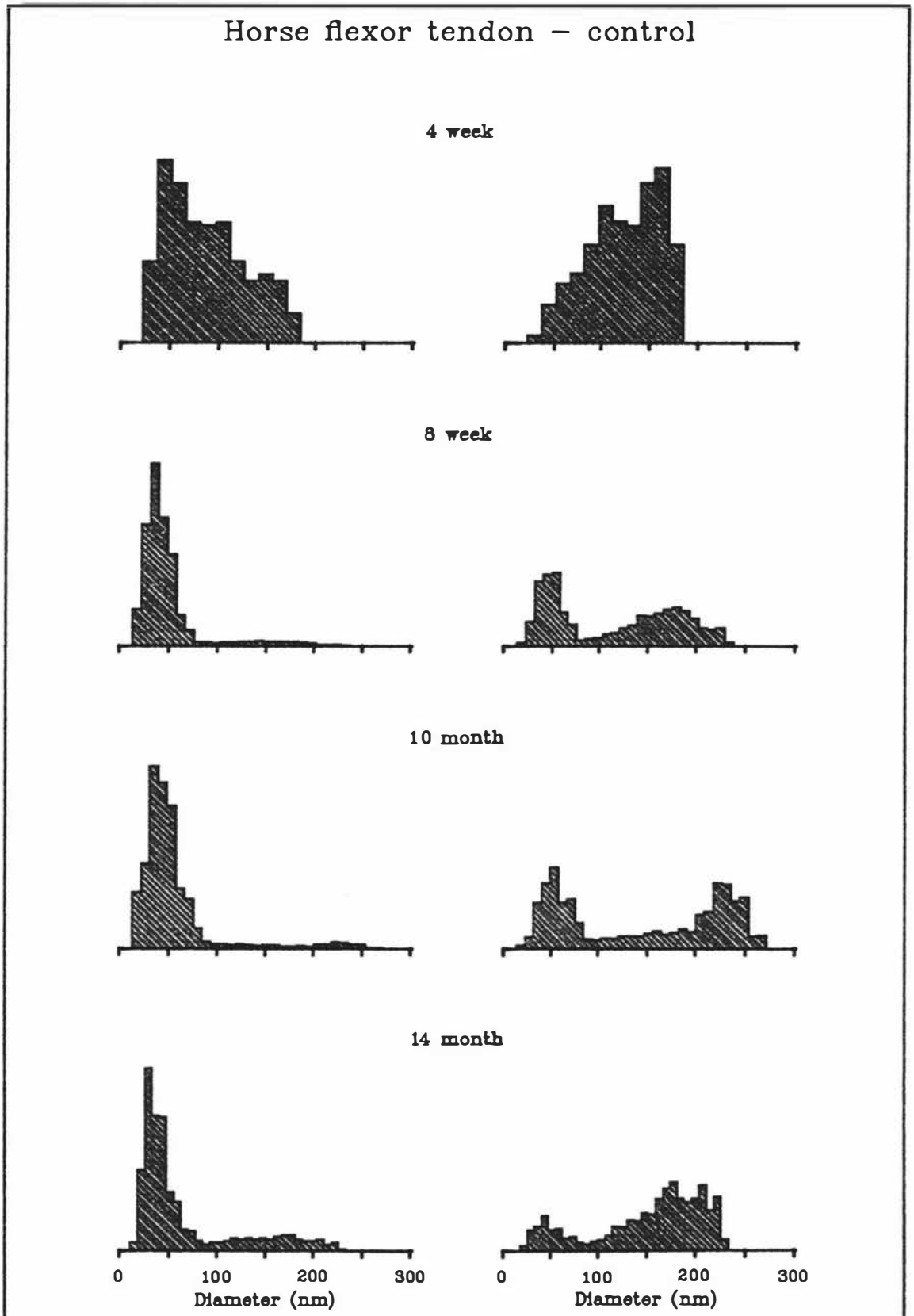

Figure 5.7 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from the contralateral flexor tendons of horses whose flexor tendons had been treated with bacterial collagenase. The times after bacterial collagenase treatment are indicated.**

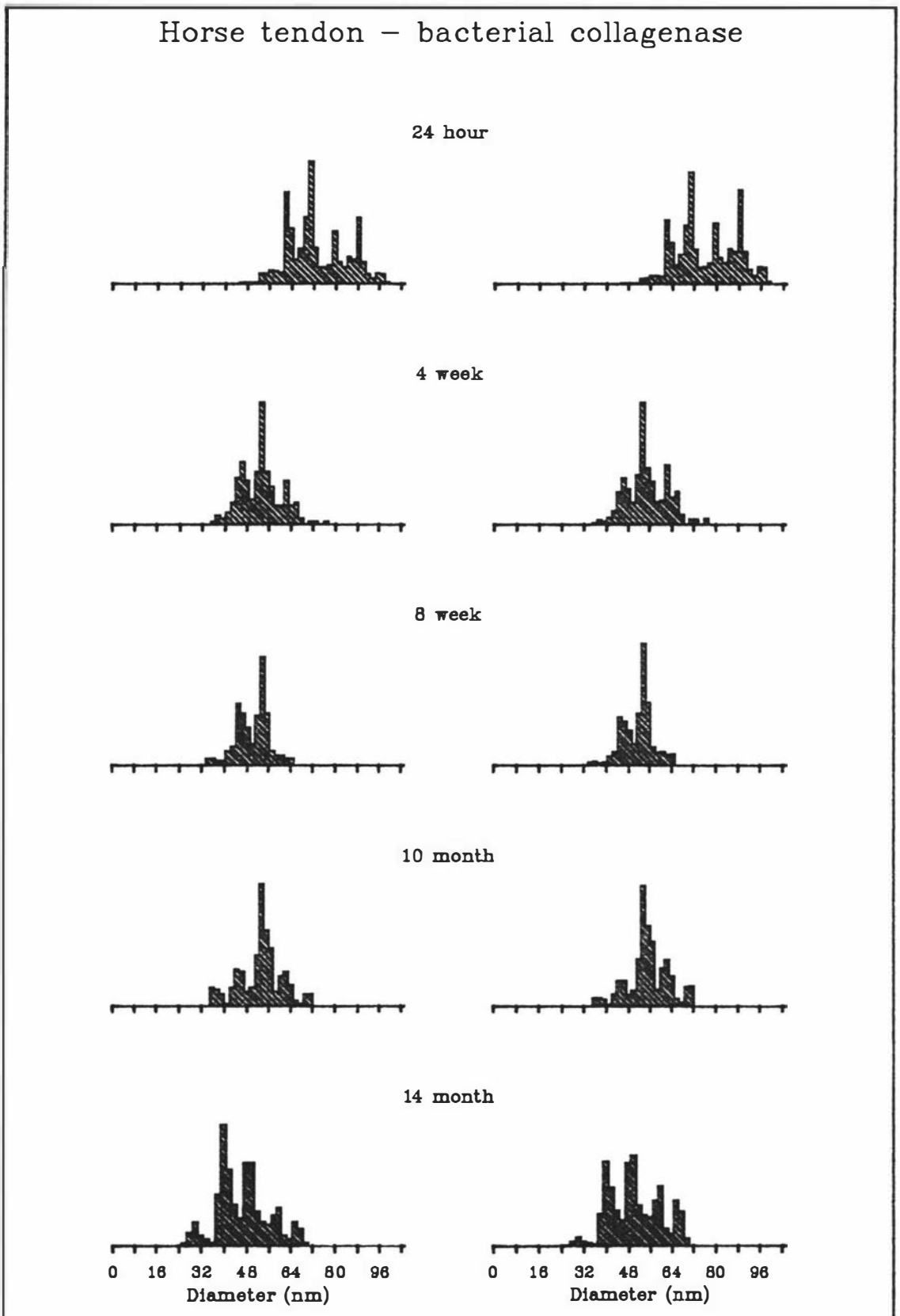

Figure 5.8 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from the superficial digital flexor tendons of horses at various times after treatment with bacterial collagenase.**

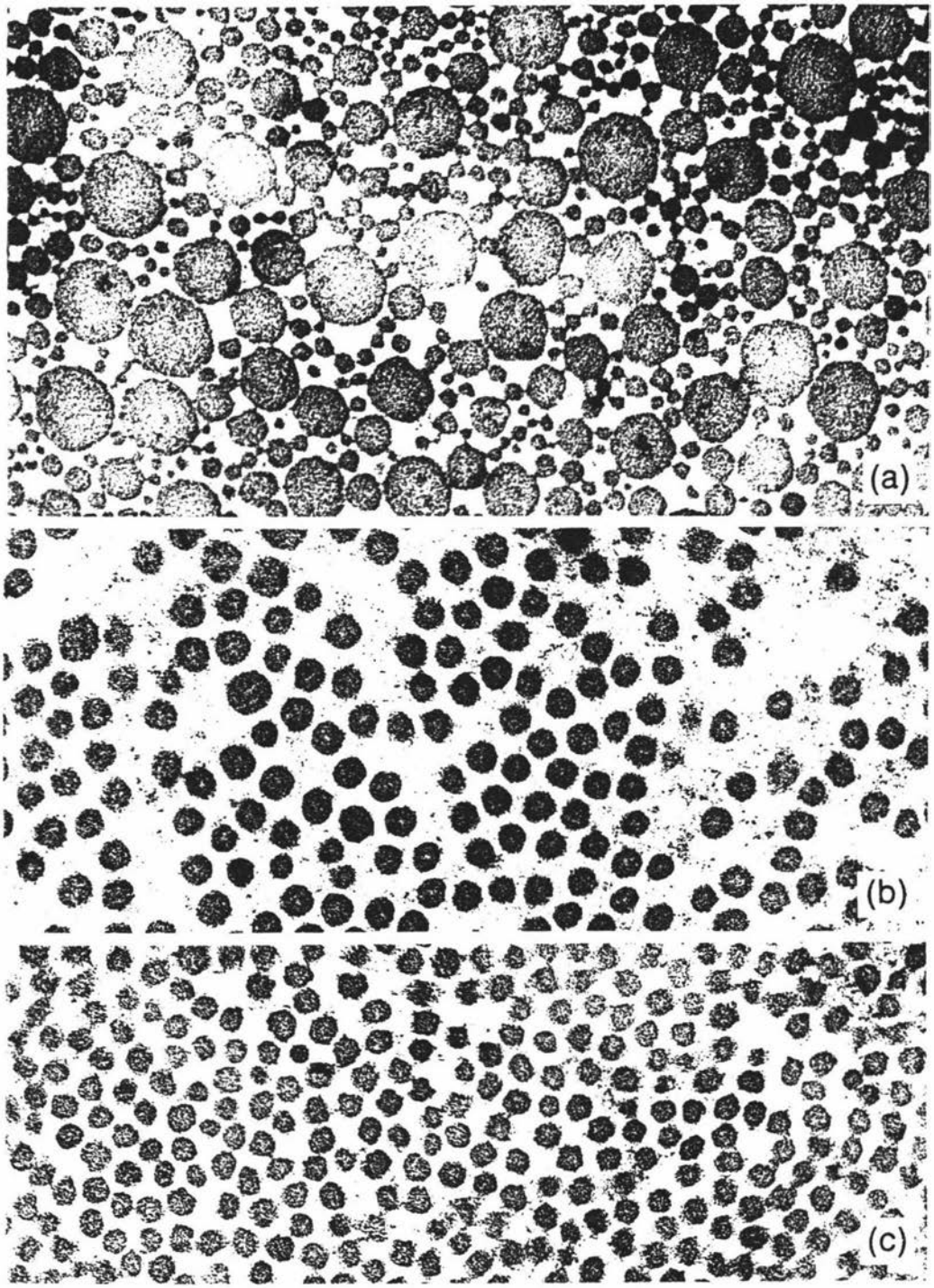

Figure 5.9 Electron micrographs of horse superficial flexor tendon showing transverse sections of collagen fibrils. Magnification: 65 000 X.
(a) Flexor tendon from a normal horse. The collagen fibril diameter distribution is markedly bimodal (mean diameter ~60 nm; mass-average diameter ~165 nm; see Figure 5.7).
(b) Flexor tendon 24 hours after treatment with bacterial collagenase. The collagen fibrils now have a narrow range of small diameter fibrils (mean diameter 73 nm; mass-average diameter 76 nm; see Figure 5.8).
(c) Flexor tendon 4 weeks after treatment with bacterial collagenase. The tissue now has collagen fibrils of even smaller diameters (mean 53 nm; mass-average 57 nm) and appear to retain these diameters up to at least 14 months after treatment (see Figure 5.8).

TABLE 5.1

COLLAGEN FIBRIL DIAMETER DISTRIBUTION DATA FROM HORSE SUPERFICIAL DIGITAL FLEXOR TENDON AFTER TREATMENT WITH BACTERIAL COLLAGENASE

Time After Treatment	Experimental Animal Mean Diam.* ± std.devn. (nm)	Experimental Animal Mass-av. Diam. (nm)	Control Animal Mean Diam. ± std.devn. (nm)	Control Animal Mass-av. Diam. (nm)
24 h	73.1 ± 10.7	76.7	---	---
4 w	52.9 ± 7.3	57.2	87.9 ± 41.4	123.3
8 w	50.4 ± 6.0	51.8	49.4 ± 34.5	113.5
10 mo	53.2 ± 7.7	55.4	56.1 ± 43.1	143.9
14 mo	46.7 ± 9.4	50.4	67.3 ± 54.2	151.0

* Can be resolved into sub-populations (see Table 5.2)

TABLE 5.2

RESOLUTION OF SUBPOPULATIONS OF COLLAGEN FIBRILS IN HORSE SUPERFICIAL DIGITAL FLEXOR TENDON TREATED WITH BACTERIAL COLLAGENASE

Time After Treatment	Mean Diam. Population ± std.devn. (nm)	Nominal Fibril Diameter (nm)*	% Fibrils in Population
24 h	55.4 ± 1.6	56	6
	62.5 ± 1.6	64	26
	70.3 ± 1.6	72	35
	79.6 ± 1.6	80	15
	88.2 ± 1.6	88	18
4 w	45.9 ± 2.0	48	31
	53.6 ± 2.0	56	49
	62.6 ± 2.0	64	20
8 w	40.7 ± 1.7	40	6
	46.0 ± 1.7	48	35
	53.8 ± 1.7	56	52
	61.5 ± 1.7	64	7
10 mo	36.5 ± 2.0	40	8
	45.2 ± 2.0	48	18
	54.0 ± 2.0	56	52
	61.7 ± 2.0	64	17
	70.7 ± 2.0	72	5
14 mo	29.7 ± 2.0	32	8
	40.2 ± 2.0	40	39
	48.8 ± 2.0	48	32
	58.2 ± 2.0	56	14
	66.2 ± 2.0	64	7

* Fibril diameter expressed as multiple of 8 nm closest to observed value.

significantly smaller diameter than those from the specimen taken 24 hours after treatment. The samples from the one week and 11 month treated tendons had fibril diameter distributions which were unchanged from the controls and it can only be assumed that the small electron microscope samples did not come from an area affected by the bacterial collagenase injections.

The fibril diameter distribution in tendon 24 hours after injury is different to that in either the contralateral tendon or the tendon at a later stage of healing. The most likely interpretation of the observations made is that during the 24 hours following collagenase injection the small diameter collagen fibrils (mean diameter ~35 nm) were totally reduced by proteolytic digestion leaving only partially degraded fibrils corresponding to the large fibrils initially present. Since these partially degraded fibrils have diameters which are multiples of ~8 nm these results provide the first evidence in support of the idea that fibril breakdown is quantized in the same manner as fibril growth.

At some stage beyond a day but less than four weeks after treatment proteolysis probably leads to the complete disappearance of fibrillar collagen. Beyond this point (and most probably within a few days of treatment) the healing proceeds with the synthesis of new collagen (including Type III) and this, in turn, gives rise to a unimodal population of small to intermediate size fibrils whose mean diameters are about 50 nm. However, up to 14 months after treatment the mean diameter of the collagen fibrils does not alter significantly, suggesting that the repair processes are incapable of restoring the collagen fibril diameter distribution to that expected of a tendon of that particular type and age.

Recently acquired biochemical data (I.F.Williams, personal communication) has shown that the healing tendon contains high proportions of Type III collagen and reducible crosslinks. This evidence strongly favours the hypothesis outlined in which the original fibrils were considered to have totally broken down and new fibrils formed which contained elevated levels of Type III collagen. Type III containing fibrils are invariably small in diameter in other connective tissues (see this thesis and Parry and Craig, 1984). The reason why the fibrils fail to attain a diameter distribution similar to that of a mature tissue 14 months after treatment cannot as yet be stated with any certainty. However, it may be speculated that the GAG composition of the mature tissue inhibits fibril growth beyond that observed. A mechanism by which fibril growth may be regulated is described in detail in Chapter 8; this hypothesis relates GAG composition to fibril size in a strictly sequential scheme of fibrillogenesis.

5.3.2 Acquired disorders

An acquired connective tissue disorder studied was Dupuytren's disease. This is a disorder, common among middle-aged Caucasians of northern European stock, and is characterized by the progressive fibrosis of the cutaneous and subcutaneous tissues of the palm. This results in localized palmar thickenings referred to as nodules. (see, for example, Flint *et al.*, 1982). Such nodules may spontaneously regress but more commonly the disease progresses to extend through the distal palm to produce tendon-like fibrous bands between the nodules and more proximal parts of the fingers. Late stages of development of the disease give rise to a progressive flexual contracture of one or

more fingers and the disorder is then commonly referred to as "Dupuytren's contracture" or "trigger-finger". Although no mechanical data have been reported for Dupuytren's disease the gross physical deformity resulting from the disorder clearly indicates a substantial change in the mechanical properties of the tissue. It is therefore of considerable interest to see if this is reflected in the collagen fibril diameter distribution and/or GAG content and composition of the affected tissues.

Analysis of the glycosaminoglycan contents of nodules and fibrous bands from sufferers of Dupuytren's disease along with analyses of normal palmar fascia have been recorded (Flint et al., 1982) and these authors have supplied material for electron microscope evaluation of the constituent collagen fibrils. The distributions of collagen fibril diameters for Dupuytren's nodules and fibrous bands and for normal palmar fascia are plotted in Figure 5.10 as both number and volume distributions. It can be seen that the normal tissue has fibrils with a bimodal distribution of diameters with populations centred about 45 and 150 nm, with the total distribution having a mass-average diameter of 140 nm; such a distribution is that exhibited by many normal flexor tendons. Conversely, the diseased tissues lack the larger diameter fibrils and the distribution reverts to that of a more immature or embryonic tissue. The collagen fibrils in the Dupuytren's tissue have a sharp unimodal distribution of diameters centred about 45 nm (mass-average diameter 47 nm).

The molecular species of collagen present and the types of reducible crosslinks predominating in Dupuytren's disease have been studied by Bailey et al., (1977) and Bazin et al., (1980). These authors have shown that the palmar aponeurosis in normal tissue is

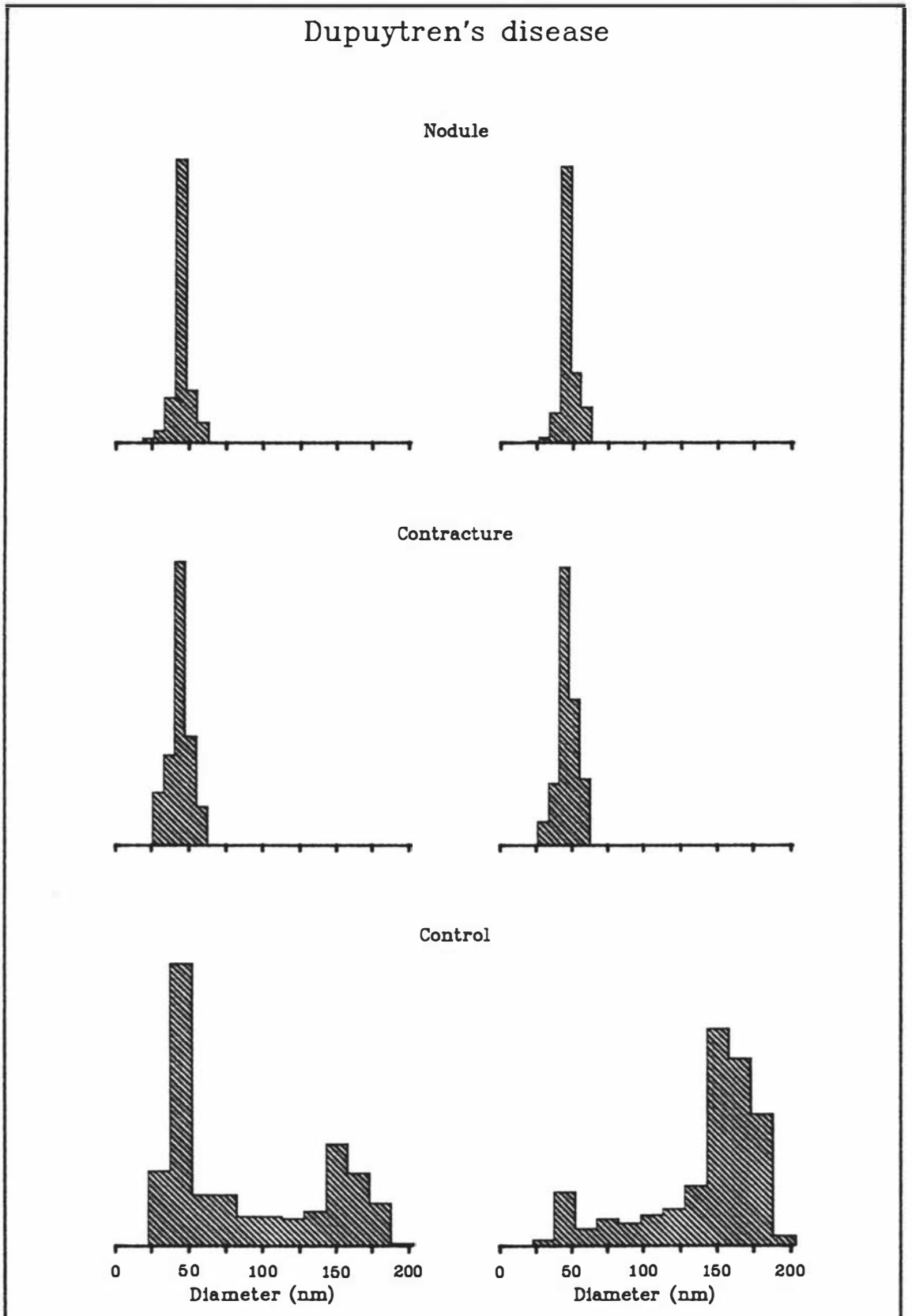

Figure 5.10 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters from the normal palmar fascia of man and from the nodule and contracture of a patient suffering from Dupuytren's disease.**

composed almost exclusively of Type I collagen, but that both affected (contracted) and unaffected areas of the aponeurosis, along with the granulation-tissue-like nodules, contain a significant proportion of Type III collagen. Further, the changing proportion of the reducible crosslinks indicated that "the nodules were newly formed collagen, the contracture mainly newly synthesized collagen with some mature collagen; the unaffected aponeurosis contained mainly mature collagen, with some newly synthesized, and the normal aponeurosis contained entirely mature collagen." (Bazin et al., 1980). These results directly parallel the electron microscope observations previously summarized in Chapter 4 where it was indicated that immature tissues contain small diameter fibrils and mature tissues contain large diameter fibrils. The small diameter collagen fibrils (mass-average diameter ~45 nm) present in the nodule and contracture are consistent with the presence of newly formed collagen, a significant proportion of which is Type III; the broad bimodal distribution of collagen fibrils (mass-average diameter ~140 nm) present in the normal aponeurosis is consistent with the presence of mature Type I collagen. There is a marked similarity between these observations and those obtained from the bacterial collagenase treated tendon. In each case the collagen fibrils have a narrow distribution of diameters (mean ~45 - 50 nm), an elevated content of Type III collagen and an increased fraction of reducible crosslinks. It would seem, therefore, that the bacterial collagenase treatment system in the horse could well prove to be a valuable model for Dupuytren's contracture in man.

Another example of an acquired disorder is that known as "contracted tendon". The aetiology of this disorder in the horse is

largely unknown but it is believed to be associated with abnormal bone growth rather than any physiological decrease in the length of the tendon (B.E.Goulden, private communication). The disease manifests itself by the permanent flexure of the metacarpal or metatarsal joints which causes the animal to walk on its fetlock if the contracture is severe. Previous work by the author had shown that the collagen fibril diameter distribution was unimodal in contrast to the bimodal distribution expected from a tendon of this type (Parry et al., 1978b). However, the total collagen content of the diseased and normal specimens appeared to be similar when calculated as the area covered by the collagen fibrils in transverse section. In longitudinal section, however, it was seen that many fibrils were ruptured and exhibited D-periods (40 - 90 nm) differing significantly from the commonly accepted value (~67 nm). Further, the tendon seemed largely devoid of elastic fibres.

These results once again demonstrate the marked changes that occur in the distribution of collagen fibril diameters when functional loadings are changed within tissues. These altered distributions and the glycosaminoglycan concentrations and composition analysed by Flint et al., (1982) will be discussed further in Chapter 8.

5.4 Conclusions

The observations recorded in this chapter support the hypothesis outlined in Chapter 7 that mechanical properties of connective tissues are directly related to the form of the collagen fibril diameter distribution in a tissue. Further, the structural integrity of the fibril must be preserved if the mechanical properties of a tissue are to be maintained. Altered mechanical properties, such as increased

fragility and elasticity, together with increased fibre disorganization, have been qualitatively demonstrated in the skins of dermatosparactic animals (Lenears et al., 1971; Bailey and Lapiere, 1973) and in the dysplastic greyhound dermis (Cahill et al., 1980). Both tissues are seen to contain collagen fibrils which are no longer circular in section and it may be inferred that this reflects some irregularities in the molecular packing and organisation. Although such "abnormalities" in these cases are known to occur through hereditary or acquired pathology it has been shown previously that the collagen fibrils of ageing (senescent) animals often display similar abnormalities (Parry and Craig, 1984). These could be a consequence of the changing mechanical properties of the tissue with age resulting, for instance, from the changing ratio of "stable" to reducible crosslinks (Light and Bailey, 1979) or the changing GAG and water composition of the matrix (Parry et al., 1982; Parry and Craig, 1984; see also Chapter 8). It should be emphasised, however, that the strengths of stable and reducible crosslinks are similar and that any change in fibril morphology must be associated with the lateral component of the stable crosslink between molecules in axial register. Further, modification of the GAGs which are thought to be associated with the interfibrillar linking of collagen fibrils and thus with the tissue's ultimate tensile strength (Miller, 1976; see also Parry et al., 1978a), is clearly likely to be implicated in morphological changes of the fibril structure.

The example cited of irregular profiles in collagen fibrils of the Cuvierian tubules of the sea cucumber (an invertebrate) would however appear anomolous in that it is a naturally occurring tissue which contains a highly elevated content of GAGs together with

collagen fibrils lacking the stable (covalent) crosslinks (Bailey et al., 1982). That the fibrils in this tissue lack a uniformity of profile and have very low tensile strength is supportive of the hypothesis outlined.

It has also been shown that surgical intervention (wound healing in the rat), biochemical disruption (bacterial collagenase treatment of tendons) and pathology leading to immobilisation of tissues (Dupuytren's contracture) all give rise to collagen fibril diameter distributions which differ markedly from the norm. All of these data support the hypothesis (described in Chapter 7) that the mechanical properties of a connective tissue are closely related to the integrity of the collagen fibril and to the maintenance of the appropriate collagen fibil diameter distribution.

CHAPTER SIX

INTERPRETATION AND SIGNIFICANCE OF COLLAGEN FIBRIL DIAMETER DISTRIBUTION DATA

6.1 Considerations of the Limitations Imposed by Electron Microscopy

Attempting to obtain quantitative electron microscope measurements from thin sections of biological material is fraught with difficulties. Such difficulties include the failure of fixation to preserve the features of the *in vivo* assembly, shrinkage and distortion artefacts induced during preparative procedures, and artefacts introduced by sectioning methods. Other factors that must be considered in an assessment of the accuracy of data obtained include section thickness, grain size and intensity of staining, artefacts caused by irradiation of the beam, objective lens astigmatism and focus, methods of recording the information photographically, the stereographic interpretation of the recorded two-dimensional image, the magnification of the image and, finally, the methods of measurement from the micrographs.

That electron microscope fixatives do not always preserve the structure known to exist *in vivo* has been well documented for a wide variety of biological tissues. Modifications to the duration of fixation and the temperature at which it is carried out, or to the pH, osmolarity and buffer content of common fixatives may overcome some of these problems (see, for example, Hayat, 1970; Glauert, 1975; Arborgh *et al.*, 1976). Dimensional changes of specimens during preparation for electron microscopy have also been well established (see, for example, Hayat, 1970; Glauert, 1975; Gusnard and

Kirschner, 1977) but references to shrinkage generally refer to an isomorphic shrinkage of isotropic specimens (Boyde and Franc, 1981). However, many of the connective tissues studied in this thesis are clearly anisotropic. Orientated collagenous tissues in general and collagen fibrils in particular are structurally anisotropic, and there seems no good reason to believe that shrinkage in such systems will proceed at the same rate in both lateral and longitudinal directions. Indeed, the evidence strongly suggests that when collagen fibrils are fixed for electron microscopy they sustain a shrinkage which is much greater laterally than longitudinally. Electron microscope measurements made from thin-sectioned specimens of collagen fibrils from 17 tendons from the rat and the horse (Parry and Craig, 1978; Parry et al., 1978b) had a mean D-period of 67.2 ± 1.8 nm. This value is within 1% of that obtained from X-ray studies (D = 66.8 nm, Miller and Parry, 1973; see also Brodsky and Eikenberry, 1982) and would indicate that processing tissues for electron microscopy produces only small longitudinal shrinkage in collagen fibrils. It is of interest to note here that the D-period of collagen fibrils measured for dehydrated tendon by X-ray diffraction methods is typically about 63.5 nm (see, for example, Bear, 1942, 1944; Rougvie and Bear, 1953; Tomlin and Worthington, 1956), a value ~5% less than that of the hydrated tissue. X-ray diffraction studies, however, suggest that the lateral shrinkage of collagen fibrils in foetal and immature tissues processed for electron microscopy could be as high as 35% (see Section 6.5). Further, the values for the diameters of corneal collagen fibrils reported from freeze-fracture studies (Ruggeri et al., 1979; Klein et al., 1981) are considerably higher than those reported for sectioned epoxy-embedded material (see Chapter 3). If the former values are indeed representative of the in vivo situation then lateral

shrinkages of the order of 45 - 65% must have occurred (see Section 3.2). Conversely these very high values could be accounted for, in part, by swelling of the fibrils caused by the use of cryoprotectants. Finally, direct electron microscope evidence that fixation affects the diameters of collagen fibrils was presented by Parry and Craig (1977) who dehydrated and embedded both fixed and unfixed specimens of rat tail tendon and showed that the mean diameter of the collagen fibril diameter distributions was 25% lower in the fixed specimen.

When epoxy resin blocks are sectioned for electron microscopy the action of the knife causes the section to be sheared from the block-face, and those elements which once lay perpendicular to the face now lie at some angle other than perpendicular to the plane of the section. This causes a reduction in the length of the section relative to that of the block-face and is commonly, though incorrectly, referred to as "compression". Three methods employed for the relief of this shearing artefact are (a) the cutting of sections on solvent-water mixtures (5 - 40% acetone, see Hayat, 1970), (b) the exposure of cut sections to high temperatures with the aid of a "heat-pen" designed for the purpose (Roberts, 1970) and (c) the exposure to chloroform vapour of sections floating on the water surface (Sotelo, 1957; Satir and Peachey, 1958). It was the latter method which was used in this work and measurements showed that the length of the sections normally return to > 90% of the vertical dimension of the block face. This residual "compression" is apparent when comparing measurements of the D-period from longitudinal sections of fibrils cut perpendicular to their long axes with measurements made from longitudinal sections of fibrils cut parallel to their long axes. A shortening of the D-period by as much as 10% is invariably

associated with the latter conditions, as can be seen from the early work of Parry and Craig (1977). In that work the mean D-period of collagen fibrils from tail tendons of five rats was shown to be 60.9 ± 1.5 nm, a value 9% lower than that of all subsequent work (D = 67.2 ± 1.8 nm) in which axial regularity was only investigated in sections cut perpendicular to the long axes of the fibrils. Confirmation of this observation has also been made by Flint and Merrilees (1977) in their study of the Tendo-achilles of the rabbit. Finally, experience has shown that highly collagenous tissues from ageing animals are much more difficult to cut than are the same tissues from younger animals. This may reflect the increasing ratio of "stable" to reducible crosslinks in ageing collagen (Allain et al., 1978; Light and Bailey, 1979; Bailey and Etherington, 1980).

Section thickness can be assessed in a resin of known refractive index by observation of the interference colour when the section is viewed in white light. In this work, sections were routinely cut which were of a pale gold interference colour, a colour associated with sections ~80 nm thick. The importance of this value is that it is large relative to the resolution desired in the specimen and hence relevant to a discussion of the problems associated with the correct stereographic interpretation of the image (see below).

The stains most commonly employed for the positive staining of thin sections are salts of uranium and lead. The grain size of these stains being ~2 nm (Beer and Zobel, 1961; Crewe, 1971; Hayat, 1975) is adequate for the medium resolution studies undertaken here, but can be limiting in very high resolution work. More importantly, optimum staining of tissues must be chosen such that "over-staining" does not affect image quality. Sections from which quantitative measurements

were taken were picked up on plastic support films on copper grids. Such sections, after floating or immersing in stain solutions, become stained on their free surface alone and it is important that this surface be lowermost in the electron microscope column. This may be achieved by placing the grid with its stained surface uppermost in the specimen holder and by its subsequent rotation through 180° after insertion into the microscope column.

Artefacts caused by irradiation of the beam can be severe. If beam currents are employed which are excessive, volatilization of substances within biological specimens will occur. Under normal operating conditions, using accelerating voltages ~100 kV and emission currents ~10 μA, power densities of up to 10^{12} W m^{-2} can be projected on to the irradiated portion of the specimen. In attempts to stain specific bases in water-spread DNA it has been shown that uranyl ions, when exposed to high intensity electron beams, can migrate on the specimen by distances much greater than their molecular diameter (Langmore et al., 1974). These authors emphasise that the electron dosages required for clear imaging of atoms are many times more than enough to break chemical bonds, and that this migratory behaviour is attributable to the thermal diffusion of atoms which are normally held to their sites by relatively small binding energies. Finally, the secondary electron emission resulting from the interaction of the beam with the support film and the thin section leads to both becoming highly positively charged. This can give rise to gross electrostatic deformations of these highly plastic materials. To avoid this phenomenon the support films were coated with a thin layer of carbon (~5 nm) which acts as a conductor and prevents any appreciable charge accumulation (Bradley, 1954; Watson, 1955).

Interpretation of high resolution images can only be achieved if the objective lens astigmatism is minimised and the degree of focus or underfocus of this lens is taken into account. Astigmatism correction is routinely checked by observation of Fresnel fringes about holes in formvar films. By this method both the cleanliness of the objective aperture and the general performance of the electron microscope can be assessed. Micrographs of a hole in a support film are illustrated in Figure 6.1. These micrographs, printed at 830 000 X, show overfocus and underfocus fringes and no detectable astigmatism can be seen. The astigmatism must also be checked with the specimen in place in the microscope. Local contamination on the specimen can produce electrostatically-induced astigmatism which can be electromagnetically compensated using the objective stigmator controls. In this work most micrographs were taken with the objective lens ~0.5 - 1.0 μm underfocus, a value which improves contrast but does not seriously affect the dimensions of the image relative to the resolution sought.

6.2 Analysis of the Diameter Distributions that are Sharp and Unimodal

X-ray diffraction studies of orientated specimens of rat tail-tendon (Miller and Wray, 1971; Miller and Parry, 1973) revealed the presence of Bragg reflections in the equatorial and near-equatorial regions that could be accounted for by a tetragonal unit cell of side 7.55 nm. Subsequent work showed that units of diameter 3.8 nm were similar in form to the microfibril structure originally proposed by Smith (1968) and could be packed together in groups of four such that they were related to one another by a 4_3 screw-axis. Some years later Fraser *et al.*, (1979a) pointed out that if the structure of crystalline collagen fibrils is indeed based

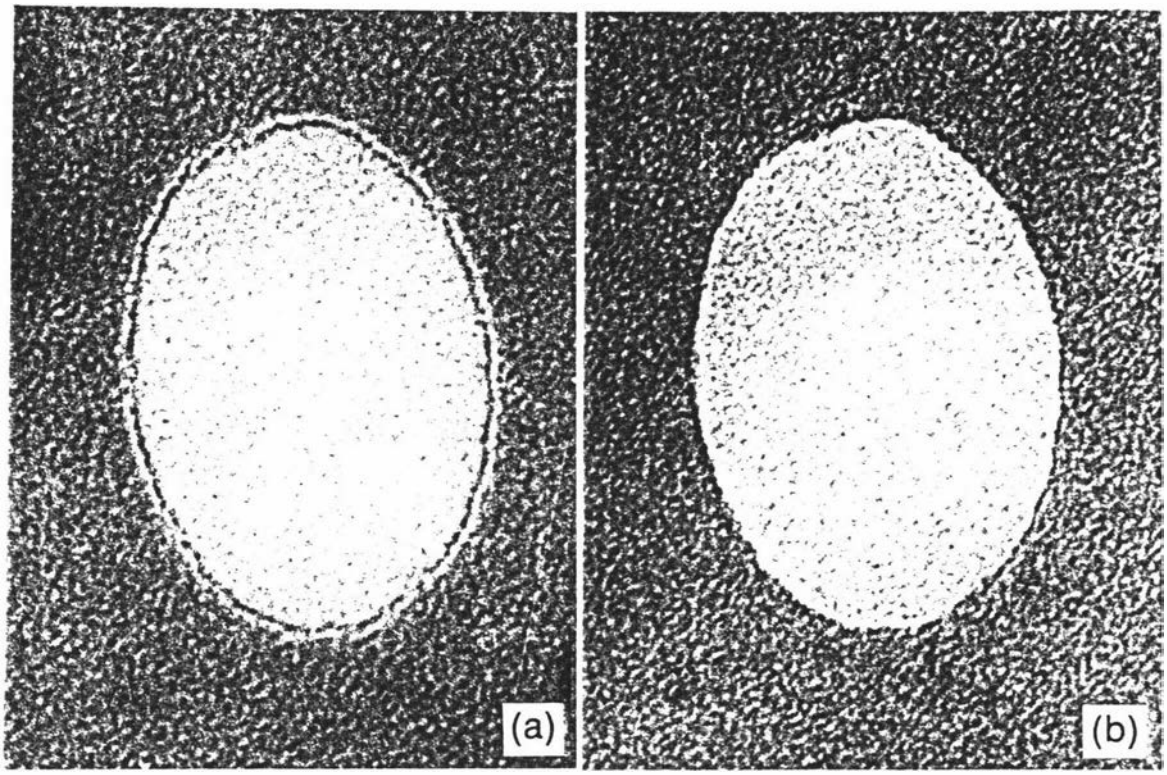

Figure 6.1 Electron micrographs of a small hole in a Formvar film supported on a thin carbon substrate; (a) and (b) show both over-focused and under-focused Fresnel fringes respectively. The fringes show a uniformity of profile about the circumference of the hole and thereby indicate the excellent degree of objective lens astigmatism correction attainable with the instruments used in this study. Magnification: 830 000 X.

upon this lateral (7.55 nm) repeating unit then the smallest fibrils observed in foetal tissues would be likely to be single groups of four microfibrils. Such a group of microfibrils, if they were to appear circular in cross-section, would be expected to have a diameter of about $7.55 \times 2/\sqrt{\pi}$ (~8.5 nm) and the diameter of all other fibrils would be multiples of this value. In other words collagen fibrils would be expected to "grow" in incremental steps of ~8.5 nm, i.e. their diameters would be "quantized".

In general, the wide range of fibril diameters in mature connective tissues does not readily allow this postulate to be tested. However, in cornea and in foetal connective tissues the collagen fibrils have been shown to have extremely uniform sizes, the mean diameters of which can be measured with some accuracy. Jackson (1956) measured collagen fibril diameters in developing chick metatarsal tendons and showed them to have diameters of 8, 12, 17, 25, 31 and 40 nm at 8, 11, 11 - 13, 14, 16 and 20 days foetal respectively, while Greenlee and Ross (1967) in their study of the rat flexor digital tendon measured fibril diameters of 16, 20 - 25 and 40 nm in 15 - 16 day foetal, 18 day foetal and neonate animals. Further, the collagen fibril diameter measurements made by Parry and Craig (see Parry and Craig, 1984 for a summary of the data and the original references) and those measurements made here for small diameter fibrils from corneal stromata and embryonic tissues, have strengthened the hypothesis formulated that collagen fibrils do indeed increase in diameter by about 8 nm increments (Figure 6.2, and see also Parry and Craig, 1979; Parry et al., 1980; Craig and Parry, 1981a, 1981b; Parry and Craig, 1981b). In essence, Parry and Craig (1979) proposed that all connective tissue collagen fibrils are constructed in a similar

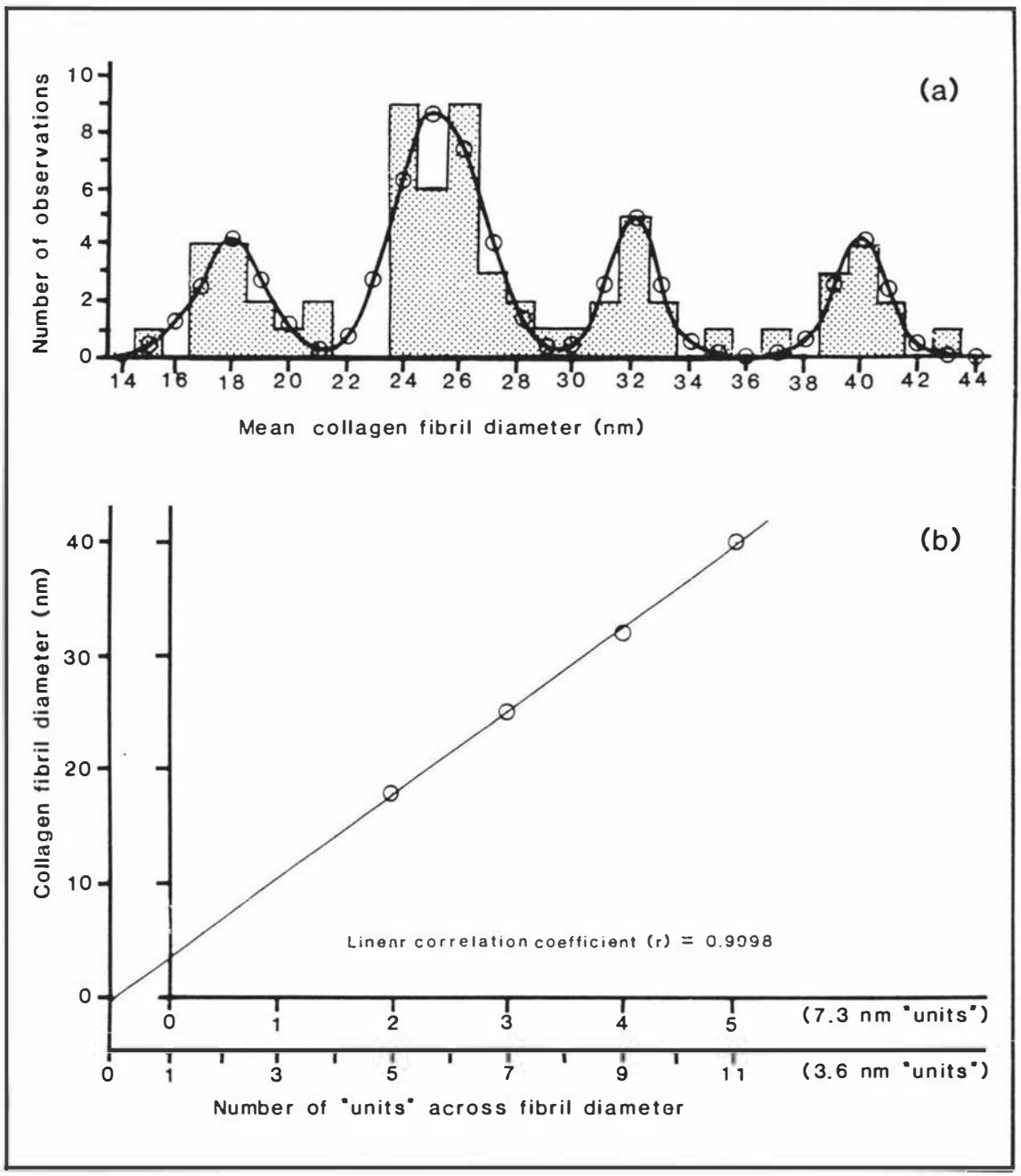

Figure 6.2 (a) Histogram showing the distribution of the mean diameters of collagen fibrils measured from sharp unimodal populations (standard deviations < 3.5 nm). These data represent all those obtained from corneas and from foetal/immature tissues. Using a computer program for the deconvolution of multimodal populations these data were resolved (smooth continuous line) into four populations with means of 17.9, 25.2, 32.0 and 39.8 nm.
(b) Mean fibril diameters plotted against the integers representing possible number of "units" traversing a fibril diameter (see text for details).

manner; that each fibril is built from ~8 nm units or a peripheral layer of ~4 nm units and that each unit is comprised of four of the five-stranded microfibrils related to each other by a 4_3 (or near 4_3) screw-axis.

To arrive at this conclusion these authors used the criterion that if the measured standard deviations of the collagen fibril diameter distribution was less than 3.5 nm, then the distribution could be considered as a relatively homogeneous population of collagen fibrils whose mean diameter was a good estimate of the diameter of the majority of fibrils. If we consider the simplest situation in which there are just two populations of collagen fibrils, the first having a mean diameter d, standard deviation σ and containing a fraction f of the fibrils and the second having a mean diameter $d + \Delta$, the same standard deviation σ and containing a fraction $(1 - f)$ of the fibrils then the observed mean (μ) and standard deviation (s) of the two population distribution are given by the expressions

$$\mu = d + (1 - f)\,\Delta \qquad (21)$$

and

$$s = \left[\sigma^2 + f(1 - f)\,\Delta^2\right]^{0.5} \qquad (22)$$

The standard deviation calculated for the fibril diameter distributions from specimens containing a single population of fibrils has a typical value ~2.5 nm. With this value assigned to σ and with Δ as 8 nm, a distribution containing 10% of fibrils from a neighbouring population ($f = 0.1$) leads to an overall standard deviation for the distribution of about 3.5 nm. This results in an overall mean (μ) differing from the mean diameter of the dominant population of fibrils by 0.8 nm. Such a shift would not disguise the fact that fibril diameters lie close to multiples of 8 nm which affirms that an

analysis of diameters of small collagen fibrils can reasonably include those distributions having values of $s < 3.5$ nm.

In this work most of the specimens have been prepared for electron microscopy using the same preparative techniques (see Chapter 2); thus any percentage decrease in fibril diameter arising from fixation artefacts should not obscure the clustering of the measured fibril diameters in discrete groups provided, of course, that quantization of collagen fibril diameter does indeed occur. Although the mean diameter of each cluster of diameters would depend on the percentage shrinkage which had occurred, each value should remain an integral multiple of the average separation of the clusters.

Cylindrical collagen fibrils which are not cut in a truly transverse plane appear elliptical in section, with the degree of ellipticity being dependent on both the obliquity of the fibril in the section and the section thickness. The effects that these variables have on the observed fibril dimensions can be appreciated by a consideration of Figure 6.3. Here d_{fib} is defined as the true fibril diameter and the angle ϕ as the deviation of the fibril axis from the normal to the section. The major diameter of the ellipse produced by the obliquity of the fibril in the section, and the maximum diameter of the fibril observed in the electron microscope are defined as d_{maj} and d_{max} respectively. Assuming that the section is uniformly stained throughout its thickness (t), the diameter d_{max} results from the projection of the image of the major diameter of the ellipse (d_{maj}) through the section.

It can thus be seen that

$$d_{maj} = d_{fib} \sec \phi \qquad (23)$$

and

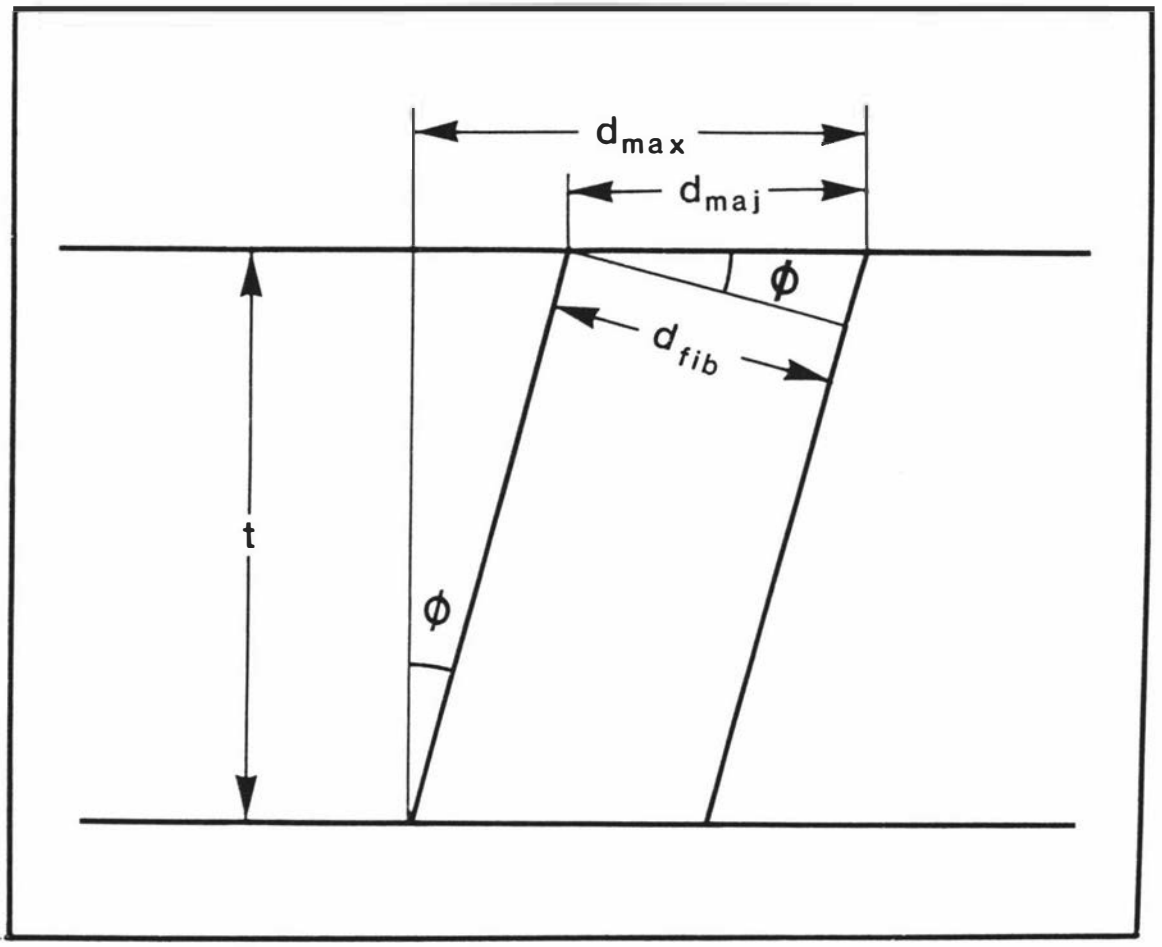

Figure 6.3 The diagram illustrates a collagen fibril (of diameter d_{fib}) which is tilted by an angle of ϕ in a section of thickness t. The symbol d_{maj} refers to the major diameter of the ellipse produced by the obliquity of a cylindrical fibril seen in section and d_{max} refers to the maximum projected diameter observable in the electron microscope.

$$d_{max} = d_{fib} \sec \phi + t \tan \phi \qquad (24)$$

For an appreciation of the effects of these factors consider a 30 nm diameter fibril inclined at 15°, to the normal in an 80 nm thick section. The increase in the major diameter of the ellipse due to the obliquity of the fibril will be 0.93 nm (3%) whilst the increase resulting from section thickness will be 19.4 nm (65%). Further examination shows that for fibrils with diameters in the range 10 - 100 nm which are tilted by angles of up to 20° in 80 nm thick sections, it is the section thickness rather than the fibril obliquity which makes the major contribution to the ellipticity of the observed image. In this work obliquity effects were largely eliminated by either measuring only those fibrils which were truly circular in section or by measuring only the minor axis of the fibrils. For some specimens use was made of a rotating goniometer stage which allowed the sections to be tilted in the microscope until they were circular in section. The magnification of the electron microscope was also determined for each set of micrographs taken by reference to a cross-grating replica, thus reducing any source of error in this respect to about 1 - 2%. For the reasons described above the systematic errors encountered in this work are unlikely to be a major factor affecting the collagen fibril diameter measurements.

The main source of random error probably lies in the procedures used to measure the collagen fibril diameters from the electron micrographs. As there is no sharply defined "membrane" delineating the fibril periphery the specification of the boundary of the collagen fibril becomes a subjective choice. However, the mean diameters of sharp unimodal populations of small diameter collagen fibrils measured in this thesis and in earlier work (Parry and Craig, 1979; Parry et

al., 1980; Parry and Craig, 1981b) were shown to be reproducible to within about 3%. The collagen fibril diameters in all of the tissues studied were measured by two observers (the author and DADP) and the results of those measurements (shown graphically in Figure 6.4) indicate (a) that the mean collagen fibril diameters measured for foetal and immature tissues are indeed clustered in groups separated by multiples of ~8 nm and (b) that the second observer (DADP) consistently makes measurements that are about 1 nm lower than the corresponding values recorded by the author.

Assessment of the boundary of a collagen fibril will necessarily differ slightly between observers. This will lead to an absolute, rather than a percentage, difference in mean fibril diameter. However the means of the groups of fibril diameter should still be separated by about 8 nm even though the recorded values may not be multiples of ~8 nm for each observer. Since the diameters of small collagen fibrils quoted in the literature are often significantly different from multiples of ~8 nm, it would seem that great care must be taken when comparing values quoted by different authors.

The results of this thesis pertaining to small collagen fibrils (Figure 6.2) clearly show that the diameters of these fibrils do not have a normal distribution of sizes but rather are grouped into populations with preferred sizes close to a multiple of 8 nm. The data supporting this statement are shown graphically in Figure 6.2a which has been constructed by plotting a frequency distribution (histogram) of all of the mean diameters of the sharp unimodal populations of collagen fibrils measured. Further, this distribution has been subjected to a computer analysis for resolving multimodal distributions into discrete populations. No constraints were imposed

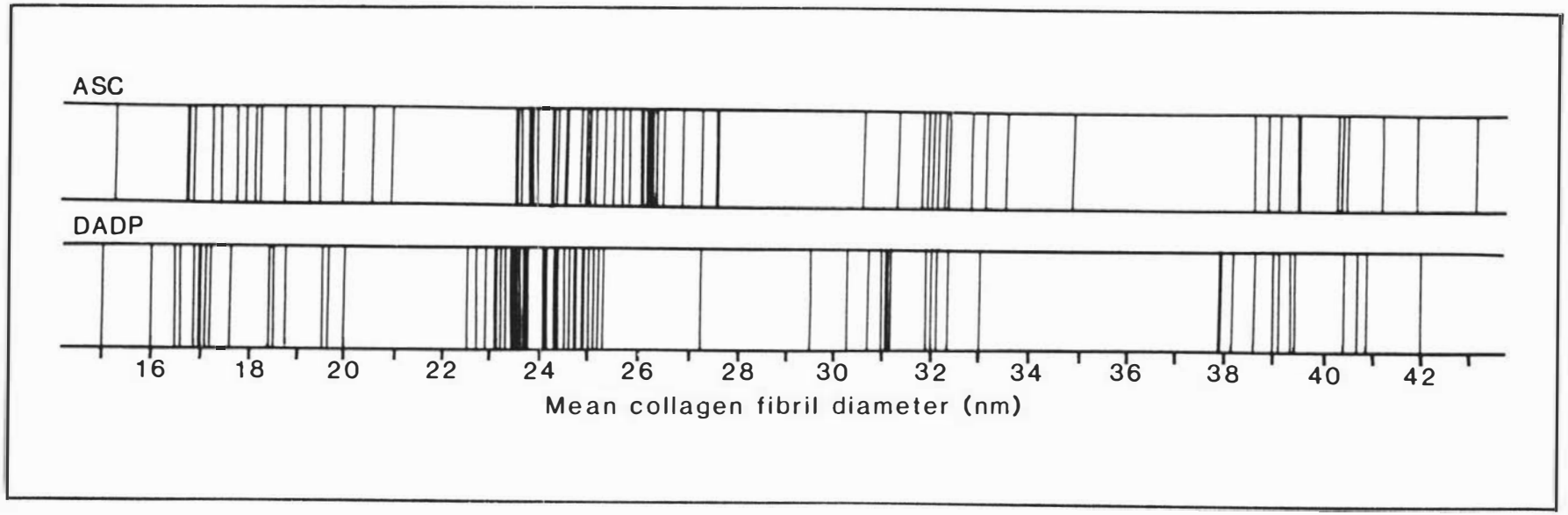

Figure 6.4 Bar diagram showing the spread of mean collagen fibril diameters recorded from sharp unimodal distributions and the differences of measurement made by two observers (ASC and DADP). The diagraam shows that both observers measure mean diameters which are clustered in groups of ~8 nm apart - but that measurements made by DADP tend to be about 1.2 nm lower than those made by ASC.

on the mean diameters of the resolved populations or on their standard deviations. To a first approximation the separation of the means is ~8 nm and thus the mean diameters, determined computationally at 17.9, 25.2, 32.0 and 39.8 nm, correspond to about 2, 3, 4 and 5 of these ~8 nm increments respectively. When these data are plotted (Figure 6.2b) the straight line graph obtained intercepts the diameter axis at 3.24 nm and has a slope of 7.28 nm. The equation of the line is thus given by the expression:

$$\begin{aligned} \text{diameter} &= 7.28\ m + 3.24 \qquad (m \geqslant 0) \\ &= 3.64\ m' + 3.24 \qquad (m' = 2m) \end{aligned} \qquad \textbf{(25)}$$

One interpretation of this observation is that each fibril contains a "core" of diameter ~3.2 nm, and that fibril growth occurs by the peripheral accretion of 3.6 nm (7.2/2 nm) units that are similar in size to the triclinic unit cell proposed from X-ray diffraction studies. Whilst there is no evidence that such a "core" would be collagenous, it has been noted that its diameter approximates that of the peripheral increment just described. Hence the diameter of any fibril could be given by the expression:

$$\begin{aligned} \text{diameter} &= 3.6\ (m' + 1) \qquad (m' = 2m) \\ &= 3.6\ (2m + 1) \qquad (m \geqslant 0) \end{aligned} \qquad \textbf{(26)}$$

Prior to these studies it had been suggested that the peripheral accretion of 4 nm units gave rise to fibrils whose diameters were multiples of ~8 nm (the diameters thus being <u>even multiples</u> of ~4 nm). However, the new interpretation of the data presented here suggest that fibril growth occurs by the peripheral accretion of 3.6 nm units about a core of similar size - the diameter of the fibrils thus being an <u>odd multiple</u> of 3.6 nm. In either case the fibrils will grow by increments of about 7 - 8 nm as previously reported. The difference, however, lies in the absolute diameters that the fibrils may attain.

It is of interest to note here that some electron micrographs of small diameter collagen fibrils indicate that the fibrils have an electron translucent "core". This is not observed in most preparations but in the tissues studied in this thesis it was most pronounced in the developing lamprey (macrophthalmia) skin (Figure 6.5). However, the diameters of these cores (~7 nm) do not relate well to the 3.2 nm diameter proposed, neither are they in agreement with the ~2 nm Ruthenium-red-positive cores shown by Nakao and Bashey (1972).

As will be discussed in Section 6.5 the incremental value of 7.2 nm in dehydrated tissues is probably closer to 9.5 nm in vivo. This latter value, obtained from X-ray diffraction studies of native tissues, indicates that the preparative procedures for electron microscopy have resulted in a systematic shrinkage of about 15 - 20%, a value commonly encountered in this work for a wide variety of other connective tissues.

6.3 Analysis of Diameter Distributions that are Heterogeneous

Some of the distributions of collagen fibril diameter measured for immature tissues from the chick, sheep and rat are clearly heterogeneous in form, i.e. they contain more than a single population of fibril sizes. These distributions, which were measured from the highest quality micrographs obtained, were subjected to an analysis using a computer program for non-linear least-squares decomposition of mixtures of populations. By this method the values for the mean diameters of collagen fibrils for each population within the distribution could be determined. The values so obtained, together with the percentage of fibrils in each population, are listed in Tables 4.2, 4.6 and 4.9.

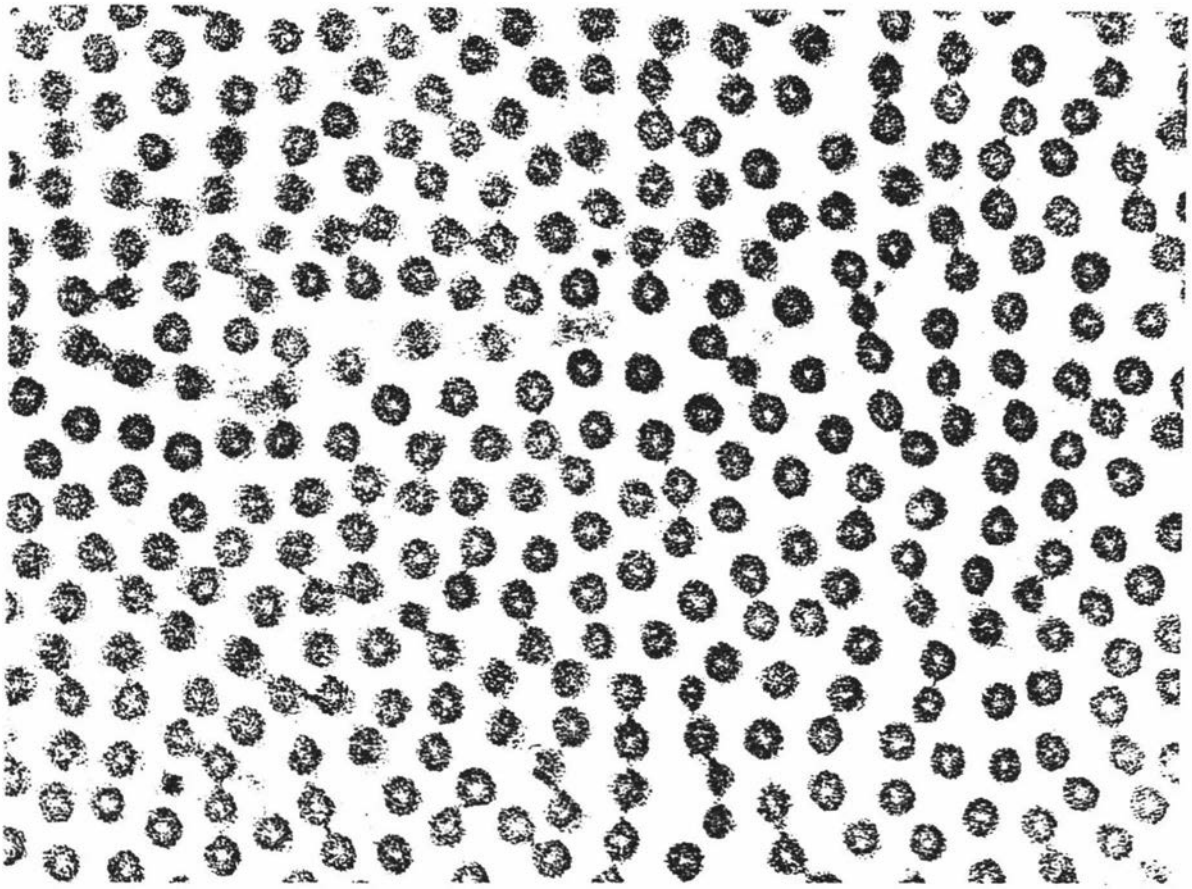

Figure 6.5 Electron micrograph of transverse section through the collagen fibrils from the skin of the developing lamprey (Macrophthalmia stage). Micrograph shows uniform diameter fibrils (~45 nm diameter) which appear to have an electron translucent "core". Magnification: 110 000 X.

The results show that the mean diameters of the fibrils in each population were clustered about multiples of 8 nm as indeed they were for the sharp unimodal populations of collagen fibrils previously discussed. It was also reported in Chapter 5 that the bacterial collagenase treated superficial flexor tendon from horse gave rise to a similar heterogeneous population of fibril sizes as has been noted here with chick, rat and sheep. The five-population distribution observed for the 18 day foetal chick metatarsal tendon has mean fibril diameters for each population which do not lie on multiples of 8 nm; rather they are displaced between such multiples. The mean separation of these values, however, is 8.4 nm lending further support to the concept of a structural unit in collagen with a diameter ~8 nm or, alternatively, to accretions of 4 nm units about the periphery of a fibril. The collagen fibril diameter distribution of this tissue was also measured by two other observers (DADP and NPM) and their results, together with those of the author, are plotted graphically for comparison in Figure 6.6. In each case the distributions were resolved into discrete populations of fibrils with diameters separated by about 8 nm.

6.4 Analysis of Broad Distributions of Collagen Fibril Diameter

Collagen fibril diameter distributions which are neither sharp (standard deviations < 3.5 nm) nor composed solely of small diameter fibrils (maximum diameter < 80 nm) have been considered as "broad" distributions in this thesis. In these cases the smallest, but not the largest fibrils may be resolvable into populations whose diameters are multiples of 8 nm. Indeed this is not unexpected, for to resolve the difference between say 24 and 32 nm diameter fibrils all errors

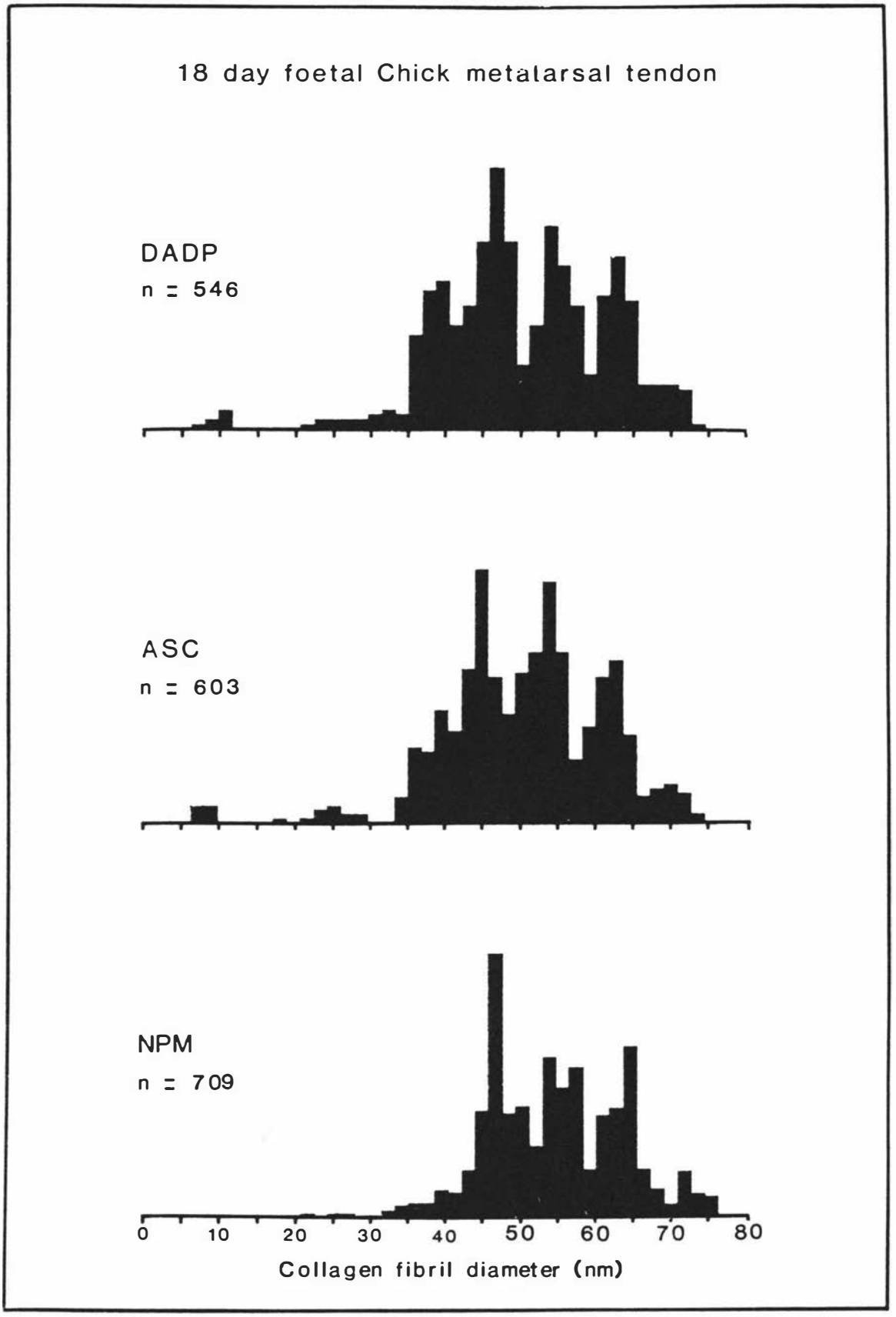

Figure 6.6 Frequency distributions (left) and mass distributions (right) of collagen fibril diameters in 18 day foetal chick metatarsal tendons as measured by three independent observers (DADP, ASC and NPM).

arising from measurements must be less than half the difference between the values to be resolved. In this case the errors must be less than about 16%, a value generally achievable within the uncertainties of the experimental method. However, in order to resolve the difference between 72 and 80 nm diameter fibrils the accuracy of the measurements must still be better than 4 nm i.e. an error of < 5%. This is unlikely to be achieved within the uncertainties of the experimental method commonly employed by electron microscopists.

6.5 Correlation between Electron Microscope and X-ray Data

The lateral dimensions of small collagen fibrils having a sharp unimodal distribution of diameters has been measured indirectly in a hydrated (physiological) state by analysis of the low angle X-ray diffraction patterns of orientated specimens. This aspect of the work was undertaken by Drs. E.F.Eikenberry and B.Brodsky at the Department of Biochemistry, Rutgers Medical School, New Jersey. In collaboration with them, the collagen fibrils of many of the tissues studied were also directly measured in a dehydrated (non-physiological) state using the electron microscope. The tissues studied in this and other work include rabbit and frog corneas (Sayers et al., 1982; Inouye and Worthington, 1983a), lamprey notochord (Eikenberry et al., 1984) lamprey skin (Eikenberry et al., in preparation), chick metatarsal tendon (Eikenberry et al., 1982a, 1982b) and rat endoneurium (Inouye and Worthington, 1983b).

The Fourier transform of a single cylinder of radius *a* is given by the expression $J_1(x)/x$, where J_1 is a first order Bessel function, x is given by the expression $2\pi aR$ and R is the lateral reciprocal lattice coordinate measured from the X-ray diffraction pattern. The positions of the maxima and minima in the low angle transform allow, in principle, the diameter of the cylinders to be calculated. In practice, however, the problem is complicated by the presence of an interference function arising from the influence of locally ordered arrays of fibrils. This problem has been discussed in detail by Eikenberry *et al.*, (1982a, 1982b) who have shown that the effects of fibrillar interference are not significant at points beyond the first maximum in the transform.

Taking account of these limitations it has still proven possible to calculate the diameters of hydrated fibrils with a high degree of accuracy and the values obtained from these X-ray studies along with the corresponding electron microscope estimates are listed in Table 6.1. It can be seen that the X-ray values are consistently higher than those obtained using electron microscopy and although there is some variation in the discrepancies between the corresponding values it is clear that the specimens prepared for electron microscopy have diameters which are, on average, only about 80% of those obtained using X-ray diffraction techniques. The limited data available at present suggest that the degree of shrinkage varies with tissue type and age. In particular the constituent collagen fibrils of the corneal stroma appear to suffer the greatest degree of shrinkage, with the values determined by electron microscopy being approximately 65% of those obtained by X-ray diffraction. Further, using the X-ray estimates of diameter which are more likely to correspond to the

TABLE 6.1

DIAMETERS OF COLLAGEN FIBRILS AS DETERMINED BY ELECTRON MICROSCOPE AND X-RAY DIFFRACTION STUDIES

Tissue	Diameter (nm) X-ray	EM	EM / X-ray (x100%)
Ox vitreous humour	9.3 (1)	--	--
Lamprey notochord sheath	17 (2)	15.0	88
Ox cornea	37 (3)	27.0	73
Frog endoneurium	38 (4)	--	--
Frog cornea	38 (5)	23.9	63
Ox cornea	39 (5)	27.0	69
Rabbit cornea	40 (5)	26.2	65
13 day foetal CMT*	46 (6)	44.9	98
14 day foetal CMT*	48 (6)	39.4	82
Rabbit endoneurium	50 (4)	--	--
Rat endoneurium	50 (4)	31-47 (8)	62-94
17 day foetal CMT*	58 (6)	41.2	71
Lamprey skin	67 (7)	51.9	77

(1) Gross et al., 1955a; (2) Eikenberry et al., 1984; (3) Sayers et al., 1982; (4) Inouye & Worthington, 1983b; (5) Inouye & Worthington, 1983a; (6) Eikenberry et al., 1982a, 1982b; (7) Eikenberry et al., (in prepn.); (8) Parry et al., 1980.

* CMT, Chick metatarsal tendon.

values found *in vivo*, it can be seen that the collagen fibril diameters are more consistent with a quantization of about 9.5 nm, a value again reflecting the degree of shrinkage suffered by specimens prepared for electron microscopy.

In addition to their diameters, the separation of the fibrils in certain tissues can be measured. In particular, the separations of fibrils in several foetal specimens of chick metatarsal tendon have been calculated from the position of the interference maximum in the low angle X-ray transform. This can be compared with the value determined from the radial distribution function calculated directly from the electron micrographs (see Chapter 2; also Table 4.3). Once again the two values differ by about 20% and it would therefore appear that the specimens prepared for electron microscopy suffer shrinkage at both the fibrillar and interfibrillar levels.

It is known from quantitative X-ray studies (Fraser *et al.*, 1983) that collagen fibrils contain ~50% water. As the longitudinal period of the collagen fibril has been shown to be largely unaffected by dehydration (shrinkage < 5% and usually < 1%), it is reasonable to assume that the total removal of this water by electron microscope preparative procedures would lead to a collapse of the molecular lattice and a reduction of the cross-sectional area of a collagen fibril to ~50% of its *in vivo* value. Such a reduction would lead to a change in diameter to about 70% of that *in vivo*, i.e. a shrinkage of ~30%. As the shrinkage observed is of this order it seems reasonable to suppose that procedures employed in electron microscopy have indeed removed the water *in toto* and that the embedding medium has failed to fill this niche. It is not clear whether the effect would be as pronounced, or indeed would occur at all, in more mature tissues

containing larger diameter fibrils.

6.6 Conclusions

It is clear from the results reported here that many factors affect the confidence with which an electron microscope observation may be held. For many collagenous tissues, especially those from foetal and immature tissues which have low proportions of the so-called "stable cross-links", shrinkage has been shown to be a major problem when absolute values of collagen fibril diameters are sought. The magnitude of this shrinkage may also vary with tissue type and age. It is not clear at present whether this problem will also be important for mature tissues which contain large diameter fibrils with a broad distribution of sizes. Unfortunately such tissues do not lend themselves to diameter analyses by X-ray diffraction methods and so no estimates of shrinkage can be made. It may be speculated, however, that the higher proportion of stable cross-links in these mature tissues may help to inhibit such large dimensional changes.

Previous studies of cornea, which are summarised in Chapter 3, have illustrated the diversity of diameters reported by different workers. These variations may be due, in part, to the different procedures used to prepare the specimens and also to the methods used to quantitate the data from micrographs. In addition to the shrinkage effects described here it is also of paramount importance that sufficient measurements be taken to determine the true shape of the collagen fibril diameter distribution. For higher resolution studies it is also necessary to measure the diameter distribution of fibrils from the original micrographs rather than from positive prints where unknown dimensional changes and photographic artefacts will contribute

to measurement errors. In this work the use of a binocular microscope has been shown to be a satisfactory method for measuring fibril diameters (< 70 nm) directly from micrographs. For lower resolution work (fibril diameters 70 - 550 nm), measurements made from prints prepared to a standard magnification (65 000 X) were quite sufficient for the shape of the collagen fibril diameter distribution to be determined. The comparative studies of the corneal stroma have indicated that meaningful correlations can be achieved when similar techniques of tissue preparation and micrograph mensuration are applied to all specimens. Whilst the absolute values determined are clearly significantly less than those found using X-ray diffraction techniques, the conclusions of the comparative study are still valid.

Much of this work is concerned with trends in fibril diameters and fibril diameter distributions. Provided that the possible sources of error are understood and that the artefacts are similar for all sets of specimens the conclusions ultimately reached will bear direct comparison. Absolute values of lateral dimensions in collagen fibrils may, however, be underestimations of the true values by as much as 20 - 30%.

CHAPTER SEVEN

THE GROWTH AND DEVELOPMENT OF CONNECTIVE TISSUES AND THE RELATIONSHIP BETWEEN COLLAGEN FIBRIL DIAMETER DISTRIBUTIONS AND MECHANICAL PROPERTIES

7.1 Introduction

As connective tissues have predominantly mechanical roles it is of importance to understand how the constituent chemical components - collagen, GAGs, elastic fibres, minerals, water - provide the tissue with its mechanical properties. The mechanical roles played by connective tissues vary markedly. For instance tendon, a connective tissue designed to have a high tensile strength, has a high collagen content, orientated fibrils and a low GAG content, whilst cartilage, a connective tissue primarily designed for its ability to withstand compression, has a lower collagen content, local ordering of fibrils and a much higher concentration of GAGs than does tendon. To a first approximation it may be assumed that the tensile properties of the tissue are provided by the collagen fibrils and that the compressive properties result from the hydrated proteoglycans and other components of the matrix. This concept is necessarily an oversimplification since evidence has been presented that tendon treated with hyaluronidase has reduced tensile properties (Partington and Wood, 1963). This may be explained if the GAGs contribute to the mechanical properties of the tissue by linking the collagen fibrils to one another (Miller, 1976). Some part of the tensile properties of a tissue are thus likely to be attributable to the components of the matrix.

Other specialized tissues, such as the ligamentum nuchae (prominent in grazing animals), are rich in elastic fibres and their mechanical properties are therefore greatly influenced by this component. Its dominance in the ligamentum nuchae and its high levels in arterial walls implies that its importance lies with its ability to give a tissue both resilience and extensibility. These factors should not be confused with elasticity in the physical sense. The modulus of elasticity of elastin has been recorded as ~6 x 10^5 N m^{-2} and it is known that the elastic modulus of collagen (~8 x 10^7 N m^{-2}) is two orders of magnitude greater. Collagen is therefore more elastic than elastin, but less extensible, breaking at a strain of about 8 - 10% (Rigby et al., 1959). Elastin shows long-range reversible extensibility and does not creep when loaded for extended periods of time; purified samples of ligamentum nuchae break at about twice their resting length (Wainwright et al., 1976). Elastic ("pliant") composites may be considered as a three-phase system, with the collagen fibrils and the proteoglycan components arranged in parallel with the elastic fibres. Because the elastin component can be described as a "network polymer in the plateau region of its response curve" the strain rate of this three-phase system will arise mainly from the interactions of the collagen fibrils and the proteoglycan components. If the viscous interactions of the latter components with the material as a whole are not too large then the system will have resilience properties similar to those of a pure "protein rubber" and the composite will be able to function as an energy-storing system for relatively high speed cyclic processes as is the case in the vertebrate arterial wall (Wainwright et al., 1976). Most of the connective tissues studied in this work (skin and tendon) have high collagen contents and low elastic fibre contents. It is therefore a

reasonable assumption that the tensile properties of the tissue are predominantly provided by the collagen fibrils. It is on this assumption that the results presented in this chapter have been largely based.

The orientation of the collagen fibrils within a specific tissue is also of importance. Examples of extreme cases are the visco-elastic fluids such as vitreous humour, in which the collagen fibrils are randomly orientated, and tendon, a tissue subjected to uni-directional loading, where the collagen fibrils are all strictly aligned with their long axes parallel to the direction of the applied load. Further, a crimp in the fibre bundles (Diamant et al., 1972; Gathercole and Keller, 1975; Fraser et al., 1979a) is present in some connective tissues (such as tendon) and acts as a compliance mechanism which allows for the rapid initial extension of the tissue (~3 - 4%) without stretching the constituent collagen fibrils. The spatial arrangements of the collagen fibrils in tissues of intermediate architecture such as skin, cartilage, bone, dentine, paratenon, arachnoid membrane, sclera and tapetum fibrosum have also been reviewed (Parry and Craig, 1984) whilst the structure of the cornea is detailed in Chapter 3.

The results of the electron microscope data presented in Chapter 4 will be analysed here and correlated with the eight conclusions made by Parry et al., (1978a) who established correlations between the collagen fibril diameter distribution, the growth and development of the tissue and its mechanical properties. One of the conclusions made was "that collagen fibril diameter distributions are a function of both the applied stress and its duration, that the duration and level of stress in a tissue is communicated to the cells which control the

biosynthesis of the glycosaminoglycans, that these glycosaminoglycans determine the ultimate size of the collagen fibrils, and that the distribution of the collagen fibril diameters may continually change in line with the mechanical and functional requirements of the connective tissue". This conclusion is elaborated in detail in Chapter 8, whilst the other seven are cited in turn at the beginning of the sections numbered 7.2.1 to 7.2.7. The data listed in Table 7.1 summarize the gross forms of the collagen fibril diameter distributions (unimodal or bimodal) for the foetal, neonatal, mature and senescent stages of life in a diverse range of connective tissues studied in this and previous work (see Parry and Craig, 1984).

7.2 Correlations between Collagen Fibril Diameter Distributions and Tissue Attributes

7.2.1 Foetal Development

"The collagen fibrils from connective tissues at birth and in the foetal stage of development have unimodal distributions of diameter."

Of the 34 tissues studied in this thesis 22 were taken from foetal or perinatal animals. Without exception all of these tissues were seen to have unimodal distributions of collagen fibril diameter (see Table 7.1), although the standard deviations of these distributions vary markedly over the range 1.5 - 50 nm (see Section 7.2.2 and Table 7.2). The tissues studied included tendons, ligaments, skins, notochord sheath, sclera, heart valve, fibrocartilage, bone and Wharton's jelly, from a diverse group of animals including lamprey, trout, chicken, rat, guinea pig, sheep, dog, ox and human. The presence of a sharp unimodal distribution of collagen fibril diameters is also found in the corneas in each of the

TABLE 7.1

FORM OF THE COLLAGEN FIBRIL DIAMETER DISTRIBUTION IN FOETAL TO SENESCENT TISSUES

Tissue	MAD * (nm) at maturity	Form of distribution ** F	B	M	S
Rat tail-tendon (1,2)	340	U	U	B	B
Chick metatarsal tendon	240	U	U	B	-
Horse digital extensor tendon (3)	240	U	U	U	B
Other mammalian tendons / ligaments	120-210	U	U	B	B
Skin (rat-tail and trout)	130-195	-	-	B	-
Skin (chick and mammalian)	70-130	U	U	U	-
Other tissues: paratenon / sclera endoneurium / fibrocartilage (4,5)	10-70	(U)	(U)	U	-
Skin (lamprey)	50	(U)	(U)	U	-
Cornea (most vertebrates)	25	U	U	U	U
Cornea (bony fish)	17	(U)	(U)	U	-

* MAD = Mass-average diameter

** F = Foetal, B = Birth, M = Maturity, S = Senescence.
B = Bimodal (or right skewed), U = Unimodal, (U) = Presumed unimodal.

(1) Parry and Craig, 1977; (2) Parry and Craig, 1978; (3) Parry et al., 1978b; (4) Parry et al., 1978a; (5) Parry et al., 1980.

TABLE 7.2

MEAN AND MASS-AVERAGE DIAMETERS OF COLLAGEN FIBRILS IN TENDONS, LIGAMENTS AND SKINS IN PERINATAL ANIMALS

ANIMAL Tissue	Age	Mean diam. ± std.dev. (nm)	Mass-av. (nm)	
RAT				
Forelimb flexor tendon	0 d	30.6 ± 1.6	30.7	
Dorsal abdominal skin	2 d	30.0 ± 3.8	30.9	
Hindlimb flexor tendon	0 d	31.1 ± 1.5	31.2	
Tail skin	2 d	31.0 ± 4.3	32.2	
Ventral abdominal skin	0 d	32.0 ± 3.4	32.8	
Flexor digitorum longus	5 d	40.6 ± 3.2	41.0	(1)
Central tendon of diaphragm	0 d	40.4 ± 7.4	43.1	
Tibial collateral ligament	5 d	46.6 ± 5.5	47.8	(1)
Tail tendon	0 d	48.5 ± 2.5	49.0	(2)
Achilles tendon	5 d	50.6 ± 5.4	51.6	(1)
RABBIT				
Achilles tendon	0 d	34.6		(3)
HUMAN				
Skin	5 d	67.4 ± 10.0	70.1	
CHICKEN				
Skin	0 d	48.4 ± 6.4	50.1	
Metatarsal tendon	0 d	45.6 ± 11.6	50.5	
GUINEA PIG				
Central tendon of diaphragm	0 d	50.0 ± 12.1	55.3	
Footpad skin	0 d	70.5 ± 9.8	73.1	
Body skin	0 d	75.6 ± 15.5	81.4	
Digital extensor tendon	0 d	72.5 ± 26.3	88.9	
Digital flexor tendon	0 d	87.6 ± 24.8	99.7	
SHEEP				
Suspensory ligament	120 d F	69.7 ± 22.1	82.2	
Superficial digital flexor	120 d F	74.4 ± 21.1	85.3	
Skin	0 d	82.7 ± 15.0	87.6	
Common digital extensor	120 d F	71.2 ± 29.8	93.4	
HORSE				
Superficial digital flexor	9 mo F	96.0 ± 33.3	115.7	(4)
Common digital extensor	9 mo F	103.1 ± 45.9	137.6	(4)
Suspensory ligament	9 mo F	106.5 ± 46.9	142.9	(4)

(1) Parry et al., 1980; (2) Parry and Craig, 1977; (3) Ippolito et al., 1980; (4) Parry et al., 1978b.

seven perinatal animals studied; frog, rat, guinea pig, human, hippopotamus, and two species of monkeys (see Table 3.3). These data are all supportive of the supposition as outlined.

7.2.2 Modes of Collagen Fibril Development for Altricious and Precocious Animals

"The form of the collagen fibril diameter distribution at birth reflects the degree of development of the animal at this stage of life; the collagen fibril diameter distributions at birth are sharp for altricious animals and broad for precocious animals."

The relevant data from Tables 4.1, 4.5, 4.7, 4.8, 4.10 and 4.11 are summarized in Table 7.2. along with other data for tissues from perinatal rats and horses, previously published (Parry et al., 1978b; Craig and Parry, 1981b). The precocious animals analysed were chick (18 day foetal to birth; incubation period 21 days), guinea pig (birth), sheep (120 day foetal to birth; gestation period 140 days) and horse (9 months foetal; gestation period 11 months). Since the postulate suggests that the collagen fibril diameter distributions will be broad in these animals at birth the inclusion of tissues from precocious animals prior to birth is also justifiable if the distributions from such tissues are already broad. Similarly the inclusion of sharp collagen fibril diameter distributions measured in tissues from altricious animals at some time after birth can also be justified on the assumption that such distributions must also be sharp at birth.

For all of these distributions the mean collagen fibril diameters and their standard deviations, and the mass-average diameters of the distributions are listed in Table 7.2. It can be seen that the mass-average diameters of the collagen fibrils in various tissues from the altricious mammals (rat, rabbit and man), lie in the range 25 to 70 nm. These distributions are all fairly sharp, having standard deviations of 1.5 - 10 nm. Conversely, perinatal tissues from the precocious animals (chick, guinea pig, sheep and horse) have mass-average diameters which are generally larger (50 to 140 nm) and distributions of diameters which are broader, the standard deviations of the means lying between 10 and 47 nm.

The two newborn chicken tissues studied (metatarsal tendon and skin) contain collagen fibrils with mass-average diameters lying in the region of overlap between the altricious-precocious classification suggested by the preceding discussion (i.e. mass-average diameters 50 to 70 nm). However, the small standard deviations of the recorded mean diameters for these two tissues suggest a better fit with the altricious rather than with the precocious classification. Similarly, human skin at birth has a collagen fibril mass-average diameter of 70 nm but also a standard deviation of the mean (~10 nm) befitting the sharp distributions displayed by altricious animals at birth.

The three intermediate values obtained for chicken and human tissues are indicative of an uncertainty which must be placed on such an altricious-precocious classification being based on whether the animal is capable of walking within a few hours of birth (see Parry and Craig, 1978a). Rather it would appear that the mass-average diameter of collagen fibrils from tendon and skin could be better equated to the degree of development of a neonate animal indicated by

its body weight at birth expressed as a percentage of the adult body weight. Data supporting this alternative are listed in Table 7.3.

7.2.3 Post-natal Development

"The mass-average diameter of the collagen fibrils in all tissues other than corneal stromal lamellae increases from birth to maturity."

Further verification for this postulate is provided by an evaluation of the data obtained for various tendinous tissues; chick metatarsal tendon (Table 4.1), rat forelimb flexor tendon (Table 4.8), and the extensor, flexor and diaphragmatic tendons of the guinea pig (Table 4.7). Further, from an evaluation of the data on skins (Table 4.10), it can be seen that human skin, guinea pig dorsal abdominal skin, rat dorsal and ventral abdominal skins and tail-skin, and chicken skin are also shown to comply with the established pattern. However guinea pig ventral abdominal and foot pad skins have collagen fibril mass-average diameters which do not alter significantly between birth and maturity and sheep skin appears to contain collagen fibrils with a maximum-mass average diameter of ~90 nm which occurs at about 120 day foetal. The mass-average diameters of the two adult sheep skin specimens studied were ~65 and ~73 nm respectively, both of which are less than that found prior to birth.

In summary, of the 14 new tissues examined 11 have collagen fibril mass-average diameters which increase between birth and maturity, two have unchanging distributions throughout the time period studied and one (sheep skin) appears anomalous.

TABLE 7.3

BIRTH-MASS OF ANIMALS EXPRESSED AS PERCENTAGES OF ADULT-MASS				
Animal	Approx. Birth mass (kg)	Approx. Adult mass (kg)	$\frac{\text{Birth mass}}{\text{Adult mass}} \times 100\%$	Collagen fibril mass-av.diam. (nm) (mean perinatal)
Rat	0.005	0.3	1.7	38
Rabbit	0.065	3.8	1.8	38
Chicken	0.040	1.6	2.5	50
Man	3	70	4.3	70
Sheep	4	75	5.3	87
Guinea pig	0.080	1	8.0	80
Horse	45	450	10.0	132

7.2.4 Form of the Collagen Fibril Diameter Distribution at Maturity

"Most, and possibly all, orientated type I collagenous tissues suffering long-term high-stress levels have a bimodal distribution of collagen fibril diameters at maturity."

The forms of the collagen fibril diameter distributions for all tissues studied are listed in Table 7.1. This table summarizes all data recorded for this thesis and those distributions previously determined for rat and horse tissues (Parry and Craig, 1977, 1978; Parry et al., 1978a, 1978b, 1980). With the exception of the horse digital extensor tendon the form of the collagen fibril diameter distribution is bimodal for all tendons and ligaments studied at maturity. All of the tendons studied from rat, chick, guinea pig, dog, sheep, horse and ox have both locomotory and postural roles, the exception being the central tendon of the diaphragm which has a continually varying load arising from its respiratory function. All these tissues are considered to suffer long-term high-stress levels and their bimodal distributions of collagen fibril diameter are therefore in accordance with the stated postulate. The common digital extensor tendon from the horse, however, endures short-term or intermittent stress (Barnes and Pinder, 1974) and the collagen fibril diameter at maturity is unimodal (Parry et al., 1978b).

The remaining tissues, fibrocartilage, sclera, vitreous humour, notochord sheath and cornea are all considered low-stress tissues and have unimodal populations of collagen fibril diameters. Skins are also generally considered to fit into this classification (see for instance lamprey skin and rat skin; Figures 7.1a, 7.1b and 7.2).

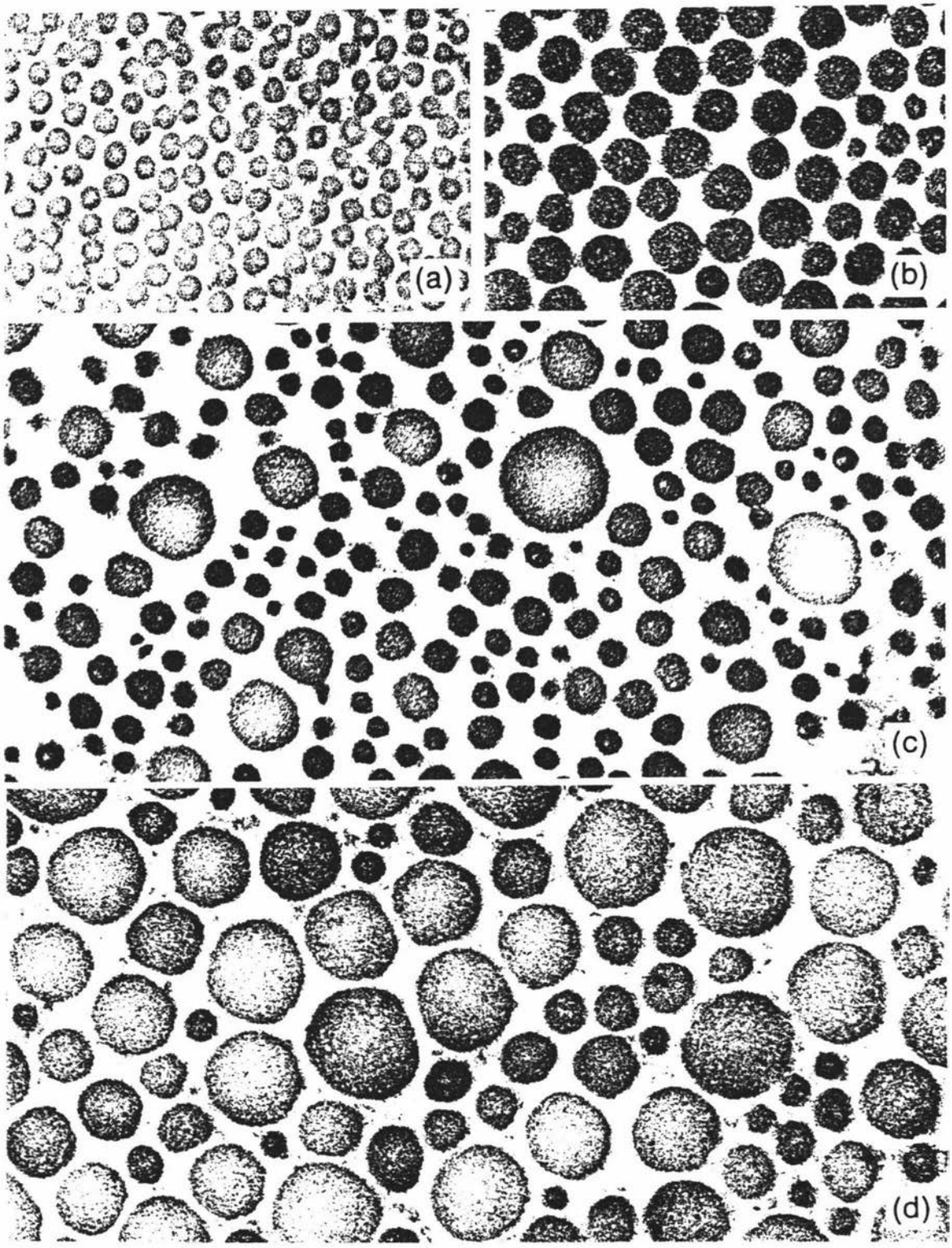

Figure 7.1 Electron micrographs of transverse sections of collagen fibrils from the skins of (a) lamprey, (b) rat, (c) and (d) trout. The two micrographs taken from the trout skin are from the stratum spongiosum (c) and stratum compactum (d). Magnification 70 000 X.

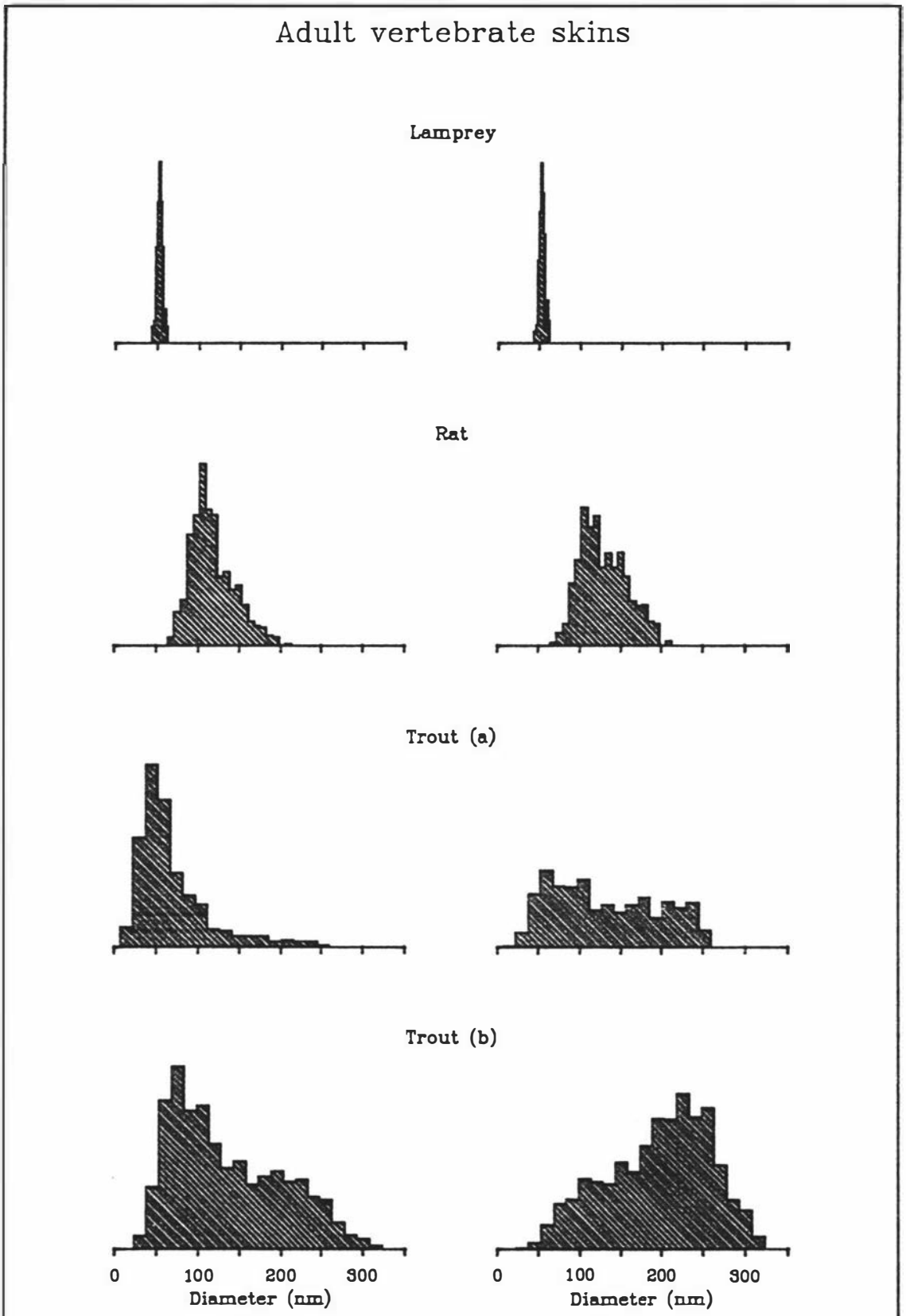

Figure 7.2 **Frequency distributions (left) and mass distributions (right) of collagen fibril diameters in skins of lamprey, rat and trout as shown in the electron micrographs in Figure 7.1.**

However two exceptions have been found in this work - the skin of the bony fish (trout; Figures 7.1c, 7.1d and 7.2) and the skin from the tail of the rat. It has been shown from studies of the skin of the shark (Wainwright et al., 1978) that the myotome-derived musculature of the body is as firmly attached to the skin as it is to the underlying skeletal system, and that this is of considerable importance in the locomotory movements of the fish. The fish skin thus acts as an "exotendon", storing and transmitting muscular force and displacement to the tail whilst the fish is actively swimming. On the basis of the hypothesis proposed the presence of a bimodal or right-skewed distribution of collagen fibril diameters in trout skin indicates that the skin effectively functions in a tendinous manner, in addition to its normal function as a boundary between the animal and its environment. It is therefore proposed that rat-tail skin, with its bimodal population of collagen fibril diameters at maturity, is also likely to perform some exotendinous function and could be likened to the skin of the fish. Indeed this idea is not unreasonable since the rat uses its highly flexible prehensile tail for balance during a variety of locomotory movements.

Lamprey skin, although displaying a unimodal population of collagen fibril diameters typical of most vertebrate skins, has features which appear unique. Both the mean diameter and the standard deviation of the distributions for lamprey skin (51.9 $\pm$ 3.4 nm) are significantly less than those recorded for other skins studied. Further the collagen fibrils are organized into lamellae (Figure 7.3) which resemble those found in the stroma of the cornea. It is interesting to speculate that this "constancy" of fibril diameter and lamellar organization could indicate a possible photoreceptive role

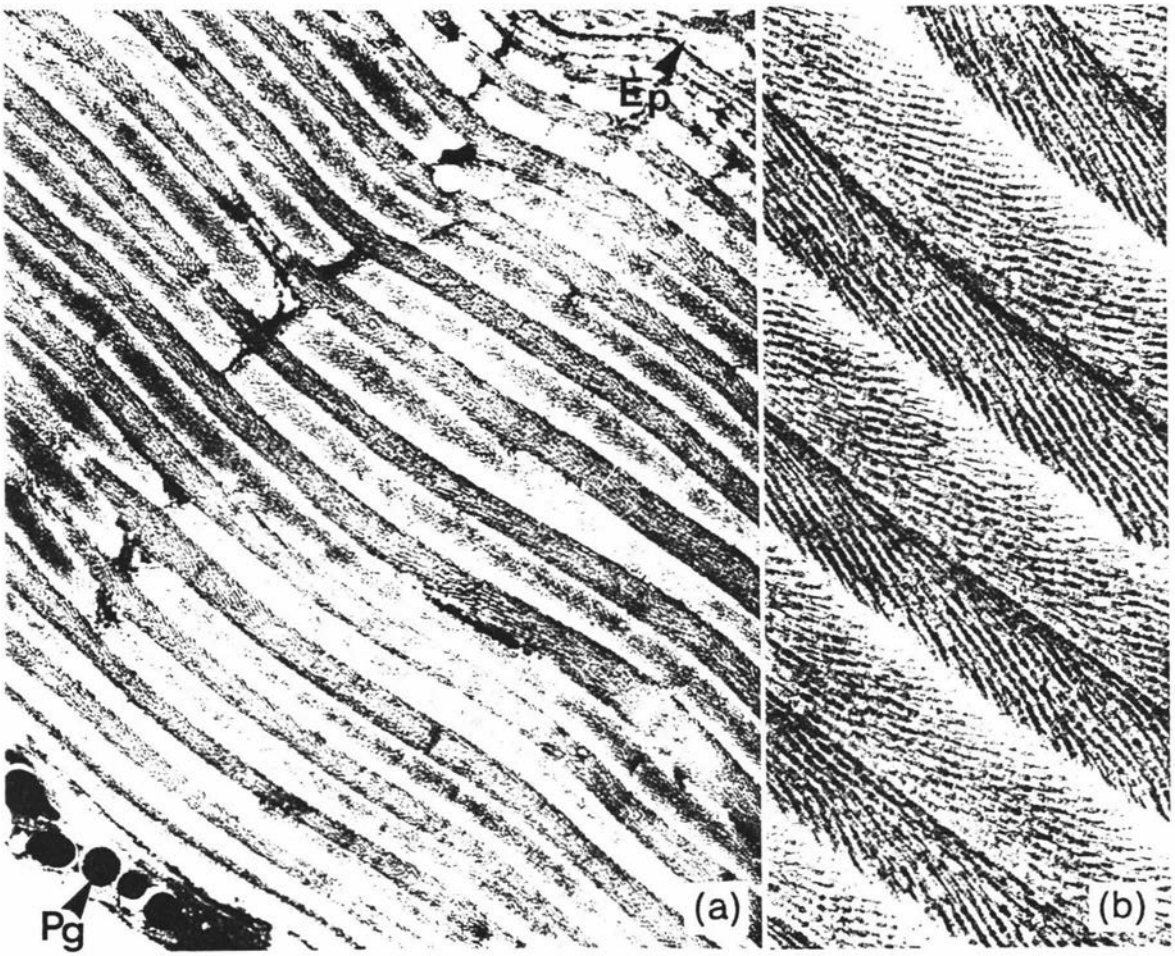

Figure 7.3 Electron micrographs showing the lamellar arrangement of the dermal collagen fibrils in the Ammocoete stage of the developing lamprey.
(a) Micrograph shows collagen lamellae traversing the extent of the dermis from the epithelium (Ep) to the underlying subdermal cells which are rich in pigmented inclusions (Pg). Magnification: 8 000 X.
(b) Details of arrangement of fibrils in adjacent lamellae showing the quasi-orthogonal arrays. Magnification: 28 000 X.

for lamprey skin.

7.2.5 Form of the Collagen Fibril Diameter Distribution at Senescence

"In all tissues other than cornea and cartilage, the mean and mass-average diameters of the collagen fibrils at late maturity are smaller than at the onset of maturity."

In this work only two non-corneal tissues were examined from very old animals, namely the skins from a 70-year-old man and a 2-year-old rat (see Table 4.10). In these cases the mass-average diameters of the constituent collagen fibrils decreased from 132 nm at maturity to 97 nm at senescence in the rat and from 95 nm at maturity to 79 nm at senescence in man. The limited data presented here thus provide further support for the postulate.

7.2.6 Correlation Between Mass-average Diameter and Ultimate Tensile Strength

"The ultimate tensile strengths of tendon, skin and cartilage are positively correlated with the mass-average diameters of the collagen fibrils."

Reliable mechanical data for connective tissues are difficult to obtain and the data which have been collected are thought to seriously underestimate, possibly as much as ten-fold, the *in vivo* functional strengths of connective tissues (Harkness, 1979; Cusack and Miller, 1979). Trends clearly show, however, that the tensile strengths of connective tissues increase manyfold between birth and maturity, but tend to decrease a little at senescence. Such trends have been

observed experimentally by Vogel (1974, 1978, 1979) who has measured the tensile strength of skin, tendon, bone and aortic wall from the rat. That such a trend of changing tensile strength with age parallels the mass-average diameter of the collagen fibrils in the tissues can be seen in Figure 7.4a, where tensile strengths (Vogel, 1974) and mass-average diameters of rat skin (data from this thesis, see Table 4.10) are plotted as a function of age, and in Figure 7.4b where tensile strengths (Vogel, 1978) and mass-average diameters of rat tail-tendon (Table 8.2) are similarly plotted. Such data clearly support the concept that increasing collagen fibril diameter gives rise to increasing tensile strengths of tissues.

7.2.7 Form of the Diameter Distribution and the Mechanical Properties of the Tissue

"The mechanical properties of a connective tissue are strongly correlated with the collagen fibril diameter distribution."

This postulate was based on the idea that the collagen fibril diameter distribution may be a function of two opposing factors. For example, if a tissue required a high tensile strength then the constituent collagen fibrils needed to be large in order to maximize the density of intrafibrillar covalent crosslinks (Parry *et al.*, 1978a, 1980). Alternatively, if it is necessary for a connective tissue to return to its original shape when *in vivo* stresses are removed then the collagen fibril network must have sufficient interfibrillar crosslinks to inhibit non-recoverable creep. It was not considered, however, that these crosslinks need be covalent. Electrostatic interactions between the collagen fibrils and the glycosaminoglycans or between collagen fibrils and the "ruthenium-red

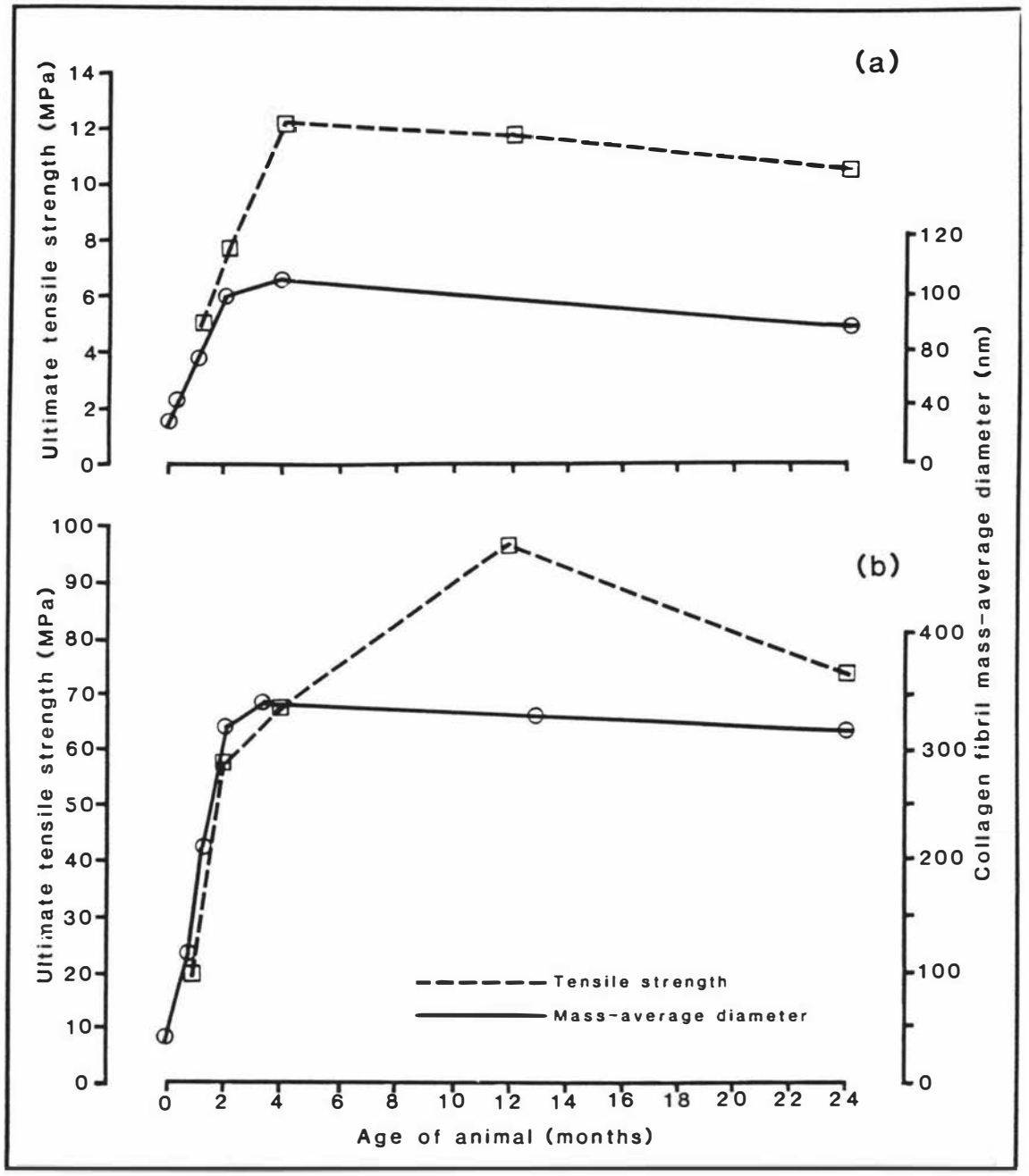

Figure 7.4 Graphs of (a) tensile strength and mass-average collagen fibril diameter versus age for rat skin, and (b) tensile strength and mass-average collagen fibril diameter versus age for rat-tail tendon.

staining glycoproteins (Myers et al., 1969, 1971; Myers, 1976, 1980) were thought to be sufficient to provide the required creep-resistant properties (Parry et al., 1978a, 1980). Such interactions were potentially more numerous if the collagen fibrils were small since their surface area per unit mass was increased. This can be appreciated from a consideration of a single collagen fibril of diameter d_1 being divided into a number (n) of small collagen fibrils each of diameter d_2. As the total volume of the collagen must stay constant it follows that $n\,\pi(d_2/2)^2 = \pi(d_1/2)^2$ and hence that $d_2 = d_1/(n)^{0.5}$. The total surface area per unit length of the small collagen fibrils is therefore $n\pi d_2 = (n)^{0.5}\,\pi\, d_1$. As πd_1 is the surface area of the original fibril the surface area per unit length of the n small diameter collagen fibrils would be $(n)^{0.5}$ times greater than for a single fibril of the same volume. Consequently the interface between fibrils and matrix is greatly enhanced if the connective tissue has a high proportion of small fibrils.

It had been suggested earlier (Parry et al., 1980) that the tensile strength of a large diameter collagen fibril is greater than that of one of smaller diameter. In that analysis it was assumed that the collagen fibrils were constructed from an array of microfibrils and that each microfibril could make a fixed number of covalent linkages with its neighbours. The number of such interactions would therefore be maximal when the microfibrils were located within an infinite array. However in a finite array, as would occur in collagen fibrils, the microfibrils comprising the peripheral layer of the fibril were unable to make the full number of lateral covalent crosslinks. The percentage of potential interactions would therefore increase from 0% for a fibril of size 4 nm x 4 nm to a value close to

100% for fibrils in excess of 100 nm diameter. However, the concept of a microfibril now no longer seems tenable, since recent X-ray diffraction data (Hulmes and Miller, 1979; Fraser et al., 1983) has suggested that collagen molecules are packed on a triclinic lattice in a quasi-hexagonal manner. The analysis presented earlier (Parry et al., 1980) is equally applicable, however, to a model for the collagen fibril in which the individual molecules are packed on such a lattice. Thus the idea remains that tensile strength is related to fibril diameter and is a consequence of the increasingly significant depletion of crosslinks around the periphery of the fibril as its diameter decreases.

The maximum mass-average diameter of the collagen fibrils in the connective tissues studied in this work, together with other published data, are listed in Table 7.4. This table clearly shows that the tissues having the greatest tensile requirements (tendons and ligaments) have the largest diameter collagen fibrils (mass-average diameters ~150 - 340 nm). Conversely, those tissues with the smallest tensile loadings (for example paratenon and vitreous humour) contain only small diameter collagen fibrils (mass-average diameters ~10 - 50 nm). Intermediate tensile loadings for cartilage, "passive skins" and "active skins" also parallel the the maximum mass-average diameters of collagen fibrils for these tissues (20 - 80 nm, 50 - 130 nm and 130 - 200 nm respectively).

TABLE 7.4

MAXIMUM MASS-AVERAGE DIAMETER OF COLLAGEN FIBRILS IN ADULT CONNECTIVE TISSUES

Tissue	Mass-average diam. (nm)	
TENDONS AND LIGAMENTS		
Rat tail-tendon	340	(1)
Chick metatarsal tendon	240	
Horse common digital extensor tendon	240	(2)
Guinea pig digital extensor tendon	224	
Rat Achilles tendon	214	
Dog Achilles tendon	213	
Rabbit Flexor digitorum profundus	204	(3)
Rat digital flexor tendon	204	
Ox Achilles tendon	198	
Horse suspensory ligament	197	(2)
Horse superficial flexor tendon	187	(2)
Rabbit tibial collateral ligament	173	(4)
Dog superficial digital flexor tendon	152	
Guinea Pig digital flexor tendon	122	
SKINS (a: ACTIVE)		
Trout skin	130-195	*
Rat tail-skin	155	
SKINS (b: PASSIVE)		
Rat ventral abdominal skin	132	
Guinea pig dorsal abdominal skin	120	
Human skin	96	
Chick skin	94	
Guinea pig ventral abdominal skin	93	
Greyhound skin	92	(5)
Sheep skin	88	
Guinea pig foot-pad skin	73	
Lamprey skin	52	
CARTILAGE		
Human hyaline cartilage	66	(6)
Rat fibrocartilage	57	(4)
Rat ear elastic cartilage	23	(7)
Rat femoral articular cartilage	83	(7)
Rat tracheal hyaline cartilage	32	(7)
Rat knee-joint meniscus cartilage	71	(7)
OTHER TISSUE		
Rat tail-tendon paratenon	40-50	(8)
Rat endoneurium	48	(4)
Most vertebrate corneas	25	
Bony fish cornea	17	
Vitreous humour	10	

* Values for two different specimens of trout skin

(1) Parry and Craig 1978; (2) Parry et al., 1978b; (3) Ippolito et al., 1980; (4) Parry et al., 1980; (5) Cahill et al., 1980; (6) Dahmen, 1973; (7) Zambrano et al., 1982; (8) Parry et al., 1978a.

CHAPTER EIGHT

THE RELATIONSHIP BETWEEN GLYCOSAMINOGLYCAN COMPOSITION AND COLLAGEN FIBRIL DIAMETERS: A POSSIBLE MECHANISM FOR FIBRILLOGENESIS

8.1 Introduction

The mechanism of collagen fibrillogenesis is still obscure. It is known, however, that intracellular synthesis produces collagen molecules that possess both amino- and carboxy-propeptides and that these are removed by specific proteases some time after the release of the procollagens into the matrix (Fessler and Fessler, 1978; Miyahara et al., 1982). Although immunofluorescent staining studies (Nowack et al., 1976, Timpl et al., 1977) have shown that the amino-propeptides are present in the matrix it was considered that these were no longer attached to the collagen molecules when fibrillogenesis commenced. Indeed, low levels or activities of amino-propeptidase in skin, with the resulting retention of the terminal peptide, leads to the malformation of collagen fibrils in the disease known as dermatosparaxis (see Section 5.2.1). More recently, however, it has been shown that the amino-propeptide probably remains attached to the collagen molecule in the early stages of fibrillogenesis (Fleischmajer et al., 1981, 1983) but the timing of the carboxypeptide cleavage is still uncertain. It is believed, however, to precede that of the aminopeptide cleavage (Miyahara et al., 1982).

That collagen fibrils grow to characteristic diameters in different connective tissues has been discussed at length in Chapters 3 - 6. The ranges of fibril diameter and forms of fibril diameter distributions have been described for a large number of tissues; this chapter will concern itself with the possible mechanisms of extracellular fibrillogenesis, and the possible mechanisms of control which must be manifest in order to maintain such stringent spectra of collagen fibril diameters. The main part of this discussion, however, will concern itself with a possible relationship between collagen fibril diameter distribution and the glycosaminoglycan composition from a diversity of connective tissues. Further a fibril diameter regulatory mechanism afforded by the glycosaminoglycans is postulated. The data presented in the following section arise from the original electron microscopy conducted for this thesis and the results of extensive glycosaminoglycan analyses undertaken by M.H. Flint and G.C. Gillard, at the University of Auckland Medical School, as part of a collaborative research programme.

Technological considerations have limited this analysis to the glycosaminoglycan composition and not to the true functional unit, the proteoglycan. Proteoglycans are the molecular assemblies which consist of sulphated glycosaminoglycans covalently linked to protein cores. In some specialized connective tissues, such as cartilage and aorta, proteoglycans bind periodically to hyaluronic acid molecules, thus generating massive assemblies (~1 - 5 μm) which determine many of the mechanical attributes of the tissue as well as its transport properties (Mathews and Lozaityte, 1958; Rosenberg *et al.*, 1970; Wellauer *et al.*, 1972). Evidence has also been presented by Fessler

and Fessler (1978) that the collagen-proteoglycan interactions are stronger than those between the collagen and the individual GAGs and this may be the reason for this additional level of aggregation. However there is no reason to believe that the relative importance of any particular GAG, whether part of a proteoglycan or a more massive assembly involving hyaluronic acid, will be significantly altered by the degree of aggregation involved. It is a reasonable premise, therefore, to postulate a model for fibrillogenesis based on GAG rather than on proteoglycan data, and it is on this assumption that the hypothesis discussed in Section 8.2.3 is formulated.

8.2. Do Glycosaminoglycans Mediate Control?

8.2.1. Previous Concepts

The earliest observations on glycosaminoglycan-collagen interactions were those of Meyer and Smyth (1937) and Meyer et al., (1937) who showed that chondroitin sulphate formed insoluble complexes with gelatin. They attributed the complex formation to the interaction of the basic amino groups of the gelatin with the sulphate and carboxyl groups of the chondroitin sulphate. This observation has since been confirmed using a variety of methods (see, for example, Mathews, 1965), and it is now considered that the reaction is primarily that of electrostatic binding between the ester sulphate groups on the glycosaminoglycans and lysyl and arginyl residues of the collagen molecule (Podrazky et al., 1971). These arginyl and lysyl residues in collagen, which account for only about 8% of the total amino acid content of the molecule, are known to be grouped at sites which have a degree of spatial regularity along the length of the

molecule (Doyle et al., 1974b, Meek et al., 1979). The distribution of the charged residues produce about 60 stained bands in segment long spacing (SLS) collagen (Bruns and Gross, 1973), whereas in fibrillar collagen, where the molecules are in a modified "quarter-staggered" array, 12 intraperiod bands have been visualised in positively stained tissues. These bands have been designated a1 - e2 (i.e. a1, a2, a3, a4, b1, b2, c1, c2, c3, d, e1 and e2) by Hodge and Schmitt (1960) after the earlier notation of a five band a - e system by Schmitt and Gross (1948). Subsequent work has shown that the positively-staining banding patterns for both SLS and native collagen can be quantitatively accounted for by the specific staining of the acidic and basic residues of the collagen molecules (Bruns and Gross, 1974; Doyle et al., 1974b; Tzaphlidou et al., 1982a, 1982b).

Electron microscope studies have attempted to determine the most likely proteoglycan binding sites on the D-periodic collagen fibrils. Smith et al., (1967, 1969), using bismuth nitrate staining on bovine nasal cartilage and cornea, claim that proteoglycan bridges between collagen fibrils terminate at a point between the a and b bands; Nakao and Bashey (1972) using Ruthenium-red staining on rabbit heart valve claimed a and d bands to be the sites of attachment; Doyle et al., (1975) interpreted the micrographs of Torp et al., (1975) as showing ruthenium-red positive material associated with the a and d bands in rat tail tendon, whilst Myers (1976), using ruthenium red staining of synovium and ear cartilage favoured the a bands as the sites of attachment. Thus histochemical localization of glycosaminoglycans (strictly proteoglycans) in collagenous tissues in vivo would appear to favour the a band as the prime contender for the

site of major electrostatic interaction between collagen and proteoglycan.

More recent work however (Scott and Orford, 1981), using rat tail tendon stained with a cationic phthalocyanin dye, "Cupromeronic Blue", has shown the proteoglycan to be distributed about the collagen fibrils in an orthogonal array, the transverse elements of which are located almost exclusively at the d band in the gap region of the fibril. This does not agree with the observations of Doyle et al., (1975) and others who claim that the c bands and the "a-region" of the fibril, being the most cationic, would be the most likely regions for the highly anionic sulphated glycosaminoglycans to attach. Intra-molecular and inter-molecular interactions, however, are thought to account for the neutralization via salt linkages of most of the charged moieties in the collagen molecule and the nett charge at any position in the fibril is probably very small. Further, Scott and Orford (1981) claim that their Cupromeronic Blue staining, based on the "critical electrolyte concentration" method of Scott (Scott, 1973, 1980; Scott et al., 1981) is conducted at near-neutral pH, is more specific, and by virtue of its much better contrast than that of earlier methods is able to localize the glycosaminoglycans "unambiguously" with respect to the collagen banding pattern. Whatever the outcome of this debate, it is clear from electron microscope evidence that glycosaminoglycans interact with collagen fibrils in vivo. It is not clear, however, what role the collagen-GAG interaction has in determining the mechanical attributes of a connective tissue. That such interactions may have some regulatory control over the extracellular assembly of collagen fibrils has been

suggested by a variety of *in vitro* experiments. Although the ability to form fibrils is an inherent property of monomeric collagen (Gross *et al.*, 1955b) it was suggested by Gross (1956) that glycosaminoglycans might affect the organization of collagen fibrils. Kinetic studies of collagen fibrillogenesis *in vitro* (Gross and Kirk, 1958; Bensusan and Hoyt, 1958; Wood and Keech, 1960) have shown that there is a "lag" or "nucleation" phase (during which there is no increase in the turbidity of the collagen solutions) followed by a "growth" phase (with concomitant rapid increase in turbidity). It is considered that these nucleation and growth phases reflect the linear and lateral accretion of collagen molecules respectively during fibril growth (Silver *et al.*, 1979; Silver and Trelstad, 1979; Silver, 1981, 1983). Both of these phases are thought to be influenced by the presence of proteoglycans or glycosaminoglycans; the latter may accelerate or retard fibrillogenesis and hence affect ultimate fibril diameter, depending on which species of glycosaminoglycan dominates (Wood 1960, Keech 1961, Obrink 1973). It has been postulated that the duration of the lag (nucleation) phase would directly affect the number-density of collagen fibrils formed; large numbers of small diameter collagen fibrils would result if the nucleation phase was long, and small numbers of fibrils (which ultimately grow to large diameters) would form when the nucleation phase was short (Toole and Lowther, 1968a, 1968b; Toole, 1969; Oegema *et al.*, 1975).

8.2.2. Observations

The mass-average diameters of the collagen fibrils and the GAG contents of skins (Table 8.1) and tendons (Table 8.2) are listed for a variety of animals at different stages of development. The electron microscope results clearly show trends of fibril growth during early development and the tendency for diminishing diameters at senescence; these data have been independently discussed in Chapter 6. The GAG compositions provided were often taken at a larger selection of ages than those sampled for electron microscopy. These are all included in the table as, due to the experimental difficulty of measuring GAG composition, the trends are often more obscure than are those of changing collagen fibril diameter and the extra values allow the existing data to be more critically assessed.

The quantity of material required for GAG analyses makes such determinations difficult for foetal tissues of laboratory animals, but it is generally considered that hyaluronic acid is the predominant GAG in these tissues (Pessac and Defendi 1972, Mathews 1975). Hyaluronic acid also predominates in Wharton's jelly of the umbilical cord (Fessler, 1960), in paratenon (Reid and Flint, 1974), synovial fluid (Comper and Laurent, 1978) and the vitreous humour (Gross et al., 1955a; Comper and Laurent, 1978; Swann et al., 1981) in adult animals. These highly hydrated connective tissues all have small diameter collagen fibrils; ~8 - 10nm in vitreous humour and synovial fluid and ~40 - 50 nm in Wharton's jelly and paratenon (see preceding references). The high values of hyaluronic acid recorded at the earliest stages of tissue development e.g. ~70 - 80% in embryonic hog skin (Loewi and Meyer, 1958) have been substantiated in this work with

TABLE 8.1

GLYCOSAMINOGLYCAN CONTENT AND MASS-AVERAGE COLLAGEN FIBRIL DIAMETER IN SKIN AS A FUNCTION OF AGE

ANIMAL Tissue	Age	Mass-av. diam.(nm)	Total GAG (% of dry wt.)	% of total GAGS * DS	CS	HA
RAT						
Dorsal skin	1 d		0.297	19.5	20	60.5
	~2 d	30.9	0.330	17.5	17	65.5
	5 d		0.681	9	31.5	59.5
	8 d		0.408	18.5	36	45.5
	15 d		0.474	16.5	33	50.5
	4 w	63.4	0.493	26.5	36.5	37
	10 w		0.353	46	26	28
	3 mo	128.9	0.426	43	26.5	30.5
	3.5 mo		0.353	48	20	32
	4 mo		0.400	39	30	31
	5 mo	153.4				
	~12 mo		0.233	46	32	22
Ventral skin	5 d	38.7	0.678	17	34.5	48.5
	8 d		0.534	16	35	49
	15 d		0.577	23	33.5	43.5
	~4 w	101.3	0.447	21	38	41
	10 w		0.437	24	31.5	44.5
	3 mo	112.0	0.397	27	33	40
	3.5 mo		0.359	33	17	50
	4 mo		0.427	29	24	47
Tail skin	~3 d	32.2	0.633	14	42	44
	8 d		0.514	17	43	40
	15 d		0.490	36.5	38	25.5
	1 mo	102.5	0.621	33	32	35
	2 mo		0.427	42	36	22
	10 w		0.427	43.5	34	22.5
	~3.5 mo	110.5	0.389	63.5	14	22.5
	5 mo	153.4				
	1 y	154.8		63		
GUINEA PIG						
Dorsal skin	~4 d	60.3	0.294	37	32	31
	1 w		0.286	28	45	27
	2 w		0.358	32	33	35
	3 w		0.250	38	39	33
	3 mo	113.7		36.5	26	37.5
	6 mo		0.270	41	26	33
	18 mo		0.220	48	26	26

Continued next page.

Table 8.1 continued.

GUINEA PIG						
Ventral skin	~2 d	81.4	0.481	16	43	41
	1 w	91.9	0.274	35	29	36
	~2 w	81.7				
	2 mo	92.6	0.350	31	28	41
	18 mo	74.5	0.252	31.5	27	41.5
Footpad skin	~2 d	73.1	0.465	27	34	29
	1 w		0.364	28	36	36
	3 w		0.265	35	30	35
	2 mo	62.0				
	6 mo		0.349	38	19	43
	18 mo	62.9	0.340	40.5	19	40.5
HUMAN						
Abdominal skin	14 w F	26.7	0.843	21	14	65
	24 w F	58.6				
	0-6 mo	70.1	0.698	56	10	34
	5 y			43	8	49
	~20 y	95.4	0.350	48	3	49
	70 y	79.3		46	8	46

* DS = Dermatan sulphate; CS = Chondroitin sulphate; HA = Hyaluronic acid

The GAG analyses reported here were all performed by Drs. M.H.Flint and G.C.Gillard (Auckland Medical School).

TABLE 8.2

GLYCOSAMINOGLYCAN CONTENT AND MASS-AVERAGE COLLAGEN FIBRIL DIAMETER IN TENDON AS A FUNCTION OF AGE

ANIMAL Tissue *	Age	Mass-av. diam.(nm)	Total GAG % of dry wt.	% of total GAGS ** DS	 CS	 HA
RAT						
Tail tendon	1 d	49.0	2.38	25	41	34
	5 d		1.44	29.5	37	33.5
	8 d		1.25	32	37	31
	15 d	115.0	0.94	34	37	29
	4 w		0.50	45	25.5	29.5
	5 w	210.0				
	2 mo	320.0	0.31	70	7	23
	3 mo	340.0		75	3	22
	~12 mo	333.0	0.26	82	3	15
RABBIT						
Achilles	Adult	203.5	0.153	74.5	4	21.5
S-region FDP	Adult	150.0	~2.8	20	60	20
WALLABY						
Achilles	Adult		0.166	71.5	0	28.5
OX						
Achilles	Adult	197.9	0.413	82	10	8
DOG						
Achilles	Adult	219.1	0.426	68	18	14
Flexor FDS	Adult	151.8	0.350	61	26	13
Sesamoid FDS	Adult	48.4	1.994	18	75	7
CHICKEN						
Flexor CMT	~9 d F			(1) 7.5	25	67.5
	11 d F	33.6				
	~13 d F	37.4		(1) 12	31	57
	18 d F	55.0	0.696	37.5	49	13.5
	6 w		0.747	45	50	5
	12 mo	239.5	0.558	60	33	7
HUMAN						
Tibialis post.	5 y		0.570	77	6	17

* FDP, Flexor digitorum profundus; FDS, Flexor digitorum sublimis; CMT, Chicken metatarsal tendon.

** DS = Dermatan sulphate; CS = Chondroitin sulphate; HA = Hyaluronic acid

The GAG analyses reported here were all performed by Drs. M.H.Flint and G.C.Gillard (Auckland Medical School), with the exception of those marked (1) which are data from Reid (1974).

recorded hyaluronic acid levels of ~65% in human skin, rat dorsal skin and chicken tendon (see Tables 8.1 and 8.2).

The skins studied were from human (body skin), guinea pig (foot pad skin and dorsal and ventral aspects of body skin) and rat (tail skin and dorsal and ventral aspects of body skin). It is known that GAG composition alters with depth in the dermis and that differential functional loading across the body of an animal results in local variations in the fibril diameter distribution. It has been shown that in human and pig (Flint, 1971) and in calf (Tajima and Nagai, 1980) that the uppermost (papillary) layer of the dermis has smaller diameter collagen fibrils, a higher GAG content and a greater ratio of hyaluronic acid to dermatan sulphate than occurs deeper within the dermis. Establishing a correlation between collagen fibril diameter and GAG composition in skin is thus inherently difficult but despite this some general trends are apparent. At the age at which the mass-average diameter of the collagen fibrils in skin is maximal it can be seen that the level of hyaluronic acid is ~40% (range 30 - 45%) with similar levels of dermatan sulphate ~40% (range 30 - 45%). The mass-average diameters of the collagen fibrils in these tissues all lie in the range of 60 - 135 nm. There exists, however, one notable exception - that of rat tail skin. Here the mass-average diameter of the collagen fibrils (155 nm) tends towards those of tensile (tendinous) tissues and the dermatan sulphate level has reached 65%, a value much greater than that recorded for any of the other skins. Further, the hyaluronic acid content has dropped to ~20% and it would appear that the tissue has passed through an intermediate chondroitin sulphate rich stage early in post-natal development.

In the advancing stages of Dupuytren's contracture (a pathological condition of the human palmar subdermis and fascia) the mass-average diameter of the collagen fibrils is much smaller than that of the normal palmar fascia (~40 - 50 nm in Dupuytren's; ~140 nm in control) and both dermatan sulphate (~60%) and chondroitin sulphate (~30%) levels have become quite high. This parallels the conditions reported for hypertrophic scarring (Kischer and Shetlar, 1974) in which the majority of collagen fibrils are of small diameters and are associated with greatly increased levels of chondroitin sulphate and decreased levels of hyaluronic acid relative to the surrounding normal tissue.

Mature rat tail tendon contains the largest collagen fibrils (mass-average diameter ~340 nm) yet recorded for any connective tissue. Here the hyaluronic acid content is ~35% during the latter stages of development but drops off to ~15% in the mature animal. The chondroitin sulphate content reaches ~40% perinatally and drops to ~5% at maturity, while the dermatan sulphate content increases from ~25 - ~80% over the same time interval. The development of chick metatarsal tendon parallels that of rat-tail tendon. In the earliest stages studied (9 - 13 day foetal), the hyaluronic acid content was extremely high (~57 - 67%) but dropped rapidly with increasing age and reached a level as low as 5 - 7% at maturity. In contrast the chondroitin sulphate level, which was initially ~25 - 30% rose to ~50% near birth then dropped to ~30 - 35% at maturity. Dermatan sulphate on the other hand was about 10% in the youngest tissues studied before peaking at about 60% at maturity. Thus hyaluronic acid, chondroitin sulphate and dermatan sulphate reached their maximal levels at

sequential stages of development. Chick tendon at maturity contained the second largest mass-average diameter fibrils (~240 nm) of any tissue yet studied. In addition, mature dog, rabbit and ox tendoachilles all have high dermatan sulphate contents (~68, 74 and 82% respectively) and collagen mass-average diameters of ~200 nm (range 198 - 213 nm).

As described in Chapter 5 the development of rat skin after wounding has been studied by electron microscopy and biochemical analysis. The GAG analyses revealed that peak levels of hyaluronic acid and chondroitin sulphate were reached at four weeks in the longitudinal wound and at about seven weeks in the transverse wound, whilst the proportion of dermatan sulphate peaked at about ten weeks in both wounds, (M.H. Flint, personal communication). The collagen fibril diameter distributions contained a small percentage of larger diameter fibrils - a feature not observed in normal skin.

A graphical representation of the trends in changing levels of the individual glycosaminoglycans as a function of the mass-average diameters of collagen fibrils is shown in Figure 8.1. All data are plotted and the histograms have been constructed as the means of such data grouped in collagen fibril mass-average diameter intervals of 30 nm. Figure 8.1 illustrates the trends that; (1) hyaluronic acid levels are high in those tissues whose collagen fibrils are small, but fall to ~20% in those more mature tissues which have collagen fibril mass-average diameters >150 nm; (2) dermatan sulphate levels are low (~15%) in tissues having small diameter collagen fibrils, but rise to a value ~80% in those mature tissues which have collagen fibril mass-average diameters >150 nm; (3) chondroitin sulphate levels are

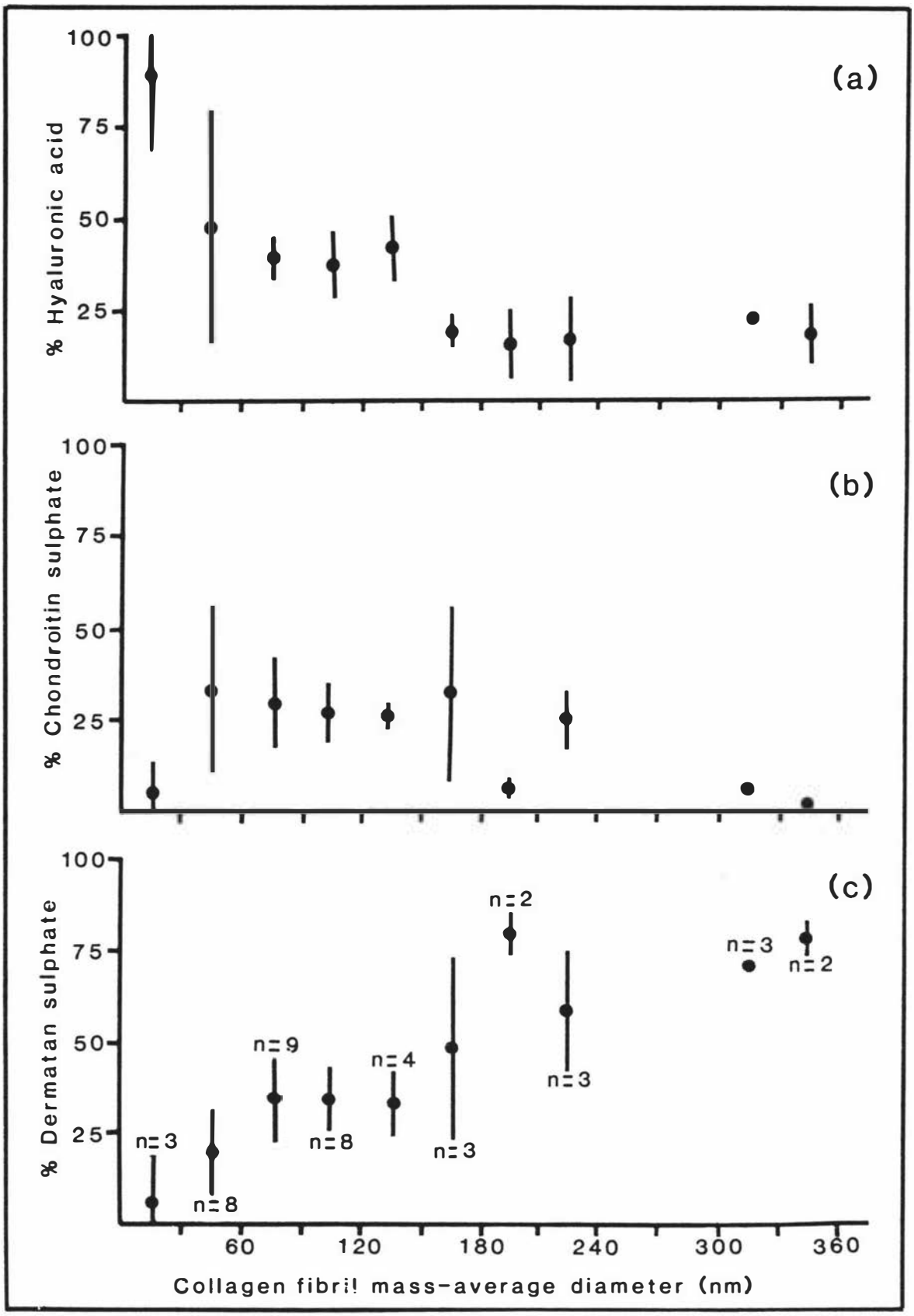

Figure 8.1 Graphs of tissue percentage contents of hyaluronic acid, chondroitin sulphate and dermatan sulphate versus collagen fibril mass-average diameter. Vertical heights of bars represent $\pm$ 1 standard deviation; n = number of tissues investigated.

rather disparate but are ~25 - 30% when the mass-average diameters of the collagen fibrils lie in the range of 30 - 180 nm and ~10% when the mass-average diameters reach the sizes observed in mature tendinous tissues. These results, taken with other data reported in the literature, form the basis for the following hypothesis.

8.2.3 Hypothesis

The data recorded have not revealed any simple (linear) relationship between the GAG composition of a tissue and the mass-average diameter of its constituent collagen fibrils. However, when the individual GAGs are expressed as a percentage of the total GAGs present it can be seen that those tissues which contain the smallest diameter fibrils usually have the highest hyaluronic acid levels. Tissues containing fibrils of intermediate size (mass-average diameter ~60 - 150 nm) frequently have an elevated percentage content of chondroitin sulphate, whereas those tissues with the largest diameter fibrils exhibit the highest percentage content of dermatan sulphate that have been experimentally observed. This hypothesis has been developed in conjunction with Drs. M.H.Flint and G.C.Gillard (see Parry *et al.*, 1982).

It is well known that hyaluronic acid is the predominant GAG synthesised by foetal connective tissue cells both *in vivo* and *in vitro* (Pessac and Defendi, 1972; Mathews, 1975) and in connective tissue regeneration and remodelling following wounding (Bentley, 1969; Toole and Gross, 1971; Mathews, 1975). Consequently it is believed that hyaluronic acid may facilitate the migration of cells to sites of connective tissue development or repair (Flint, 1972; Toole, 1976; Merrilees and Scott, 1980). As these sites also represent the domains of collagen synthesis, it is possible that hyaluronic acid, as a

consequence of its very large excluded volume (i.e. its water-inclusion properties), could also facilitate the movement and distribution of newly synthesised collagen molecules and fibrils. Thus in the primary stage of fibril growth this hypothesis would postulate that the transverse or circumferential growth of collagen fibrils is limited by the hyaluronic acid-rich matrix such that only small fibrils less than about 60 nm in diameter are formed. However, while hyaluronic acid may inhibit lateral growth of the fibrils beyond a diameter of about 60 nm, the hypothesis would suggest that there is no comparable inhibition of longitudinal growth during this stage of fibrillogenesis (Silver and Trelstad, 1979). Indeed, such growth would be highly favoured. Thus the tissue may lengthen and grow without the inherent rigidity imposed by the presence of thicker fibrils. This mechanism does not imply that hyaluronic acid has a direct role as a nucleating agent; it has already been shown that hyaluronic acid does not bind to collagen under physiological conditions (Obrink, 1973; Greenwald et al., 1975; Comper and Laurent, 1978; Lindahl and Hook, 1978) and that neither hyaluronic acid nor the other GAGs are essential for initiating the formation of collagen fibrils in vitro.

Between birth and maturation, the hyaluronic acid content of many connective tissues generally decreases quite rapidly whereas the chondroitin sulphate and dermatan sulphate contents increase from the low levels normally present during early foetal development. During this second stage of fibril development, the hypothesis postulates that the inhibition of the lateral growth of fibrils imposed by the hyaluronic acid is removed by the proportionate increase of

chondroitin sulphate (Scott et al., 1981) and/or dermatan sulphate synthesised preferentially by the cells in response to their changing mechanical or micro-electrical environment (Flint et al., 1980; Flint, 1981). In contrast to hyaluronic acid, chondroitin sulphate makes weak ionic interactions with collagen under conditions encountered in vivo. This hypothesis considers that this allows the development of larger diameter collagen fibrils. However it is also postulated that chondroitin sulphate itself will have an inhibitory effect on fibril growth beyond a diameter of about 150 nm.

Other tissues subjected to high tensile stresses, such as tendons and some skins, experience a third stage of fibril development. The mass-average diameters of the collagen fibrils increase to values which are typically ~200 nm and the dermatan sulphate concentrations increase significantly to about 60 - 80%. Since dermatan sulphate is known to make strong ionic interactions with collagen under physiological conditions, the hypothesis speculates that the increasing proportion of dermatan sulphate will remove the inhibition on fibril growth imposed at the earlier stages of development and hence allow the collagen fibrils to grow laterally (Silver and Trelstad, 1979) to those sizes required for their increased tensile loading (Parry et al., 1978a) (mass-average diameter >150 nm and typically ~170 - 240 nm). It should be noted that although the dermatan sulphate and chondroitin sulphate molecules have equal charges per unit length, the charges on the dermatan sulphate seem more accessible to interactions with collagen. It has been speculated (Obrink, 1973), that this "charge-availability" may be due to the presence of L-iduronic acid - a hexuronic acid present in dermatan

sulphate but not in chondroitin sulphate.

The scheme of fibrillogenesis as postulated is strictly sequential; the tissues containing the largest diameter collagen fibrils must pass through a hyaluronic acid rich stage, a chondroitin sulphate and/or dermatan sulphate rich stage but ultimately a dermatan sulphate rich stage. It should be mentioned, however, that although tissues with collagen fibrils of mass-average diameter ~200 nm can be predicted to have a high dermatan sulphate content, the converse cannot necessarily be assumed true; a dominance of dermatan sulphate does not favour the presence of large diameter collagen fibrils unless the hyaluronic acid rich and the chondroitin sulphate and/or dermatan sulphate rich stages have occurred previously and in that order. The three stages of development as postulated have clearly occurred sequentially in rat tail tendon and in chicken flexor tendon, and the fibrils in these tissues have indeed grown to the largest diameters yet observed.

The third stage of development is never reached by body skin and the collagen fibrils in this tissue grow only to intermediate sizes (~60 - 140 nm). However in mature rat tail skin, where dermatan sulphate levels reach ~65% and the hyaluronate content drops to ~20%, the chondroitin sulphate peaks in early post-natal development. The increased fibril growth for this skin (mass-average diameter ~155 nm) beyond that observed for normal body skin (mass-average diameter ~90 nm) provides further support for the hypothesis as postulated. The perivascular and perifollicular zones of the dermis, which both have high hyaluronic acid contents, contain only small diameter collagen fibrils. These observations are again in agreement with the

hypothesis.

The small diameter collagen fibrils (~40 - 50 nm) found in Dupuytren's contracture, like another human pathological condition - hypertrophic scarring (Kischer and Shetlar, 1974) - are associated with high levels of both chondroitin and dermatan sulphate. In both cases the majority of the collagen fibrils have small diameters and are associated with greatly increased proportions of chondroitin sulphate but decreased levels of hyaluronic acid. It can therefore be speculated that the large decrease in the hyaluronate level from ~40% to ~10% may have occurred at the onset of the disorder and that the omission of a hyaluronate rich stage has hindered the normal processes of tissue repair. This in turn would appear to have prevented the formation of collagen fibrils with diameters greater than ~60 nm.

Evidence supporting the hypothesis includes the observations on paratenon, vitreous humour, umbilical cord and synovial fluid. Each of these tissues contains a high concentration of hyaluronic acid (typically > 60%) and only small diameter fibrils (mass-average diameters in the range 10 to 50 nm). Bone and cartilage, which contain high concentrations of chondroitin sulphate at maturity, have collagen fibrils of intermediate size (~40 to 80 nm). Additional support for the hypothesis comes from the work of Gillard et al., (1979) and Flint et al., (1980). They showed that the rabbit flexor digitorum profundus tendon (which curves around the back of the ankle) is chemically and structurally distinct on the concave and convex sides. On the concave (pressure) side of the tendon the GAG content is about 2.3 - 3.5%, of which 60% is chondroitin sulphate and the collagen fibrils have mass-average diameters of ~150 nm. On the

convex (tensional) side of the tendon, the GAG content is much lower (~0.2%), contains about 70% dermatan sulphate, and has fibrils with a mass-average diameter of ~200 nm (Merrilees and Flint, 1980). Furthermore, it has been shown that if the tendon is moved away from the bone so that it no longer turns through an angle of about 90°, it becomes subject only to tensional forces, and this results in major biochemical and morphological changes (Gillard et al., 1977, 1979; Flint et al., 1980). Specifically, the pressure-bearing region of the tendon, which normally contains small diameter collagen fibrils and high levels of chondroitin sulphate, is gradually replaced by normal tension-transmitting tendon with closely packed large diameter collagen fibrils associated with small amounts of GAG, of which a major fraction is now dermatan sulphate. This process can be reversed by relocating the tendon about the bone at an appropriate time after initial translocation. Thus a reversible and concomitant change in fibril diameter and GAG composition accompanies any change in the physical environment of the cells. These results provide further support for the concept that glycosaminoglycans have an important role in fibril growth and remodelling.

Cornea, a highly specialized connective tissue, appears to be anomalous with regard to the hypothesis as postulated since both corneal keratan sulphate and chondroitin sulphate are synthesised in high proportions after the initial hyaluronic acid rich stage of development. Throughout post-natal development the cornea maintains uniform and small diameter collagen fibrils (~17 or 25 nm; Craig and Parry, 1981a), in spite of high proportions of chondroitin sulphate present. This feature may be related to the unique properties of

corneal keratan sulphate, a GAG which appears to restrict fibril growth absolutely. Corneal keratan sulphate is similar to hyaluronic acid in that neither interact with collagen under conditions found *in vivo* (Obrink, 1973; Greenwald *et al.*, 1975; Lindahl and Hook, 1978). It is also interesting that corneal scars, in which corneal keratan sulphate is largely replaced by dermatan sulphate, contains larger diameter fibrils than are found in normal cornea (Anseth, 1965; Mathews, 1975).

8.3 Conclusions

The hypothesis relating fibril size distribution to GAG composition poses almost as many questions as it answers. For instance what importance should be attached to the previously reported results that fibrillogenesis and fibril growth are related (i) to the extent of co-polymerization of various collagen types (Henkel and Glanville, 1982; Hendrix *et al.*, 1982); (ii) to the amount of the amino-terminal propeptides retained in a growing fibril (Fleischmajer *et al.*, 1981, 1983); (iii) to the interactions between collagen and fibronectin (Kleinman *et al.*, 1981); (iv) to the degree of glycosylation of the collagen molecule (Mathews, 1975); (v) to parameters such as pH, temperature and ionic strength of the medium in which the collagen fibril is assembled (Wood and Keech, 1960; Cassel, 1966; Fessler and Tandberg, 1975; Williams *et al.*, 1978; Bornstein and Traub, 1979) and (vi) to the procollagen aggregates, intermediate aggregates and intracellular subassemblies (Trelstad *et al.*, 1976; Trelstad and Hayashi, 1979; Bruns *et al.*, 1979; Hulmes *et al.*, 1983; Gross and Bruns, 1984)? Also why should the relative amount of the

different glycosaminoglycans present be more important than the absolute amount in the tissue? Further, how can bimodal collagen fibril diameter distributions (present in some mature tendons) be explained in terms of a specific GAG composition?

With regard to the first point it would now seem apparent that fibrillogenesis is highly complex and is likely to involve more than a single factor such as glycosaminoglycan composition (see, for example, Furthmayr and Madri, 1982; Gross and Bruns, 1984). It must thus be emphasized that the results presented here are neither all-inclusive nor mutually-exclusive of other ideas and that this new hypothesis for the mechanism of fibrillogenesis does not necessarily detract from alternative hypotheses elaborated elsewhere. It can be stated, however, that the trends established for fibril diameter distribution and for GAG composition in a wide variety of connective tissues at different ages are based on a substantial body of evidence and that any theory must take these results into consideration.

The second point is harder to explain. No clear trends in the absolute contents of any particular GAG as a function of fibril size have been detected. It could be argued that the hypothesis would have appeared more plausible if it had been proposed that fibril diameter was limited by the absolute amounts of the particular GAGs present rather than by the percentages of GAGs in the tissue. Any relationship between absolute GAG content and collagen fibril diameter could have been envisaged in terms of a stoichiometric binding (for example electrostatic interaction) between the surface of a collagen fibril and the GAGs in the surrounding matrix. This possibility, although studied extensively, failed to reveal any consistent or

significant trends in all of the tissues studied and thus was not considered as a viable alternative to the scheme proposed. The implication of the hypothesis, therefore, is that it is the ratio rather than the amount of the different GAGs which determines the fibril size distribution. This could be accomplished through competitive interactions between collagen and the different GAGs. The hypothesis thus describes a feedback mechanism valid even for the oldest tissues, where the total GAG levels are often extremely low compared to those found in foetal or immature tissues. It therefore remains a matter of speculation as to how interactions between collagen and the different GAGs can be truly competitive, or indeed how such interactions are able to affect the growth and development of the collagen fibrils at varying stages of development. An alternative hypothesis has been suggested by Scott et al., (1981) in which they suggest that the amount of chondroitin sulphate in rat-tail tendon may limit the diameters of the constituent collagen fibrils, and they speculate that a similar mechanism may be appropriate for all connective tissues. However, the results presented in this thesis do not support the broader outlines of their hypothesis although the data obtained for several individual tissues are indeed consistent with their interpretation of the rat-tail tendon data.

The third point, which concerns the occurrence of bimodal populations of collagen fibril diameters, is one that is not explicable in terms of our current state of knowledge. It could be speculated that there is local variation in the composition of GAGs - i.e. their distribution is not homogeneous. Such variations do indeed occur, as for example in the rabbit flexor digitorum profundus tendon (which turns through an angle of about 90°), and in skins. In both cases the GAG content and composition have been shown to vary with location in the tissue (Flint, 1971; Tajima and Nagai, 1980;

Flint et al., 1980) and it is therefore conceivable that local variations could occur in all tissues. It would seem unlikely, however, that such variations would be sufficiently localized to account for bimodal fibril diameter distributions that are constant in form throughout the tendon.

This apparent anomaly in the framework of the hypothesis indicates that there is at least one factor, other than GAG composition, which has some control over the ultimate sizes to which collagen fibrils may grow. It could be speculated that the co-polymerization of genetically distinct molecular species of collagen is the remaining factor. Broad bimodal populations of collagen fibrils all arise from distributions which are sharp and unimodal in the foetal and immature tissues. This means that within the matrix many fibrils have grown to large diameters whilst others have not developed to the same extent. Thus it would appear that something within or on the fibril, rather than within the matrix, has had a regulatory effect on the growth of some of the fibrils. Factors previously discussed, such as the degree of glycosylation, the amount of amino-terminal propeptide retention or molecular species co-polymerization, could be candidates for this regulatory role and there is some evidence that it may be the latter (co-polymerization). It has already been discussed at length (Chapter 5) that high levels of Type III collagen are found in Dupuytren's disease and in horse tendons previously treated with bacterial collagenase, that both of these tissues contain small diameter fibrils (~50 nm) and that such fibrils have a very small range of diameters. It is possible that these fibrils are composed of the polymerization product of Types I and III collagens. Further, the uniform diameter collagen fibrils observed in cornea (Chapter 3) may be composed of the polymerization product of Types I and II collagens, and that the GAG-mediated control

outlined in the hypothesis is only effective on collagen fibrils composed of a single molecular species (specifically Type I fibrils).

Type III collagen is known to exist in significant proportions in many foetal and immature tissues and to fall to low levels with increasing age. In principle, Type III collagen molecules may form fibrils solely of that molecular species (Type III fibrils) or, alternatively, may contribute to the formation of Type I/III (polymerization product) fibrils. Further, as two molecular species of collagen co-existing within a single fibril have no need to be in equal amounts, small levels of one species (for instance Type III collagen molecules) could account for a large number of Type I/III fibrils in a predominantly Type I collagen-containing tissue. It is possible that fibrils containing Type III collagen are unable to grow to large diameters but that subsequently synthesized Type I fibrils may grow to increasingly large diameters as the levels of chondroitin sulphate and dermatan sulphate rise sequentially. This could give rise to the markedly bimodal distributions of collagen fibril diameters observed.

CHAPTER NINE

SUMMARY

This thesis has primarily been concerned with the collection and interpretation of electron microscope data obtained from transverse sections of a range of connective tissues studied over various stages of tissue development. In some cases these data, which were necessarily obtained from dehydrated tissues, have been compared with those obtained from X-ray measurements on the identical tissue prior to dehydration. These results, collected in collaboration with workers in the USA, have revealed that lateral shrinkage of the tissue at both the fibril and matrix levels can be severe and hence provide a major source of artefact in work of this type. This, in turn, can lead to misinterpretation of electron microscope images unless care is taken.

A detailed study of one highly specialized class of connective tissues - the corneas - has revealed the value of comparative studies carried out by a single research worker. Previous results reported in the literature had failed to reveal any relationship between the fibril diameters in the corneas of animals from different species. Also, conflicting results on fibril diameter as a function of depth below the anterior surface of the cornea had been reported but no follow-up work had been undertaken to assess the relative merits of these works or indeed their general relevance. In addition, no studies had been undertaken on the size of the fibrils in the cornea as a function of maturity. All of these aspects were specifically studied in this thesis using, wherever possible, standardized techniques of electron microscope preparation and measurement

procedures. The results obtained clearly showed that there was no significant difference in fibril diameters in five classes of vertebrates - mammals, birds, reptiles, amphibians and cartilaginous fish. However the corneas in bony fish were shown to have collagen fibrils with diameters which were significantly smaller than those found for all the other animals studied. In addition to these results the collagen fibril diameters were studied, for a selection of six animals, as a function of position across the width of the cornea. Once again no significant differences in fibril diameter were observed except for a small number of fibrils present in the layer in closest proximity to Descemet's membrane. Corneas from foetal animals other than the bony fish revealed that the collagen fibril diameters were close to those found in the mature bony fish suggesting that corneal collagen fibrils do indeed increase in size with age up until birth. However, during active life the collagen fibrils in the corneas of all animals do not change in diameter. Whilst the absolute values of fibril diameter measured in this work are likely to be underestimates of the true (hydrated) values, the comparative values remain valid. Thus significant classifications have been revealed for cornea as a result of a consistent set of experimental protocols being maintained by a single research worker.

The extensive data collected on the distribution of collagen fibril diameters in connective tissues has revealed new details on the pattern of fibrillar growth and development. In the most immature tissues studied (i.e. those from early foetal or embryonic animals) the collagen fibrils had very sharp distributions of diameters with mean values lying close to one of a set of "quantized" diameters. Such diameters differed from one another by a multiple of about

7.3 nm. Occasionally the mean diameter measured in one fibre was different to that measured in an adjacent fibre though the feature of "quantization" was maintained. With increasing age the unimodal distribution of fibril sizes measured were often resolvable into two or more distinct populations with mean values quantized in the manner previously discussed. At birth in altricial animals and in early postnatal development in precocial animals the unimodal collagen fibril diameter distributions broadened considerably and in those cases the diameter distributions could only occasionally be resolved into discrete populations. During tissue maturation the distributions continued to broaden appreciably. In short-term low-stress tissues the diameter distributions remained unimodal in form but in the long-term high-stress tissues (such as some tendons) the distribution became bimodal or right-skewed. Beyond maturity the few tissues studied contained fewer larger diameter collagen fibrils than previously present at maturity. In addition the mean fibril size decreased significantly. These patterns of fibril growth, established for a range of connective tissues, provide the first quantitative data upon which theories of fibrillogenesis and fibril development may be proposed.

In addition the fibril size distributions have been related to the mechanical attributes of those tissues over that part of the age range accessible to study. These latter data have been reported previously by others but are now shown to parallel the mass-average diameters of the collagen fibrils reported in this work for tissues as wide ranging as skin and tendon. The theory enunciated earlier in collaboration with D.A.D.Parry and G.R.G.Barnes, which attempted to relate the presence of small diameter fibrils to creep-resistant

properties and the larger diameter fibrils to tensile attributes, has been further substantiated by the results reported here. A number of normal tissues exhibiting unusual collagen fibril diameter distributions or dysplastic tissues exhibiting collagen fibril diameters altered from the norm have also been investigated and, where possible, related to the mechanical attributes of that tissue.

In a further collaborative study with workers in Auckland (M.H.Flint, G.C.Gillard and H.C.Reilly) it has been shown that a correlation exists between the mass-average diameter of collagen fibrils in connective tissues and the relative amounts of the different glycosaminoglycans (GAGs) present. The results have lead to the hypothesis that the GAG levels influence the size of the collagen fibrils in the tissue. Thus it has been suggested that hyaluronic acid limits the lateral (but not longitudinal) growth of collagen fibrils in the early stage of tissue development. As the tissue matures the chondroitin sulphate levels increase (relative to the hyaluronic acid) and the inhibition on fibril growth may be removed thus allowing the fibrils to grow to intermediate diameters (up to ~150 nm). It is then suggested that the chondroitin sulphate concentration does not allow fibril growth beyond this intermediate diameter unless the dermatan sulphate concentration increases to a high level. If this occurs the fibrils may grow to the large diameters (~200 nm) normally found in high tensile tissues such as tendon. This hypothesis suggests that a specific size fibril only remains a stable entity provided that appropriate GAG levels are maintained. Fibril growth and breakdown may therefore be controlled by the synthesis of the different GAGs by the cells present. It is possible that if the mechanical properties of a tissue are altered the

cells may experience changes in the physical forces and stresses imposed upon them (with concomitant changes in their microelectrical environment), that this will induce different GAGs to be produced and finally that this will allow the fibrils to grow or breakdown as the case may be. The more rapid turnover rate of the GAGs relative to collagen suggests that the changes in GAG composition precede changes in collagen fibril size. Though this hypothesis does not disprove any of the theories previously put forward to explain fibrillogenesis and fibril development, it does provide new data that is likely to be important in any scheme that is finally accepted.

Overall this thesis has been devoted to the low resolution structure of connective tissue, an area which has surprisingly been neglected by most workers in the field of connective tissue research. Much more work is still required but the outlines of some of the factors involved in collagen fibril growth and development and in specifying the mechanical properties of a tissue are now becoming a little better understood. A more complete understanding of connective tissue structure and function may lead to medical applications such as in the area of wound healing. As Ross (1980) has stated "Collagen is the principal connective tissue component of the healing wound, and it will be necessary to further pursue the nature of the factors responsible for stimulating collagen formation by, and proliferation of, fibroblasts. These and other studies could provide a basis for the manipulation of the process of wound repair in terms of rapidity, the extent of scar formation, and the prevention of faulty healing." It is hoped that studies of the type undertaken in this thesis will help aspirations such as these to be realized in the forseeable future.

APPENDIX 1

GENETICALLY DISTINCT COLLAGEN TYPES
AND THEIR DISTRIBUTION IN THE BODY TISSUES *

Collagen type	Subunits	Chain composition	Tissue distribution
I	α1(I) and α2(I)	$[\alpha 1(I)]_2\ \alpha 2(I)$	Bone, dentine, tendon, skin, vessel walls, cornea, placenta, connective tissue proper.
Trimer I	α1(I)	$[\alpha 1(I)]_3$	Bone, dentine, skin.
II	α1(II)	$[\alpha 1(II)]_3$	Hyaline, elastic and fibrous cartilages, intervertebral disc, vitreous humour, notochord, embryonic corneal epithelium, neural retina.
III	α1(III)	$[\alpha 1(III)]_3$	Skin, vessel walls, connective tissue proper (reticular fibres, endomysium, endoneurium, loose areolar connective tissue, paratenon, etc.), placenta.
IV	α1(IV) α2(IV)	$[\alpha 1(IV)]_2\ \alpha 2(IV)$ or $[\alpha 1(IV)]_3$ and $[\alpha 2(IV)]_3$	Basement membranes; (capillaries, and lens capsule, glomerular basement membrane, Descemet's membrane, parietal endoderm).
V	α1(V) and α2(V)	$[\alpha 1(V)]_2\ \alpha 2(V)$ and $[\alpha 1(V)]_3$	Amnion, chorion, placenta, skin,tendon, cornea, bone, aorta,muscle, connective tissue proper.

* Table modified from von der Mark, 1981.

Note 1: Many new incompletely characterized collagens are continually being described. These include endothelial collagen, intestinal epithelial collagen, cartilage 1α, 2α collagen, cartilage disulphide-linked m-collagen, and vessel wall/placenta 40-50K short chain collagen (Chung et al., 1976; Burgeson and Hollister, 1979; Sage et al., 1980; Quaroni and Trelstad, 1980; Shimokomaki et al., 1980; Furoto and Miller, 1980, 1981; Ayad et al., 1981; Jander et al., 1981).

Note 2: There is increasing evidence that when two or more collagen types occur in the same tissue they may exist as co-polymers (see for example, Henkel and Glanville, 1982; Hendrix et al., 1982).

APPENDIX 2

DIMENSIONS OF THE PARAMETERS OF PROPOSED COLLAGEN SUB-FIBRILLAR ASSEMBLIES

Model (1)	Dimensions of unit cell (2) Diameter (nm)	a* (nm^{-1})	b* (nm^{-1})	γ^*	References
2-stranded MF	~1.8	1/5.5	1/5.5	90	Woodhead-Galloway et al., 1975.
4-stranded MF	~3.8	--	--	--	Veis and Yuan, 1975.
5-stranded MF	~3.8	--	--	--	Smith, 1968; Miller and Wray, 1971.
	~3.8	1/7.69	1/7.69	90	Miller and Parry, 1973; Fraser et al., 1974.
	~3.8	1/7.55	1/7.55	90	Fraser et al., 1979.
8-stranded MF	~3.5	1/3.84	1/3.50	90	Hosemann et al., 1974.
Liquid crystal	~1.5	--	--	--	Hukins and Woodhead-Galloway, 1977.
Quasi-hexagonal	~1.5	1/3.82	1/2.63	75.3	Hulmes and Miller, 1979; Miller and Tocchetti, 1981; Fraser and MacRae, 1981; Fraser et al., 1983.
5-stranded compressed MF	--	1/3.78	1/2.44	66.0	Trus and Piez, 1980; Piez and Trus, 1981.

(1) MF represents "microfibril".

(2) Where the model is not a microfibrillar one the diameter of the collagen molecule is quoted (~1.5 nm); a* and b* are the unit cell dimensions in reciprocal space and γ^* is the angle between these reciprocal space parameters. These data have not always been quoted in the literature cited but, rather, have been calculated from real space coordinates to complete this table.

APPENDIX 3

SOURCE OF CORNEAS FOR COMPARATIVE STUDY AND STATE OF PRESERVATION PRIOR TO PREPARATION FOR ELECTRON MICROSCOPY.

(a) Fresh Corneas:
Dogfish, elephant fish, stingray, butterfish, moki, trout, goldfish, chick, magpie, thrush, rat, rabbit, opossum, guinea pig, sheep, ox (1).

(b) Formalin Fixed Corneas:
Lamprey (2); salamander, toad, frog, turtle, snake (3); kiwi, wallaby, wallaroo, sea-lion, blackbuck, hippopotamus, diana monkey, capuchin monkey, squirrel monkey (4); foetal human (5).

(c) Frozen Corneas:
Tuatara, pigeon (3); kiwi (6).

(d) From Storage in "Cornea-bank":
Adult human (7).

(1) All animals whose corneas were taken in a fresh state were collected by the author from a variety of sources, including small animal breeding houses at Massey University and the DSIR, Foxton Fisheries, Feilding Freezing Works, fishermen and friends.

(2) Dr. P.R.Todd, Fisheries Research Division, Christchurch.

(3) Drs. R.A.Fordham and I.A.N.Stringer; Zoology Dept., Massey University.

(4) Messrs G.W.Meadows, C.F.Smith, R.Gates and M.Sibley; curator and veterinarians, Auckland Zoo.

(5) Dr. R.W.Darby, pathologist, Palmerston North Hospital.

(6) Mr. B.Reid, Wildlife Division., Dept. of Internal Affairs, Wellington.

(7) Dr. M.J.Merrilees, Medical School, Auckland.

APPENDIX 4

SUMMARY OF PROCESSED MATERIAL SUPPLIED BY OTHER RESEARCH WORKERS

Animal	Tissue (Source)	Condition
Sea cucumber	Cuvierian tubules (1)	Normal tubules
Lamprey	Notochord sheath (2)	Normal adult
	Skin (2)	Normal adult
Chick	Metatarsal tendons (2)	Foetal (11, 12, 13, 14, 17 & 18 days incubation).
Rat	Skin (3)	Scars from wounds transverse or longitudinal to Langer's lines.
Lamb	Skin (2)	2 day Dermatosparactic (and control).
	Tendon (2)	2 day Dermatosparactic (and control).
Horse	Flexor Tendons (4)	24 h, 1, 4, & 8 w, 10, 11, & 14 mo after intratendinous injection with bacterial collagenase (and controls).
Human	Umbilical cord (3)	Normal neonate.
	Palmar fascia (3)	Normal fascia, Dupuytren's contracture and Dupuytren's nodule.

(1). Dr. A.J.Bailey, Meat Research Institute, Bristol, England.

(2). Drs. B.Brodsky and E.F.Eikenberry, Rutgers Medical School, Piscataway, New Jersey, U.S.A.

(3). Drs. M.H.Flint and A.C.Poole, Dept. of Surgery, Medical School, Auckland, New Zealand.

(4). Dr. I.F.Williams, Dept. of Pathology, School of Veterinary Sciences, University of Bristol, England. (see also Appendix 5)

All tissues other than the sea cucumber Cuvierian tubules were supplied after glutaraldehyde and osmium tetroxide fixation and subsequent epoxy resin embedding in Epon 812. The Cuvierian tubule specimen was freeze-dried.

APPENDIX 5

BACTERIAL COLLAGENASE TREATED FLEXOR TENDONS FROM HORSE. EXPERIMENTAL PROTOCOL, CLINICAL AND POST-MORTEM OBSERVATIONS. *

Animals: Experimental ponies were housed in small groups in indoor pens or at grass.

Superficial Digital Flexor Tendon Injury: Acetyl promazine (Acepromazine C-Vet Ltd) 0.1 mg/kg, was administered as a tranquilizer by slow intravenous injection and provided some degree of analgesia. Regional analgesia of the forelimb distal to the carpus was obtained by blocking the lateral and medial palmar digital nerves just below the carpus, with xylocaine solution (Xylotox-Willows Francis) in each case. The hair was clipped over the flexor tendons in the mid-carpal region and the skin prepared with alcoholic chlorhexidine gluconate solution (Hibitane ICI). Injections of 0.5 ml (10 mg/ml) collagenase were made at 3 sites 1 cm apart into the centre of the superficial digital flexor tendon (SDFT) in the mid-carpal region.

Experimental Design: Fourteen animals were used in the study. Of these one was killed at 24 hours, one at 1 week, one at 1 month and one at 2 months after injury. Four animals were killed at 3 months, four at 6 months and two at 14 months after injury.

Clinical Assessment During Recovery from Injury: Within 6 - 8 h of intratendinous enzyme injection animals showed signs of clinical lameness and the inflammatory reaction continued to develop during the subsequent 18 h. The affected SDFT was expanded by inflammatory fluid and haemorrhage to between 2 - 3 times its previous size, and had a characteristic bowed appearance similar to that of naturally occuring sprains in the horse. Within a few days the heat and inflammatory swelling of the injured leg had partially subsided but the tendon remained enlarged, soft and malleable. Over the next 2 - 3 weeks the soft tissue in the injured areas was replaced by firmer scar. There was no obvious regression in the size of the scarred tendon up to 2 months after injury, but lameness was minimal at that time and had disappeared by 3 months.

Post Mortem Examination: At the appropriate time after injury the horses were mechanically stunned and exsanguinated. The limbs were disarticulated at the carpus and the tendons severed at the joint. Specimens of tendon and peritendinous tissue were taken from standard sites in relation to the injury and from the same sites in the control leg. Examination of the injured SDFT 24 h after injection revealed severe damage, extensive haemorrhage and exudation, although the deep flexor tendon remained unaffected. Most of the injured tissue and thrombus had been replaced by granulation tissue 1 week after injury and after a further 3 weeks haemorrhage and exudation had been completely organized or removed. New granulation tissue within the tendon had resulted in considerable enlargement of its cross-sectional area both 1 and 2 months after injury. The peritendinous tissues were adherent to the tendon and the appearance of the lesion was not distinguishable from that of a natural traumatic injury in a clinical case of severe tendon sprain. The morphology of tendons taken from experimental ponies 3 months after injury was very similar to that at 2 months, although the cross-sectional area of the scarred region of the SDFT was not as grossly enlarged. Thickening of the SDFT never regressed to less than twice the cross-sectional area of the contralateral (control) tendon during the 14 month period of healing under examination.

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* This appendix has been taken directly from Williams,I.F., McCullagh,K.G. and Silver,I.A. 1984. The distribution of Types I and III collagen and fibronectin in the healing equine tendon. Connect. Tiss. Res. (in press).

REFERENCES

Agar,A.W. 1974. The basic principles of the electron microscope. In: Practical methods in electron microscopy, Vol. 2: Principles and practice of electron microscope operation. (Eds: Agar,A.W., Alderson,R.H. and Chescoe,D.), North-Holland Publishing Co., Amsterdam, pp 1-37.

Allain,J.C., Le Lous,M., Bazin,S., Bailey,A.J. and Delaunay,A. 1978. Isometric tension developed during heating of collagenous tissues. Relationships with collagen cross-linking. Biochim. Biophys. Acta, 533: 147-155.

Anderson,H.C. and Sajdera,S.W. 1971. The fine structure of bovine nasal cartilage. Extraction as a technique to study proteoglycans and collagen in cartilage matrix. J. Cell Biol. 49: 650-663.

Anseth,A. 1961. Glycosaminoglycans of the developing corneal stroma. Exp. Eye Res. 1: 116-121.

Anseth,A. 1965. In: Structure and function of connective and skeletal tissue. (Eds: Jackson,S.F., Harkness,R.D., Partridge,S.M. and Tristram,G.R.) Butterworths, London, pp 506-507.

Arborgh,B., Bell,B.P., Brunk,U. and Collins,V.P. 1976. The osmotic effect of glutaraldehyde during fixation: A transmission electron microscopy, scanning electron microscopy and cytochemical study. J. Ultrastruct. Res. 56: 339-350.

Ayad,S., Abedin,M.Z., Grundy,S.M. and Weiss,J.B. 1981. Isolation and characterization of an unusual collagen from hyaline cartilage and intervertebral disc. FEBS Letters, 123: 195-199.

Bailey,A.J. 1975. Collagen: General introduction. In: Structure

of fibrous biopolymers. Colston papers No. 26. (Eds: Atkins,E.D.T. and Keller,A.) Butterworths, London, pp 115-124.

Bailey,A.J. and Etherington,D.J. 1980. Metabolism of collagen and elastin. In: Comprehensive Biochemistry, 19b (Eds: Florkin,M. and Stotz,E.H.) Elsevier North-Holland, New York, pp 299-460.

Bailey,A.J. and Lapiere,C.M. 1973. Eur. J. Biochem. Effect on additional peptide extension of the N-terminus of collagen from dermatosparactic calves on the cross-linkages of collagen fibres.

Bailey,A.J., Sims,T.J., Gabbiani,G., Bazin,S. and Le Lous,M. 1977. Collagen of Dupuytren's disease. Clin. Sci. Mol. Med. 53: 449-502.

Bailey,A.J., Light,N.D. and Atkins,E.D.T. 1980. Chemical cross-linking restrictions on models for the molecular organization of the collagen fibre. Nature (Lond.) 288: 408-410.

Bailey,A.J., Gathercole,L.J., Dlugosz,J., Keller,A. and Voyle,C.A. 1982. Proposed resolution of the paradox of extensive crosslinking and low tensile strength of Cuvierian tubules from the sea cucumber Holothuria forskali. Int. J. Biol. Macromol. 4: 329-334.

Barber,A.N. 1955. In: Embryology of the human eye. C.V.Mosby Co., St. Louis.

Barnes,G.R.G. and Pinder,D.N. 1974. In vivo tendon tension and bone strain measurement and correlation. J. Biomechanics, 7: 35-42.

Bazin,S., Le Lous,M., Duance,V.C., Sims,T.J., Bailey,A.J., Gabbiani,G., D'Andiran,G., Pizzolato,G., Browski,A., Nicoletis,C. and Delaunay,A. 1980. Biochemistry and histology of the connective tissue of Dupuytren's disease lesions. Eur. J. Clin. Invest. 10: 9-16.

Bear,R.S. 1942. Long X-ray diffraction spacings of collagen. J. Am. Chem. Soc. 64: 727-728.

Bear,R.S. 1944. X-ray diffraction studies on protein fiber. I. The large fiber-axis period of collagen. J. Amer. Chem. Soc. 66: 1297-1305.

Beer,M. and Zobel,C.R. 1961. Electron stains II. Electron microscopic studies on the visibility of stained DNA molecules. J. Mol. Biol. 3: 717-726.

Belton,J.C., Crise,N., McLaughlin,R.F. and Tueller,E.E. 1977. Ultrastructural alterations in collagen associated with microscopic foci of human emphysema. Human Pathol. 8: 669-677.

Benedek,G.B. 1971. Theory of transparency of the eye. Appl. Optics, 10: 459-473.

Bensusan,H.B. and Hoyt,B.L. 1958. The effect of various parameters on the rate of formation of fibers from collagen solution. J. Am. Chem. Soc. 80: 719-724.

Bentley,J.P. 1969. In: Repair and regeneration. (Eds: Dunphy,J.E. and van Winkle,W.) McGraw Hill, New York, pp 151-160.

Bentley,J.P. 1980. In: Wound healing and wound infection: Theory and surgical practice. (Ed: Hunt,T.K.), Appleton Century Crofts, New York.

Black,C.M., Gathercole,L.J., Bailey,A.J. and Beighton,P. 1980. The Ehlers-Danlos syndrome: An analysis of the structure of the collagen fibres of the skin. Br. J. Dermatol. 102: 85-96.

Borcherding,M.S., Blacik,L.J., Sittig,R.A., Bizzell,J.W., Breen,M. and Weinstein,H.G. 1975. Proteoglycans and collagen fibre organization in human corneoscleral tissue. Exp. Eye Res. 21: 59-70.

Bornstein,P. and Sage,H. 1980. Structurally distinct collagen

types. Ann. Rev. Biochem. 49: 957-1003.

Bornstein,P. and Traub,W. 1979. The chemistry and biology of collagen. In: The Proteins, Vol. 4. (Eds: Neurath,H. and Hill,R.L.) Academic Press, New York, pp 411-632.

Boyde,A. and Franc,F. 1981. Freeze-drying shrinkage of glutaraldehyde fixed liver. J. Microscopy, 122: 75-86.

Bradley,D.E. 1954. Evaporated carbon films for use in electron microscopy. Brit. J. Appl. Physics, 5: 65-66.

Breen,M., Johnson,R.L., Sittig,R.A., Weinstein,H.G. and Veis,A. 1972. The acidic glycosaminoglycans in human fetal development and adult life: Cornea, sclera and skin. Connect. Tiss. Res. 1: 291-303.

Brodsky,B. and Eikenberry,E.F. 1982. Characterization of fibrous forms of collagen. In: Methods in Enzymology, Vol. 82. "Structural and contractile proteins" (Eds: Cunningham,L.W. and Frederiksen,D.W.) Academic Press, New York, pp 127-174.

Bruns,R.R. and Gross,J. 1973. Band pattern of the segment-long-spacing form of collagen. Its use in the analysis of primary structure. Biochemistry, 12: 808-815.

Bruns,R.R. and Gross,J. 1974. High-resolution analysis of the modified quarter-stagger model of the collagen fibril. Biopolymers, 13: 931-941.

Bruns,R.R., Hulmes,D.J.S., Therrien,S.F. and Gross,J. 1979. Procollagen segment-long-spacing crystallites: Their role in collagen fibrillogenesis. Proc. Natl. Acad. Sci. USA, 76: 313-317.

Burgeson,R.E. and Hollister,D.W. 1979. Collagen heterogeneity in human cartilage: Identification of several new collagen chains. Biochem. Biophys. Res. Commun. 87: 1124-1131.

Cahill,J.I., Jones,B.R., Barnes,G.R.G. and Craig,A.S. 1980. A collagen dysplasia in a greyhound bitch. N. Z. Vet. J. 28: 203-204 and 213.

Cassel,J.M. 1966. Collagen aggregation phenomena. Biopolymers, 4: 989-997.

Cassidy,K., Eikenberry,E.F., Olsen,B. and Brodsky,B. 1980. X-ray diffraction investigations of collagen fibril structure in dermatosparactic lamb tissues. Lab. Invest. 43: 542-546.

Chung,E., Rhodes,R.K. and Miller,E.J. 1976. Isolation of three collagenous components of probable basement origin from several tissues. Biochem. Biophys. Res. Commun. 71: 1167-1174.

Cintron,C., Hassinger,L.C., Kublin,C.L. and Cannon,D.J. 1978. Biochemical and ultrastructural changes in collagen during corneal wound healing. J. Ultrastruct. Res. 65: 13-22.

Comper,W.D. and Laurent,T.C. 1978. Physiological function of connective tissue polysaccharides. Physiol. Rev. 58: 255-315.

Cowan,P.M. and McGavin,S. 1955. Structure of poly-L-proline. Nature (Lond.) 176: 501-503.

Cox,J.L., Farrell,R.A., Hart, R.W. and Langham, M.E. 1970. The transparency of the mammalian cornea. J. Physiol. 210: 601-616.

Craig,A.S. and Parry,D.A.D. 1981a. Collagen fibrils of the vertebrate corneal stroma. J. Ultrastruct. Res. 74: 232-239.

Craig,A.S. and Parry,D.A.D. 1981b. Growth and development of collagen fibrils in immature tissues from rat and sheep. Proc. Roy. Soc. London B. 212: 85-92.

Crewe,A.V. 1971. High resolution scanning microscopy of biological specimens. Phil. Trans. Roy. Soc. Lond. B, 261: 61-70.

Cusack,S. and Miller,A. 1979. Determination of the elastic

constants of collagen by Brillouin light scattering. J. Mol. Biol. 135: 39-51.

Dahmen,G. 1973. Polarizing and electronmicroscopic investigations of maturing and old human articular cartilage. In: Connective tissue and ageing (Ed: Vogel,H.G.) Excerpta Medica, Amsterdam pp 109-113.

Davison,P.F. and Cannon,D.J. 1977. Heterogeneity of collagens from basement membranes of lens and cornea. Exp. Eye Res. 25: 129-137.

Delvoye,P. Nusgens,B. and Lapiere,C.M. 1983. The capacity of retracting a collagen matrix is lost by dermatosparactic skin fibroblasts. J. Invest. Dermatol. 81: 267-270.

Diamant,J. Keller,A. Baer,E. Litt,M. and Arridge,R.G.C. 1972. Collagen ultrastructure and its relation to mechanical properties as a function of ageing. Proc. Roy. Soc. Lond. B 180: 293-315.

Dlugosz,J., Gathercole,L.J. and Keller,A. 1979. Cholesteric analogue packing of collagen fibrils in the Cuverian tubules of Holothuria forskali (Holothuroidea, Echinodermata). Micron, 10: 81-87.

Doyle,B.B., Hulmes,D.J.S., Miller,A., Parry,D.A.D., Piez,K.A. and Woodhead-Galloway J. 1974a. A D-periodic narrow filament in collagen. Proc. Roy. Soc. London B. 186: 67-74.

Doyle,B.B., Hulmes,D.J.S., Miller,A., Parry,D.A.D., Piez,K.A. and Woodhead-Galloway J. 1974b. Axially projected collagen structures. Proc. Roy. Soc. London B. 187: 37-46.

Doyle,B.B., Hukins,D.W.L., Hulmes,D.J.S., Miller,A. and Woodhead-Galloway,J. 1975. Collagen polymorphism: Its origins in the amino acid sequence. J. Mol. Biol. 91: 79-99.

Eikenberry,E.F., Brodsky,B., Craig,A.S. and Parry,D.A.D. 1982a. Collagen fibril morphology in developing chick metatarsal tendon. 2: Electron microscope studies. Int. J. Biol. Macromol. 4: 393-398.

Eikenberry,E.F., Brodsky,B. and Parry,D.A.D. 1982b. Collagen fibril morphology in developing chick metatarsal tendons. 1: X-ray diffraction studies. Int. J. Biol. Macromol. 4: 322-328.

Eikenberry,E.F., Childs,B., Sheren,S.B., Parry,D.A.D., Craig,A.S. and Brodsky,B. 1984. Crystalline fibril structure of Type II collagen in lamprey notochord sheath. J. Mol. Biol. (In press).

Epstein,E.H. Jr. and Munderloh,N.H. 1975. Isolation and characterisation of CNBr peptides of human Type III collagen and tissue distribution of Type I and Type III collagens. J. Biol. Chem. 250: 9304-9312.

Farrell,R.A. and Hart,R.W. 1969. On the theory of spatial organization of macromolecules in connective tissue. Bull. Math. Biophys. 31: 727-760.

Fessler,J.H. 1960. A structural function of mucopolysaccharide in connective tissue. Biochem. J. 76: 124-133.

Fessler,J.H. and Fessler,L.I. 1978. Biosynthesis of procollagen. Ann. Rev. Biochem. 47: 129-162.

Fessler,J.H. and Tandberg,W.D. 1975. Interactions between collagen chains and fiber formation. J. Supramol. Struct. 3: 17-23.

Fietzek,P.P. and Kuhn,K. 1976. The primary structure of collagen. Int. Rev. Connect. Tiss. Res. 7: 1-60.

Fjolstad,M. and Helle,O. 1974. A collagen dysplasia of collagen tissues in sheep. J. Pathol. 112; 183-188.

Fleischmajer,R., Timpl,R., Tuderman,L., Raither,L., Wiestner,M.,

Perlish,J.S. and Graves,P.N. 1981. Ultrastructural identification of extension aminopeptides of Types I and III collagens in human skin. Proc. Natl. Acad. Sci. USA, 78: 7360-7364.

Fleishmajer,R., Olsen,B.R., Timpl,R., Perlish,J.S. and Lovelace,O. 1983. Collagen fibril formation during embryogenesis. Proc. Natl. Acad. Sci. USA, 80: 3354-3358.

Flint,M.H. 1971. In: Transactions of the Fifth International Congress of Plastic and Reconstructive Surgery. (Ed: Hueston,J.) Butterworths, London, pp 730-740.

Flint,M.H. 1972. Interrelationships of mucopolysaccharide and collagen in connective tissue remodelling. J. Embryol. Exp. Morphol. 27: 481-495.

Flint,M.H. 1981. Connective tissue organization. In: The hand. Vol. 1. (Ed: Tubiana,R.) Saunders, New York, pp 552-564.

Flint,M.H. and Merrilees,M.J. 1977. Relationship between the axial periodicity and staining of collagen by the Masson trichrome procedure. Histochem. J. 9: 1-13.

Flint,M.H., Gillard,G.C. and Merrilees,M.J. 1980. The effects of local physical environmental factors on connective tissue organization and glycosaminoglycan synthesis. In: Fibrous Proteins: Scientific, Industrial and Medical Aspects, Vol. 2 (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, London, pp 107-119.

Flint,M.H. Gillard,G.C. and Reilly,H. 1982. The glycosaminoglycans of Dupuytren's Disease. Connect. Tiss. Res. 9: 173-179.

Francis,M.J.O. and Duksin,D. 1983. Heritable disorders of collagen metabolism. TIBS, 8: 231-234.

Francois,J., Rabaey,M. and Vandermeerssche,G. 1954.

L'ultrastructure des tissues oculaires au microscope electronique. Ophthalmologica, 127: 73-81.

Fraser,R.D.B. and MacRae,T.P. 1981. Unit cell and molecular connectivity in tendon collagen. Int. J. Biol. Macromol. 3: 193-200

Fraser,R.D.B., MacRae,T.P., Miller,A. and Suzuki,E. 1964. The quantitative analysis of fibril packing from electron micrographs. J. Mol. Biol. 9: 250-252.

Fraser,R.D.B., Miller,A. and Parry,D.A.D. 1974. Packing of microfibrils in collagen. J. Mol. Biol. 83: 281-233.

Fraser,R.D.B., MacRae,T.P. and Suzuki,E. 1979a. The molecular and fibrillar structure of collagen. In: Fibrous Proteins: Scientific Industrial and Medical Aspects, Vol. 2 (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, London, pp 179-206.

Fraser,R.D.B., MacRae,T.P. and Suzuki,E. 1979b. Chain conformation in the collagen molecule. J. Mol. Biol. 129: 463-481.

Fraser,R.D.B., MacRae,T.P., Miller,A. and Suzuki,E. 1983. Molecular conformation and packing in collagen fibrils. J. Mol. Biol. 167: 497-521.

Furoto,D.K. and Miller,E.J. 1980. Isolation of a unique collagenous fraction from limited pepsin digests of human placental tissue. Characterization of one of the constituent polypeptide chains. J. Biol. Chem. 255: 290-295.

Furoto,D.K. and Miller,E.J. 1981. Characterization of a unique collagenous fraction from limited pepsin digests of human placental tissue. Molecular organization of the native aggregate. Biochemistry, 20: 1635-1640.

Furthmayr,H. and Madri,J.A. 1982. Rotary shadowing of connective

tissue macromolecules. Collagen Rel. Res. 2: 349-363.

Gathercole,L.J. and Keller,A. 1975. Light microscopic waveforms in collagenous tissues and their structural implications. In: Structure of fibrous biopolymers. Colston papers No. 26. (Eds: Atkins,E.D.T. and Keller,A.) Butterworths, London, pp 153-187.

Gillard,G.C., Reilly,H.C., Bell-Booth,P.G. and Flint,M.H. 1977. A comparison of the glycosaminoglycans of weight-bearing and non-weight-bearing dermis. J. Invest. Dermatol. 69: 257-261.

Gillard,G.C., Reilly,H.C., Bell-Booth,P.G. and Flint,M.H. 1979. The influence of mechanical forces on the glycosaminoglycan content of the rabbit flexor digitorum profundus tendon. Connect. Tiss. Res. 7: 37-46.

Glanville,R.W. and Kuhn,K. 1979. The primary structure of collagen. In: Fibrous proteins: Scientific, Industrial and Medical aspects. (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, London, pp 133-150.

Glauert,A.M. 1975. Fixation, dehydration and embedding biological specimens. North Holland Publishing Co., Amsterdam.

Goldman,J.N. and Benedek,G.B. 1967. The relationship between morphology and transparency in the non-swelling corneal stroma of the shark. Invest. Ophthalmol. 6: 574-600.

Greenlee,T.K. and Ross,R. 1967. The development of the rat flexor digital tendon, a fine structure study. J. Ultrastruct. Res. 18: 354-376.

Greenlee,T.K., Ross,R. and Hartman,J.L. 1966. The fine structure of elastic fibers. J. Cell Biol. 30: 59-71.

Greenwald,R.A., Schwartz,C.E. and Cantor,J.O. 1975. Interaction of cartilage proteoglycans with collagen-substituted agarose gels. Biochem. J. 145: 601-605.

Gregory,J.D., Coster,L. and Damle,S.P. 1982. Proteoglycans of rabbit corneal stroma. Isolation and characterization. J. Biol. Chem. 257: 6965-6970.

Groot,C.G. 1981. Positive colloidal thorium dioxide as an electron microscopical contrasting agent for glycosaminoglycans, compared with ruthenium red and positive colloidal iron. Histochemistry, 71: 617-627.

Gross,J. 1956. The behaviour of collagen units as a model in morphogenesis. J. Biophys. Biochem. Cytol. 2: Supplement, pp 261-274.

Gross,J. and Bruns,R.R. 1984. Another look at fibrillogenesis. Symp. Soc. Dev. Biol. (in press).

Gross,J. and Kirk,D. 1958. The heat precipitation of collagen from neutral salt solutions: Some rate-regulating factors. J. Biol. Chem. 233: 355-360.

Gross,J. and Schmitt,F.O. 1948. The structure of human skin collagen as studied with the electron microscope. J. Exp. Med. 88: 555-568.

Gross,J., Matoltsy,A.G. and Conen,C. 1955a. Vitrosin: A member of the collagen class. J. Biophys. Biochem. Cytol. 1: 215-220.

Gross,J., Highberger,J.H. and Schmitt,F.O. 1955b. Extraction of collagen from connective tissue by neutral salt solutions. Proc. Natl. Acad. Sci. USA, 41: 1-7.

Gusnard,D. and Kirschner,R.H. 1977. Cell and organelle shrinkage during preparation for scanning electron microscopy: Effects of fixation, dehydration and critical point drying. J. Microscopy, 110: 51-57.

Hall,C.E. 1966. Introduction to electron microscopy. 2nd edition, McGraw Hill, New York.

Hall,C.E., Jakus,M.A. and Schmitt,F.O. 1942. Electron microscope observations of collagen. J. Am. Chem. Soc. 64: 1234.

Harkness,R.D. 1961. Biological function of collagen. Biol. Rev. 36: 399-463.

Harkness,R.D. 1979. Mechanical properties of connective tissue in relation to function. In: Fibrous Proteins: Scientific, Industrial and Medical Aspects, Vol. 1. (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, London, pp 207-230.

Hart,R.W. and Farrell,R.A. 1969. Light scattering in the cornea. J. Opt. Soc. Am. 59: 766-774.

Hay,E.D. 1973. Origin and role of the collagen in the embryo. Amer. Zool. 13: 1085-1107.

Hay,E.D. 1980. Development of the vertebrate cornea. Int. Rev. Cytol. 63: 263-322.

Hay,E.D. and Revel,J.P. 1969. Fine structure of the developing avian cornea. In: Monographs in developmental biology, Vol. 1. (Eds: Wolsky,A. and Chen,P.S.) Karger, Basle. pp 1-144.

Hayat,M.A. 1970. Principles and techniques of electron microscopy: Biological applications. Vol. 1. Van Nostrand Reinhold Co. New York.

Hayat,M.A. 1975. Positive staining for electron microscopy. Van Nostrand Reinhold Co. New York.

Hegreberg,G.A., Padgett,G.A., Ott,R.L. and Henson,J.B. 1970. A heritable connective tissue disease of dogs and mink resembling the Ehlers-Danlos syndrome in man. I. Skin tensile strength properties. J. Invest. Dermatol. 54: 377-380.

Helle,O. and Ness,N.N. 1972. A hereditary skin defect in sheep. Acta Vet. Scand. 13: 443-445.

Hendrix,M., Hay,E.D., von der Mark,K. and Linsenmayer,T.F. 1982.

Immunohistochemical localization of collagen Types I and II in the developing chick cornea and tibia by electron microscopy. Invest. Ophthalmol. Vis. Sci. 22: 359-375.

Henkel,W. and Glanville,R.W. 1982. Covalent crosslinking between molecules of Type I and Type III collagen. The involvement of the N-terminal, non-helical regions of the α1(I) and α1(III) chains in the formation of intermolecular crosslinks. Eur. J. Biochem. 22: 205-213.

Hodge,A.J. and Petruska,J.A. 1963. In: Aspects of protein structure. (Ed: Ramachandran,G.N.), Academic Press, New York. pp 289-306.

Hodge,A.J. and Schmitt,F.O. 1960. The charge profile of the tropocollagen macromolecule and the packing arrangement in native-type collagen fibrils. Proc. Natl. Acad. Sci. USA, 46: 186-197.

Hogan,M.J., Alvardo,J.A. and Weddell,J.E. 1971. In: The histology of the human eye. W.B.Saunders and Co., Philadelphia. pp 55-111.

Holbrook,K.A. and Byers,P.H. 1981. Ultrastructural characteristics of the skin in a form of the Ehlers-Danlos Syndrome Type IV. Lab. Invest. 44: 342-350.

Hosemann,R., Dreissig,W. and Nemetschek,T. 1974. Schachtelhalm-structure of the octafibrils in collagen. J. Mol. Biol. 83: 275-280.

Hulmes,D.J.S. and Miller,A. 1979. Quasi-hexagonal molecular packing in collagen fibrils. Nature, (Lond.) 282: 878-880.

Hulmes,D.J.S., Bruns,R.R. and Gross,J. 1983. On the state of newly secreted procollagen. Proc. Natl. Acad. Sci. USA, 80; 388-392.

Hukins,D.W.L. and Woodhead-Galloway,J. 1977. Collagen fibrils as examples of smectic A biological fibres. J. Molec. Cryst. Liq. Cryst. 41: 33-39.

Inouye,H. and Worthington,C.R. 1983a. X-ray diffraction study of the cornea. Biophys. J. 41: 285a.

Inouye,H. and Worthington,C.R. 1983b. X-ray observations on a collagen fibril lattice structure in peripheral nerve. Int. J. Biol. Macromol. 5: 199-203.

Ippolito,E., Natali,P.G., Postacchini,F., Accinni,L. and De Martino,C. 1980. Morphological, immunochemical, and biochemical study of rabbit Achilles tendon at various ages. J. Bone Joint Surg. 62A: 583-598.

Jackson,S.F. 1956. The morphogenesis of avian tendon. Proc. Roy. Soc. Lond. B, 144: 556-572.

Jakus,M.A. 1954. Studies on the cornea. I: The fine structure of the rat cornea. Am. J. Ophthalmol. 38: 40-53.

Jakus,M.A. 1956. Studies on the cornea. II: The fine structure of Descemet's membrane. J. Biophys. Biochem. Cytol. Suppl. 2: 243-255.

Jakus,M.A. 1961. The fine structure of the human cornea. In: The structure of the eye (Ed: Smelser,G.K.) Academic Press, New York, pp 343-366.

Jander,R., Rauterberg,J., Voss,B. and von Bassewitz,D.B. 1981. A cysteine-rich collagenous protein from bovine placenta. Isolation and constituent polypeptide chains and some properties of the non-denatured protein. Eur. J. Biochem. 114: 17-25.

Jarrett,A. 1973. Disorders affecting the dermo-epidermal junction. In: The physiology and pathophysiology of skin. Academic Press, London, pp 268-294.

Karnovsky,M.J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27: 137A-138A.

Kayes,J. and Holmberg,A. 1960. The fine structure of Bowman's layer and the basement membrane of the epithelium. Am. J. Ophthalmol. 50: 1013-1021.

Keech,M.K. 1961. The formation of fibrils from collagen solutions. IV. Effect of mucopolysaccharides and nucleic acids: An electron microscope study. J. Biophys. Biochem. Cytol. 9: 193-209.

Kefalides,N.A. and Denduchis,B. 1969. Structural components of epithelial and endothelial basement membranes. Biochemistry, 8: 4613-4621.

Kischer,C.W. and Shetlar,M.R. 1974. Collagen and mucopolysaccharides in the hypertrophic scar. Connect. Tiss. Res. 2: 205-213.

Klein,L., Itoh,T. and Geil,P.H. 1981. Relationship between collagen fibril and subfibril diameters as revealed by transverse freeze-fracture and -etching techniques. In: The Chemistry and Biology of Mineralized Connective Tissues. (Ed: Veis,A.) Elsevier North-Holland, New York, pp 129-132.

Kleinman,H.K., Wilkes,C.M. and Martin,G.R. 1981. Interaction of fibronectins with collagen fibrils. Biochemistry, 20: 2325-2330.

Langmore,J.P., Isaacson,M.S. and Crewe,A.V. 1974. The study of single heavy atom motion in the STEM. 32nd Ann. Proc. E. M. Soc. Amer. St. Louis, Missouri. pp 378-379.

Lenears,A., Ansay,M., Nusgens,B.V. and Lapiere,C.M. 1971. Collagen made of extended α-chains, procollagens, in

genetically-defective dermatosparaxic calves. Eur. J. Biochem. 23: 533-543.

Light,N.D. and Bailey,A.J. 1979. Covalent crosslinks in collagen. Characterization and relationships to connective tissue disorders. In :Fibrous Proteins: Scientific, Industrial and Medical Aspects, Vol. 1. (Eds: Parry,D.A.D. and Creamer,L.K.) Academic press, London. pp 151-177.

Lillie,J.H., MacCallum,D.K., Scaletta,L.J. and Occhino,J.C. 1977. Collagen structure: Evidence for a helical organization of the collagen fibril. J. Ultrastruct. Res. 58: 134-143.

Lindahl,U. and Hook,M. 1978. Glycosaminoglycans and their binding to biological macromolecules. Ann. Rev. Biochem. 47: 385-417.

Linsenmayer,T.F., Trelstad,R.L. and Gross,J. 1973. The collagen of chick embryonic notochord. Biochem. Biophys. Res. Commun. 53: 39-45.

Linsenmayer,T.F., Smith,G.N., Jr. and Hay,E.D. 1977. Synthesis of two collagen types by embroyonic duck corneal epithelium in vitro. Proc. Natl. Acad. Sci. USA, 74: 39-43.

Loewi,G and Meyer,K. 1958. The acid mucopolysaccharides of embryonic skin. Biochim. Biophys. Acta, 27: 453-456.

Mathews,M.B. 1965. The interaction of collagen and acid mucopolysaccharides. A model for connective tissues. Biochem. J. 96: 710-716.

Mathews,M.B. and Lozaityte,I. 1958. Sodium chondroitin sulphate-protein complexes of cartilage. I: Molecular weight and shape. Arch. Biochem. Biophys. 74: 158-174.

Mathews,M.B. 1975. Connective tissue macromolecular structure and evolution. Springer-Verlag, Berlin.

Maurice,D.M. 1957. The structure and transparency of the cornea. J. Physiol. 136: 263-286.

Maurice,D.M. 1969. The cornea and sclera. In: The eye (Ed: Davson,H.) Academic Press, London, pp 489-600.

Meek,K.M., Chapman,J.A. and Hardcastle,R.A. 1979. The staining pattern of collagen fibrils. Improved correlation with sequence data. J. Biol. Chem. 254: 10710-10714.

Menton,D.A. and Hess,R.A. 1980. The ultrastructure of collagen in the dermis of tight-skin (Tsk) mutant mice. J. Invest. Dermatol. 74: 139-147.

Merrilees,M.J. and Flint,M.H. 1980. Ultrastructural study of tension and pressure zones in a rabbit flexor tendon. Amer. J. Anat. 157: 87-106.

Merrilees,M.J. and Scott,L. 1980. Interaction of epithelial cells and fibroblasts in culture: Effect on glycosaminoglycan levels. Dev. Biol. 76: 396-409.

Meyer,K. and Smyth,E.M. 1937. On glycoproteins. VI: The preparation of chondroitinsulfuric acid. J. Biol. Chem. 119: 507-510.

Meyer,K., Palmer,J.W. and Smyth,E.M. 1937. On glycoproteins. V: Protein complexes of chondroitinsulfuric acid. J. Biol. Chem. 119: 501-506.

Miller,A. 1976. Molecular packing in collagen fibrils. In: Biochemistry of Collagen, (Eds: Ramachandran,G.N. and Reddi,A.H.) Plenum press, New York. pp 85-136.

Miller,A. and Parry,D.A.D. 1973. Structure and packing of microfibrils in collagen. J. Mol. Biol. 75: 441-447.

Miller,A. and Tocchetti,D. 1981. Calculated X-ray diffraction pattern from a quasi-hexagonal model for the molecular

arrangement in collagen. Int. J. Biol. Macromol. 3: 9-18.

Miller,A. and Wray,J.S. 1971. Molecular packing in collagen. Nature (Lond.) 230: 437-439.

Miller,E.J. 1971. Isolation and characterization of a collagen from chick cartilage containing three identical α-chains. Biochemistry, 10: 1652-1659.

Minor,R.R. 1978. A comparison of diseases of collagen. In: Biochemical Mechanisms and Disease Processes. (Ann. Meeting Amer. College Vet. Pathologists, San Antonio, Texas) pp 151-216.

Miyahara,M., Njieha,F.K. and Prockop,D.J. 1982. Formation of collagen fibrils in vitro by cleavage of procollagen with procollagen proteinases. J. Biol. Chem. 257: 8442-8448.

Myers,D.B. 1976. Electron microscope autoradiography of $^{35}SO_4$-labelled material closely associated with collagen fibrils in mammalan synovium and ear cartilage. Histochem. J. 8: 191-199.

Myers,D.B. 1980. Interaction of Ruthenium Red with collagen. In: Fibrous proteins: Scientific, Industrial and Medical aspects. (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, London, pp 99-105.

Myers,D.B., Highton,T.C. and Rayns,D.G. 1969. Acid mucopolysaccharides closely associated with collagen fibrils in normal human synovium. J. Ultrastruct. Res. 28: 203-213.

Myers,D.B., Highton,T.C. and Rayns,D.G. 1971. Ruthenium Red-positive filaments interconnecting collagen fibrils. J. Ultrastruct. Res. 42: 87-92.

Nakamaru,H., Kanai,C. and Mizuhira,V. 1977. An electron stain for elastic fibres using orcein. J. Histochem. Cytochem. 25: 306-308.

Nakao,K. and Bashey,R.I. 1972. Fine structure of collagen fibrils as revealed by ruthenium red. Exp. Mol. Path. 17: 6-13.

Nemetschek,Th., Grassmann,W. and Hofmann,U. 1955. Uber die hochunterteilte querstreifung des kollagens. Z. Naturforsch. 10b: 61-63.

Neufeld,E.F., Lim,T.W. and Shapiro,L.J. 1975. Inherited disorders of lysosomal metabolism. Ann. Rev. Biochem. 44: 357-376.

Newsome,D.A., Linsenmayer,T.F. and Trelstad,R.L. 1976. Vitreous body collagen. Evidence for a dual origin from the neural retina and halocytes. J. Cell Biol. 71: 59-67.

Nichols,D. 1967. Echinoderms, 3rd edition, Hutchinson, London.

Nowack,H., Gay,S., Wick,G., Becker,U. and Timpl,R. 1976. Preparation and use in immune histology of antibodies specific for Type I and Type III collagen and procollagen. J. Immunol. Methods, 12: 117-124.

Obrink,B. 1973. A study of the interactions between monomeric tropocollagen and glycosaminoglycans. Eur. J. Biochem. 33: 387-400.

Oegema,T.R., Laidlaw,J., Hascall,V.C. and Dziewiatkowski,D.D. 1975. The effect of proteoglycans on the formation of fibrils from collagen solutions. Arch. Biochem. Biophys. 170: 698-709.

O'Hara,P.J., Read,W.K., Romane,W.M. and Bridges,C.H. 1970. A collagenous tissue dysplasia of calves. Lab. Invest. 23: 307-314.

Olsen,B.R. 1963. Electron microscope studies on collagen. I: Native collagen fibrils. Z. Zellforsch. 59: 184-189.

Ozanics,V., Rayborn,M. and Sagan,D. 1976. Some aspects of corneal and scleral differentiation in the primate. Exp. Eye Res. 22: 305-327.

Parry,D.A.D. and Craig,A.S. 1977. Quantitative electron microscope observations of the collagen fibrils of rat-tail tendon. Biopolymers, 16: 1015-1031.

Parry,D.A.D. and 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. and Craig,A.S. 1979. Electron microscope evidence for an 80 Å unit in collagen fibrils. Nature (Lond.) 282: 213-215.

Parry,D.A.D. and Craig,A.S. 1981. An 80 Å unit in collagen fibrils. In: Structural Aspects of Recognition and Assembly in Biological Macromolecules. (Eds: Balaban,M., Sussman,J.L., Traub,W. and Yonath,A.), Balaban ISS, Rehovot, pp 377-385.

Parry,D.A.D. and Craig,A.S. 1984. Growth and development of collagen fibrils in connective tissue. In: The ultrastructure of the connective tissue matrix. (Eds: Ruggeri,A. and Motta,P.M.), Martinus Nijhoff, Netherlands, pp 34-64.

Parry,D.A.D., Barnes,G.R.G. and Craig,A.S. 1978a. A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. Proc. Roy. Soc. Lond. B. 203: 305-321.

Parry,D.A.D., Craig,A.S. and Barnes,G.R.G. 1978b. Tendon and ligament from the horse: An ultrastructural study of collagen fibrils and elastic fibres as a function of age. Proc. Roy. Soc. Lond. B. 203: 293-303.

Parry,D.A.D., Craig,A.S. and Barnes,G.R.G. 1980. Fibrillar collagen in connective tissue. In: Fibrous Proteins: Scientific, Industrial and Medical Aspects, Vol.1. (Eds: Parry,D.A.D. and Creamer,L.K.) Academic Press, New York, pp 77-88.

Parry,D.A.D., Flint,M.H., Gillard,G.C. and Craig,A.S. 1982. A role for the glycosaminoglycans in the development of collagen fibrils. FEBS Letters, 149: 1-7.

Partington,F.R. and Wood,G.C. 1963. The role of non-collagen components in the mechanical behaviour of tendon fibres. Biochim. Biophys. Acta, 69; 485-495.

Partridge,S.M., Elsden,D.F. and Thomas,J. 1963. Constitution of the cross-linkages of elastin. Nature, (Lond.) 197: 1297-1298.

Patterson,D.F. and Minor,R.R. 1977. Hereditary fragility and hyperextensibility of the skin of cats; a defect in collagen fibrillogenesis. Lab. Invest. 37: 170-179.

Payrau,P., Offret,G., Faure,J-P., Pouliquen,Y., Cug,G. 1964. Sur la structure lamellaire des cornees de certains poissons. Arch. Ophthal. 24: 685-691.

Payrau,P., Pouliquen,Y., Faure,J-P., Bisson,J. and Offret,G. 1965. Ultrastructure des fibres suturales de la cornee de poissons elasmobranches. Arch. Ophthal. 25: 745-752.

Pease,D.C. and Bouteille,M. 1971. The tridimensional structure of native collagenous fibrils, cytochemical evidence for a carbohydrate matrix. J. Ultrastruct. Res. 35: 339-358.

Perlman,M. and Baum,J.L. 1974. The mass culture of rabbit corneal endothelial cells. Arch. Ophthalmol. 92: 235-237.

Perlman,M., Baum,J.L. and Kay,G.I. 1974. Fine structure and collagen synthesis activity of cultures of rabbit corneal epithelium. J. Cell Biol. 63: 306-311.

Pessac,B. and Defendi,V. 1972. Cell aggregation: Role of acid mucopolysaccharides. Science, 175: 898-900.

Piez,K.A. and Trus,B.L. 1981. A New Model for Packing of Type I Collagen Molecules in the Native Fibril. Bioscience Reports, 1:

801-810.

Podrazky,V., Steven,F.S., Jackson,D.S., Weiss,J.B. and Leibovich,S.J. 1971. Interaction of tropocollagen with protein-polysaccharide complexes. An analysis of the ionic groups responsible for interaction. Biochim. Biophys. Acta, 229: 690-697.

Poschl,A. and von der Mark,K. 1980. Synthesis of Type V collagen by chick corneal fibroblasts *in vivo* and *in vitro*. FEBS Lett. 115: 100-104.

Prockop,D.J., Kivirikko,K.I., Tuderman,L. and Guzman,N.A. 1979. The biosynthesis of collagen and its disorders, I. New England J. Med. 301: 13-23.

Quaroni,A. and Trelstad,R. 1980. Biochemical characterization of collagens synthesized by intestinal epithelial cell cultures. J. Biol. Chem. 255: 8351-8361.

Ramachandran,G.N. and Kartha,G. 1955. Structure of collagen. Nature (Lond.) 176: 593-595.

Ramachandran,G.N. and Ramakrishnan, 1976. In: Biochemistry of Collagen. (Eds: Ramachandran,G.N. and Reddi,A.H.) Plenum Press, New York, Vol. 1, pp 103-183.

Reid,T. 1974. Ph.D. Thesis, University of Auckland, New Zealand.

Reid,T. and Flint,M.H. 1974. Changes in glycosaminoglycan content of healing rabbit tendon. J. Embryol. Exp. Morphol. 31: 489-495.

Rigby,B.S., Hirai,N., Spikes,J.D. and Eyring,.H. 1959. The mechanical properties of rat-tail tendon. J. gen. Physiol. 43: 265-283.

Roberts,I.M. 1970. Reduction of compression artefacts in ultra-thin sections by the application of heat. J. Microscopy 92: 57-61.

Rosenberg.L., Hellmann,W. and Kleinschmidt,A.K. 1970.

Macromolecular models of proteinpolysaccharides from bovine nasal cartilage based on electron microscope studies. J. Biol. Chem. 245: 4123-4130.

Ross,R. 1980. Inflammation, cell proliferation, and connective tissue formation in wound repair. In: Wound healing and wound infection: Theory and surgical practice, (Ed: Hunt,T.K.), Appleton Century Crofts, New York, 1-10.

Ross,R. and Bornstein,P. 1969. The elastic fiber. I: The separation and partial characterization of its macromolecular components. J. Cell Biol. 40: 366-381.

Rougvie,M.A. and Bear,R.S. 1953. An X-ray diffraction of swelling by collagen. J. Amer. Leather Chem. Ass. 48: 735-751.

Ruggeri,A., Benazzo,F. and Reale,E. 1979. Collagen fibrils with straight and helicoidal microfibrils: A freeze-fracture and thin-section study. J. Ultrastruct. Res. 68: 101-108.

Sage,H., Pritzl,P. and Bornstein,P. 1980. A unique, pepsin-sensitive collagen synthesized by aortic endothelial cells in culture. Biochemistry, 19: 5747-5755.

Satir,P.G. and Peachey,L.D. 1958. Thin sections. II. A simple method for reducing compression artefacts. J. Biophys. Biochem. Cytol. 4: 345-347.

Sayers,Z., Koch,M.H.J., Whitburn,S.B. Meek,K.M., Elliott,G.F. and Harmsen,A. 1982. Synchrotron X-ray diffraction study of corneal stroma. J.Mol. Biol. 160: 593-607.

Schmitt,F.O. and Gross,J. 1948. Further progress in the electron microscopy of collagen. J. Amer. Leather Chem. Assoc. 43: 658-673.

Schmitt,F.O., Hall,C.E. and Jakus,M.A. 1942. Electron microscope investigations of the structure of collagen. J. Cell Comp.

Physiol. 20: 11-33.

Schmitt,F.O., Gross,J. and Highberger,J.H. 1955. Tropocollagen and the properties of fibrous collagen. Exp. Cell Res. Supplement 3: 326-334.

Schuppan,D., Timpl,R. and Glanville,R.W. 1980. Discontinuities in the triple helical sequence Gly-X-Y of basement membrane (Type IV) collagen. FEBS Letters, 115: 297-300.

Schwarz,W. 1953. Elektronenmikroskopische untersuchungen uber die differenzierung der cornea- und sklera-fibrillen des meschen. Z. Zellforsch. 38: 78-86.

Schwarz,W. 1957. Morphology and differentiation of the connective tissue fibres. In: Connective tissue. (Eds: Tunbridge,R.E., Keech,M., Delafresnaye,J.F. and Wood,G.C.), pp 144-156.

Schwarz,W. 1966. Uber die feinstruktur der memschlichen cornea, mit besonderer berucksichtigung des problems der transparenz. Z. Zellforsch. Mikrosk. Anat. 73: 540-548.

Scott,J.E. 1973. Affinity, competition and specific interactions in the biochemistry and histochemistry of polyelectrolytes. Bioch. Soc. Trans. 1: 787-806.

Scott,J.E. 1980. Collagen-proteoglycan interactions. Localization of proteoglycans in tendon by electron microscopy. Biochem. J. 187: 887-891.

Scott,J.E. and Orford,C.R. 1981. Dermatan sulphate-rich proteoglycan associates with rat tail-tendon collagen at the d-band in the gap region. Biochem. J. 197: 213-216.

Scott,J.E., Orford,C.R. and Hughes,E.W. 1981. Proteoglycan-collagen arrangements in developing rat tail tendon. An electron-microscopical and biochemical investigation. Biochem. J. 195: 573-581.

Shimokomaki,M., Duance,V.C. and Bailey,A.J. 1980. Identification of a new disulphide bonded collagen from cartilage. FEBS Letters, 121: 51-54.

Siegel,B.M. 1965. The physics of the electron microscope. In: Modern developments in electron microscopy. (Ed: Siegel,B.M.) Academic Press, New York, pp 1-79.

Silver,F.H. 1981. Type I collagen fibrillogenesis in vitro. Additional evidence for the assembly mechanism. J. Biol. Chem. 256: 4973-4977.

Silver,F.H. 1983. A two step model for lateral growth of collagen fibrils. Collagen Rel. Res. 3: 167-180.

Silver,F.H. and Trelstad,R.L. 1979. Linear aggregation and the turbidometric lag phase: Type I collagen fibrillogenesis in vitro. J. Theor. Biol. 81: 515-526.

Silver,F.H. and Trelstad,R.L. 1980. Type I collagen in solution. Structure and properties of fibril fragements. J. Biol. Chem. 255: 9427-9433.

Silver,F.H., Langley,K.H. and Trelstad,R.L. 1979. Type I collagen fibrillogenesis: Initiation via reversible linear and lateral growth steps. Biopolymers, 18: 2523-2535.

Simar,L.J. and Betz,E.H. 1971. Dermatosparaxis in the calf, a genetic defect of the connective tissues. 2. Ultrastructural study of the skin. Hoppe-Seyler's Z. Physiol. Chem. 352: 13.

Sjostrand,F.S. 1967. Electron microscopy of cells and tissues. Vol. 1: Instrumentation and techniques. Academic Press, New York.

Smelser,G.K. and Ozanics,V. 1957. Distribution of radioactive sulphate in the developing eye. Am. J. Ophthalmol. 44,(II): 102-110.

Smelser,G.K. 1962. Corneal hydration. Comparative physiology of

fish and mammals. Invest. Ophthalmol. 11-31.

Smelser,G.K. and Ozanics,V. 1972. In: Symposium on the cornea. (Eds: Castroviejo,R., Devoe,A.G., Dohlman,C.H., Elliot,J.H., Fine,M. and Smelser,G.K.) Mosby, St. Louis, pp 1-19.

Smith,J.W. 1968. Molecular pattern in native collagen. Nature (Lond.) 219: 157-158.

Smith,J.W. 1969. The transparency of the corneal stroma. Vis. Res. 9: 393-396.

Smith,J.W. and Frame,J. 1969. Observations on the collagen and protein-polysaccharide complex of rabbit corneal stroma. J. Cell Sci. 4: 421-436.

Smith,J.W., Peters,T.J. and Serafini-Fracassini,A. 1967. Observations on the distribution of the protein-polysaccharide complex and collagen in bovine articular cartilage. J. Cell Sci. 2: 129-136.

Sotelo,J.R. 1957. Technical improvements in specimen preparation for electron microscopy. Expl. Cell Res. 13: 599-601.

Spurr,A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.

Squire,J.M. and Freundlich,A. 1980. Direct observation of a transverse periodicity in collagen fibrils. Nature (Lond.) 288: 410-413.

Tajima,S. and Nagai,Y. 1980. Distribution of macromolecular components in calf dermal connective tissue. Connect. Tiss. Res. 7: 65-71.

Timpl,R., Wick,G. and Gay,S. 1977. Antibodies to distinct types of collagens and procollagens and their application in immunohistology. J. Immunol. Methods, 18: 165-182.

Timpl,R., Wiedemann,H., van Delden,V., Furthmayr,H. and Kuhn,K.

1981. A network model for the organization of Type IV collagen molecules in basement membranes. Eur. J. Biochem. 120: 203-211.

Tomlin,S.G. and Worthington,C.R. 1956: Low-angle X-ray diffraction patterns of collagen. Proc. Roy. Soc. Lond. A 235: 189-201.

Toole,B.P. 1969. Solubility of collagen fibrils formed in vitro in the presence of sulphated acid mucopolysaccharide protein. Nature (Lond.) 222: 872-873.

Toole,B.P. 1976. In: Neuronal recognition. (Ed: Barondes,G.H.), Plenum Press, New York.

Toole,B.P. and Gross,J. 1971. The extracellular matrix of the regenerating newt limb: synthesis and removal of hyaluronate prior to differentiation. Dev. Biol. 25: 57-77.

Toole,B.P. and Lowther,D.A. 1968a. The effect of chondroitin sulphate-protein on the formation of collagen fibrils in vitro. Biochem. J. 109: 857-866.

Toole,B.P. and Lowther,D.A. 1968b. Dermatan sulphate-protein: Isolation from and interaction with collagen. Arch. Biochem. Biophys. 128: 567-578.

Torp,S., Baer,E. and Friedman,B. 1975. Effects of age and of mechanical deformation on the ultrastructure of tendon. In: Structure of fibrous biopolymers. Colston Papers No. 26. (Eds: Atkins,E.D.T. and Keller,A.) Butterworths, London, pp 223-250.

Traub,W. 1974. Some stereochemical implications of the molecular conformation of collagen. Isr. J. Chem. 12: 435-439.

Traub,W. and Piez,K.A. 1971. The chemistry and structure of collagen. Adv. Prot. Chem. 25: 243-352.

Traub,W., Yonath,A. and Segal,D.M. 1969. On the molecular structure of collagen. Nature (Lond.) 221: 914-917.

Trelstad,R.L. and Hayashi,K. 1979. Tendon collagen fibrillogenesis: Intracellular subassemblies and cell surface changes associated with fibril growth. Develop. Biol. 71: 228-242.

Trelstad,R.L., Hayashi,K. and Gross,J. 1976. Collagen fibrillogenesis: Intermediate aggregates and suprafibrillar order. Proc. Natl. Acad. Sci. USA, 73: 4027-4031.

Trelstad,R.L., Rubin,D. and Gross,J. 1977. Osteogenesis imperfecta congenita. Evidence for a generalized molecular disorder of collagen. Lab. Invest. 36: 501-508.

Tromans,W.J., Horne,R.W., Gresham,G.A. and Bailey,A.J. 1963. Electron microscope studies on the structure of collagen fibrils by negative staining. Z. Zellforsch. 58: 798-802.

Trus,B.L. and Piez,K.A. 1980. Compressed microfibril models of the native collagen fibril. Nature, (Lond.) 286: 300-301.

Twersky,V. 1975. Transparency of pair-correlated, random distributions of small scatterers, with applications to the cornea. J. Opt. Soc. Amer. 65: 524-530.

Tzaphlidou,M., Chapman,J.A. and Meek,K.M. 1982a. A study of positive staining for electron microscopy using collagen as a model system. I. Staining by phosphotungstate and tungstate ions. Micron, 13: 119-131.

Tzaphlidou,M., Chapman,J.A. and Al-Samman,M.H. 1982b. A study of positive staining for electron microscopy using collagen as a model system. II. Staining by uranyl ions. Micron, 13: 133-145.

Uitto,J. and Lichtenstein,J.R. 1976. Defects in the biochemistry of collagen in diseases of connective tissue. J. Invest. Dermatol. 66: 59-79.

Uitto,J., Santa Cruz,D.J., Starcher,B.C., Whyte,M.P. and Murphy,W.A.

1981. Biochemical and ultrastructural demonstration of elastin accumulation in the skin lesions of the Buschke-Ollendorff syndrome. J. Invest. Dermatol. 76: 284-287.

Vaughan,T.A. 1978. Mammalogy, 2nd edition, W.B.Saunders Co., Philadelphia.

Veis,A. and Yuan,L. 1975. Struture of the collagen microfibril. A four stranded overlap model. Biopolymers, 14: 895-900.

Venable,J.H. and Coggeshall,R. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25: 407-408.

Vogel,A., Holbrook,K.A., Steinmann,B., Gitzelmann,R. and Byers,P.H. 1979. Abnormal collagen fibril structure in the gravis form (Type I) of Ehlers-Danlos Syndrome. Lab. Invest. 40: 201-206.

Vogel,H.G. 1974. Correlation between tensile strength and collagen content in rat skin. Effect of age and cortisol treatment. Connect. Tiss. Res. 2: 177-182.

Vogel,H.G. 1978. Influence of maturation and age on mechanical and biochemical parameters of connective tissue of various organs in the rat. Connect. Tiss. Res. 6: 161-166.

Vogel,H.G. 1979. Influence of maturation and aging on mechanical and biochemical parameters of rat bone. Geront. 25: 16-23.

von der Mark,K. 1981. Localization of collagen types in tissue. Int. Rev. Connect. Tiss. Res. 9: 265-324.

von der Mark,K., von der Mark,H., Timpl,R. and Trelstad,R.L. 1977. Immunofluorescent localization of collagen Types I, II and III in the embryonic chick eye. Developm. Biol. 59: 75-85.

von der Mark,K., Sasse,J., von der Mark,H. and Kuhl,U. 1982. Changes in the distribution of collagen types during embryonic development. Connect. Tiss. Res. 10: 37-42.

Wainwright,S.A., Biggs,W.D., Currey,J.D. and Gosline,J.M. 1976. Mechanical Design in Organisms. Edward Arnold, London.

Wainwright,S.A., Vosbrugh,F. and Hebrank,J.H. 1978. Shark skin: Function in locomotion. Science 202: 747-749.

Walker, 1975. Mammals of the world. Johns Hopkins University Press, 3rd edition, 2 vols.

Watson,M.L. 1955. The use of carbon films to support tissue sections for electron microscopy. J. Biophys. Biochem. Cytol. 1: 183-184.

Watson,M.R. and Silvester,N.R. 1959. Studies of invertebrate collagen preparations. Biochem. J. 71: 578-584.

Weber,M. 1878. Terminaisons nerveuses sensitives cornee. In: Lecons d'anatomie generale. (Ed: Ranvier,L.) Faites au college de France, Paris, 1881, Libraire J.B.Bailliere et Fils.

Wellauer,P., Wyler,T. and Buddecke,E. 1972. Electron microscopic and physico-chemical studies on bovine nasal cartilage proteoglycan. Hoppe Seyler's Z. Physiol. Chem. 353: 1043-1052.

White,S.W., Hulmes,D.J.S., Miller,A. and Timmins,P.A. 1977. Collagen-mineral axial relationship in calcified turkey leg tendon by X-ray and neutron diffraction. Nature, (Lond.) 266: 421-425.

Williams,B.R., Gelman,R.A., Poppke,D.C. and Piez,K.A. 1978. Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results. J. Biol. Chem. 253: 6578-6585.

Williams,G. 1970. The late phases of wound healing: Histological and ultrastructural studies of collagen and elastic-tissue formation. J. Pathol. 102: 61-68.

Wischnitzer,S. 1973. The electron microscope. In: Principles and

techniques of electron microscopy, Vol. 3. (Ed: Hayat,M.A.) Van Nostrand Reinhold Co., New York, pp 1-51.

Witschel,H., Fine,B.S., Grutzner,P. and McTigue,J.W. 1978. Congenital stromal dystrophy of the cornea. Archive Ophthalmol. 96: 1043-1051.

Wolpers,C. 1943. Kollagenquerstreifung und grundsubstanz. Klin. Wochschr. 32: 624-625.

Wood,G.C. 1960. The formation of fibrils from collagen solutions. 3: Effect of chondroitin sulphate and some other naturally occurring polyanions on the rate of formation. Biochem. J. 75: 605-612.

Wood,G.C. and Keech,M.K. 1960. The formation of fibrils from collagen solutions. 1. The effect of experimental conditions: kinetic and electron microscope studies. Biochem. J. 75: 588-598.

Woodhead-Galloway,J., Hukins,D.W.L. and Wray,J.S. 1975. Closest packing of two-stranded coiled-coils as a model for the collagen fibril. Biochem. Biophys. Res. Commun. 64: 1237-1244.

Zambrano,N.Z., Montes,G.S., Shigihara,K.M., Sanchez,E.M. and Junqueira,L.C.U. 1982. Collagen arrangements in cartilages. Acta Anat. 113: 26-38.