ULTRASTRUCTURAL AND IMMUNOCHEMICAL STUDIES OF ELASTIN-ASSOCIATED MICROFIBRILS

by

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ABSTRACT

Elastic fibres consist of two morphologically distinct components, comprising an amorphous core of polymeric elastin surrounded by a peripheral mantle of microfibrils. Elastin-associated microfibrils possess different ultrastructural staining characteristics from amorphous elastin, on which basis they have been claimed to contain glycoprotein moieties. Many attempts have been made to differentially extract microfibrillar components, the products obtained ranging from heterogeneous protein mixtures to discrete glycoproteins. However, in no case has identity between these extracted materials and elastin-associated microfibrils been firmly established. As microfibrils precede the appearance of amorphous elastin in developing elastic tissues, a more precise understanding of the nature of microfibrils (and 'microfibrillar extracts') is essential if their role in elastogenesis is to be adequately delineated.

This study has used a combined biochemical, immunological and electron microscopic approach to study elastin-associated microfibrils. Experiments were carried out on foetal bovine tissues derived from animals in the third trimester (specifically of 200-220 days gestation) at which time elastogenesis has been shown to have reached its maximum rate. The specific aims were as follows:

i A critical re-evaluation of the extractability of elastin-associated microfibrils with reductive guanidinium chloride (GuHCl) solutions.

ii To raise an antiserum against such a 'microfibrillar antigen' extract, and study its tissue localization.

iii To examine elastin fibrillogenesis in cultures of foetal calf ligamentum nuchae fibroblasts to assess their potential as a source of a soluble form of microfibrillar antigen(s).

Finely homogenized foetal calf ligamentum nuchae was subjected to a cyclical series of extractions with saline, 6M GuHCl and chromatographically purified bacterial collagenase, in the presence of protease inhibitors. Electron microscopic monitoring

showed that microfibrils were solubilized by 6M GuHCl throughout the extraction schedule, without the need for reduction. All remaining microfibrils could be removed by further GuHCL extraction. The product of this reductive extraction, 'microfibrillar antigen' (MAg), consisted of a heterogeneous mixture of proteins, the extreme insolubility of which limited biochemical characterization.

A rabbit antiserum raised against MAg produced only a single precipitin band on immunodiffusion, but was shown by a more sensitive ELISA assay to be polyspecific. No significant cross-reactivity was found toward elastin or collagen, but some antibody activity directed against fibronectin and components of foetal calf serum was present in the crude antiserum. It was possible to remove this contaminating antibody activity by affinity chromatography, whilst retaining high titres toward the original extract. The purified antibody was later shown, however, to contain low-level activity against a new collagen-like (CL) glycoprotein, which has been isolated from elastin-rich foetal bovine tissues by Dr M A Gibson in this laboratory.

Immunohistochemical experiments demonstrated specific localization of anti-MAg antibodies to elastin-associated microfibrils in both foetal calf ligamentum nuchae and aorta, with a small amount of binding to thin filaments which form a loose network in the extracellular matrix of these tissues. Comparative studies with a highly-specific anti-(CL glycoprotein) antibody preparation revealed specific localization to these thin matrix filaments, but not to elastin-associated microfibrils. These studies have shown unequivocally that MAg contains components derived from elastin-associated microfibrils. In addition, it is clear that microfibril-related antigens can be found in GuHCl extracts of elastin-rich tissues obtained with or without reducing agents. The antigenically active components in these extracts were, however, found to be quite insoluble if the GuHCl concentrations were reduced. This precluded isolation of antigen by immunoprecipitation with anti-MAg antiserum.

Attempts were made to circumvent this problem by growing nuchal ligament fibroblasts in tissue culture in order to obtain a soluble form of 'microfibrillar antigen'. Explant cultures of foetal calf ligamentum nuchae fibroblasts were shown to

accumulate large numbers of morphologically typical microfibrils in the extracellular matrix, but no amorphous elastin was observed even after extended periods in culture. Labelling of cultures with [^{3}H]-fucose and [^{3}H]-proline demonstrated that a large number of newly-synthesized macromolecules were secreted into the medium. However, attempts to isolate microfibrillar precursors from such medium by immunoprecipitation with anti-(MAg) antiserum were unsuccessful.

These findings indicate that the 'microfibrillar' glycoproteins MFP I and MFP II, isolated by Sear and his co-workers in similar immunoprecipitation experiments, may be related to contaminating non-microfibrillar antibodies contained within the antiserum on which their work was based.

During the course of these studies, claims were made in the literature that elastin fibrillogenesis occurred in ear cartilage without the participation of identifiable microfibrils. As this seemed of major importance to determining the role of microfibrils in elastogenesis, a comparative ultrastructural study of elastic fibres in foetal calf and rabbit ear cartilage in vivo and in vitro was undertaken in order to establish the presence of elastin-associated microfibrils in these tissues.

It was shown that elastin-associated microfibrils were visible at the periphery of elastic fibres in native foetal calf and neonatal rabbit (2-day-old) ear cartilage, but appeared to be masked by surface condensation of proteoglycan-like material in young rabbit (sixteen-day-old) ear cartilage. Cultures of enzymically dissociated foetal calf and neonatal rabbit auricular chondrocytes, when maintained under scorbutic conditions, produced a scanty extracellular matrix of which microfibrils and amorphous elastin were the dominant components. In contrast, young rabbit auricular chondrocyte cultures accumulated an extensive, predominantly proteoglycan extracellular matrix with relatively few elastic fibres. As in native ear cartilage, elastin-associated microfibrils could not be identified due to the dense proteoglycan encrustation of the surface of these elastic fibres.

Ascorbate supplementation of foetal calf auricular chondrocyte cultures resulted in a massive increase in collagen fibrillogenesis with suppression of elastic fibre formation.

Immunofluorescent labelling of foetal calf auricular chondrocyte cultures with anti-MAg antibodies demonstrated the presence of fibrillar material in the extracellular matrix, implying that microfibrils produced by such cultures are antigenically similar to those found in intact tissues, and are present during elastogenesis in ear cartilage.

It is concluded that an affinity-purified polyclonal antibody preparation has been obtained which has good specificity for elastin-associated microfibrils, but which contains some residual contaminant activity, most notably against CL glycoprotein. Nevertheless, this antibody preparation provides a useful tool for monitoring tissue extracts in order to establish the presence of microfibrillar components. Further studies to elucidate the specific antigen(s) responsible for the observed anti-microfibrillar activity offer the possibility of substantial insights into the biology of microfibrils.

These studies have also shown that foetal calf ear cartilage chondrocyte cultures are a potentially useful model system for the study of elastogenesis in vitro, and have clearly demonstrated the presence of typical microfibrils in association with developing elastin in this tissue. Such cultures are technically simple to propagate and maintain, and readily produce both components of the elastic fibre, thus offering significant advantages over foetal calf ligamentum nuchae fibroblast cultures.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan.

Ian W. Prosser

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I am deeply grateful to my wife, Jenni, my family and friends, and my colleagues at the Royal Adelaide Hospital for their concern, encouragement and unselfish help during the completion of this work.

Indeed it is well said, 'in every object there is inexhaustible meaning; the eye sees in it what the eye brings means of seeing'.

Thomas Carlyle (1795-1881)

CHAPTER 1

INTRODUCTION

1.1 GENERAL

Elastic fibres are a ubiquitous component of mammalian connective tissues, and are present in virtually every organ of the body. Their physiological importance lies in the unique elastomeric properties of elastin, the fibrous protein which forms the functional component of the elastic fibre. Elastin is of particular importance to the structural integrity and function of tissues in which reversible extensibility or deformability are crucial, such as the major arterial vessels, the lung and the skin. Changes in these tissues due to senescence or involvement in disease states are associated with structural derangement and degradation of their elastic elements, perhaps accompanied by disordered resynthesis. The consequent loss of physiological functioning is exemplified by the major human 'degenerative' diseases, atherosclerosis and pulmonary emphysema.

Progress toward a comprehensive understanding of the pathogenesis of such changes in elastic fibres has, however, been inhibited by the intrinsic difficulties involved in the separation, isolation and biochemical identification of the purified protein. For this reason, pathological studies of disease states involving elastin have largely been limited to description of the structural alterations in the appearance of elastic fibres or changes in their tinctorial properties.

Recognition of the molecular nature of elastin and its soluble precursor, tropoelastin, has allowed considerable advances to be made in understanding the molecular biology of elastin. However, there are still major deficiencies in our knowledge of the factors controlling elastin biosynthesis and metabolism, particularly concerning the mechanism of elastin fibrillogenesis and its role in the morphological organization of elastic tissues. It is obvious that a more precise delineation of the physiology of elastogenesis is necessary in order to interpret the multiple changes which occur as a result of pathological processes.

The studies described in this thesis were initiated in an attempt to further our understanding of one aspect of elastin physiology, namely the role of elastin-associated microfibrils in elastin fibrillogenesis. Although the two-phase nature of elastic fibres

was clearly defined nearly twenty years ago (Fahrenbach et al., 1966; Greenlee et al., 1966), little progress has been made toward elucidating the chemical nature and structural significance of elastin-associated microfibrils. Since the studies of Ross and Bornstein (1969), many contenders for the title of 'microfibrillar protein' have been put forward. Most of these preparations, including the 'structural glycoproteins' derived from elastic tissues, remain very poorly characterized. In no instance has a clear relationship of any of these materials to elastin-associated microfibrils been established. Indeed there is still controversy as to whether elastin-associated microfibrils are a distinct entity or an ultrastructural artefact, and whether they are essential to elastin fibrillogenesis.

In view of this situation, the present study was undertaken to re-evaluate critically the extractability of elastin-associated microfibrils from developing bovine ligamentum nuchae and to study the tissue localization of antibodies raised against such a 'microfibrillar antigen' extract. Two model systems of elastogenesis in cultured cells (foetal calf ligamentum nuchae fibroblasts and auricular chondrocytes) were also studied in order to assess their potential as a source of a soluble form of microfibrillar antigen(s). The major aims and experimental plan of these investigations are outlined in section 1.9.

To place these studies in perspective, this introductory chapter will review the morphology and biochemistry of elastin and elastic tissues, with particular reference to elastin-associated microfibrils. Brief consideration will also be given to other elastic-tissue matrix glycoproteins, and to the relevant biosynthetic activities of cultured cells derived from bovine ligamentum nuchae and auricular cartilage.

1.2 ELASTIN AND ELASTIC TISSUES

1.2.1 Definitions

Elastic fibres were first defined operatively on the basis of their distinctive tinctorial properties when stained in histological preparations (see section 1.2.2). This concept has now been superseded by a more precise understanding of elastic fibres as connective tissue elements composed predominantly of the structural protein, elastin. This protein has mechanical properties similar to those of polymeric rubbers (Dorrington and McCrum, 1977), and is responsible for the long-range, reversible extensibility of elastic connective tissues. The physical properties of elastin have been reviewed by Gosline (1976).

The term 'elastic tissue' is applied to those connective tissues in which elastic fibres are the dominant fibrous components of the extracellular matrix. The ligamentum nuchae of ungulates, the vertebral ligamenta flava and vocal cords of primates, and the aorta and pulmonary arteries of most mammals have traditionally been regarded as 'elastic tissues'. It has been suggested that this definition should be expanded to include those tissues such as lung, skin and elastic cartilage in which elastic fibres, although present in small quantities, are essential to the maintenance of normal physiological function (Sandberg et al., 1981b).

Electron microscopic examination of elastic fibres has shown that they comprise two morphologically distinct components; a core of polymeric elastin, which appears amorphous and stains lightly with cationic heavy-metal stains, and a peripheral mantle of tubular-appearing microfibrils (diameter 10-14nm) which have a bead-like periodicity in longitudinal sections and display a strong affinity for cationic stains (Fahrenbach et al., 1966; Greenlee et al., 1966). Because they stain differently from the amorphous elastic material, the microfibrillar components of elastic fibres are thought to be chemically distinct from elastin, and have been claimed to be associated with a number of glycoproteins isolated from elastic tissues (Ross and Bornstein, 1969; Muir et al., 1976; Sear et al., 1981a,b). In this thesis the term 'elastic fibre' will be used to signify fibres containing both morphological components, whilst 'elastin' will be used to denote

the purified protein which lacks microfibrils. The studies reported herein concentrate almost entirely on the microfibrils of elastic fibres, and their relationship to elastogenesis. Therefore such microfibrils will be referred to as 'elastin-associated microfibrils' in order to distinguish them from morphologically similar microfibrils (apparently unrelated to amorphous elastin) which occur in non-elastic tissue (see section 1.3.5).

1.2.2 Morphology of Elastic Fibres

Elastic fibres can be recognized at light microscopic level by typical staining reactions, and at electron microscopic level by typical ultra-structural appearances.

Light Microscopy. Stains which have the ability to bind selectively to elastic fibres have been known to histologists since last century. Commonly used reagents include Gomori's Aldehyde-Fuchsin, Unna-Tanzaer's Orcein, Verhoeff's Iron-Hematoxylin and Weigert's Resorcin-Fuchsin (Pearse, 1968). Due to their apparent specificity these 'elastic stains' have found extensive use in the study of elastic tissues, and despite uncertainty as to their modes of action, staining of fibres with these dyes has often been taken as evidence for identity of composition.

Histological examination of elastic connective tissues has shown that elastin is distributed in three morphologically distinct forms:

1) In ligamentum flavum and lung, elastic fibres are found to be small, rarely exceeding 10μm in diameter, and variable in length. These rope-like fibres branch and fuse in an irregular manner. In tissues such as ligamentum nuchae, which are subjected to undirectional stresses, the elastic fibres have a preferred orientation parallel to the direction of stress. Because of this parallel arrangement, and its high elastin content, ligamentum nuchae has frequently been used in studies of elastic tissue. Wirtschafter et al. (1967) have described the histological changes which occur in developing ligamentum nuchae.

2) In the tunica media of the aorta, elastic tissue forms numerous concentric sheets, or lamellae, approximately 3μm in thickness, which are pierced by many small openings, or

fenestrae. These lamellae may be interconnected by 'bridges' of elastic tissue running between lamellae. Smaller longitudinal fibres predominate in the tunica intima, immediately subjacent to the endothelial surface, merging somewhat indistinctly with the medial layer (Ayer, 1964).

3) Elastic cartilages show a three-dimensional honeycomb arrangement of elastin, consisting of very large anastomosing fibres (Sheldon and Robinson, 1958). The morphology of elastic cartilage is described in detail in section 1.7.1.

In all elastic tissues, elastic fibres have intimate connections with collagen fibres, cells, and other matrix proteins, which possibly modify the physical properties and assembly of elastin within them. There has been some speculation that the morphological differences observed may be due to differing primary structures of elastins in each tissue. However, the compositions of both insoluble elastins (Starcher and Galione, 1976) and tropoelastins (Foster et al., 1980a) have been found to be remarkably similar in different tissues of given species. This does not preclude the possibility of differences in molecular organization, a question which is discussed in section 1.4.

Ultrastructural Studies. Elastic fibres in sections of osmicated, plastic-embedded material, stained with heavy metal salts, are seen to be composed of a light-staining core of apparently structureless, amorphous elastin, surrounded by an envelope of microfibrils (Fahrenbach et al., 1966; Greenlee et al., 1966). The homogeneous appearance of elastin in conventionally prepared specimens has been observed by a large number of investigators, and has been attributed to the blocking of staining either by embedding materials, or by fixatives (Quintarelli et al., 1973; Gotte and Volpin, 1974).

However, it has been reported that the intensity of staining of amorphous elastin decreases with the age of the animal, both in calf ligamentum nuchae (Fahrenbach et al., 1966) and rabbit ear cartilage (Cox and Peacock, 1977). Thyberg et al. (1979) have recently demonstrated that similar age-related changes in elastin staining can be observed in rat aorta using a variety of embedding media. The significance of this phenomenon is unknown, although it has been suggested that the decrease in stain uptake may correlate with increasing cross-linking of elastin (Fahrenbach et al., 1966).

Under some conditions, a fibrillar substructure has been demonstrated in fixed, embedded tissues. Fahrenbach et al. (1966) found that a tangled filamentous appearance could be demonstrated in immature elastic fibres in foetal bovine ligamentum nuchae, but not in mature elastin. Cliff (1971) showed that a fibrillar appearance could be produced in rat aortic elastin by prolonged (72 hours) treatment at 4°C with 2% osmium tetroxide before embedding. This revealed filaments with an average diameter of 4.4nm. Katsuda et al. (1982) confirmed this finding, and noted that a similar appearance could be produced in the elastic lamellae of rabbit carotid artery by extended (up to 150 hours) pre-embedding treatment of the tissue with periodic acid at 18°C.

Fibrillar structures have also been reported in purified elastin materials from both ligament and aorta, and a substantial amount of evidence has been accumulated which indicates that rather than being amorphous, elastin may have a filamentous or fibrillar substructure that is not apparent in material routinely prepared for transmission electron microscopy. These studies have, in the main, utilized either purified elastin which has been mechanically disrupted, or coacervates of hydrolyzed insoluble elastin, tropoelastin or synthetic elastin-like polypeptides, and may therefore not be representative of the situation in native elastin. Nonetheless, some interesting insights into the possible molecular organization of elastin have resulted.

Cleary and Cliff (1978), in a study of oxalic acid-hydrolyzed elastin (α-elastin), observed that negatively-stained coacervates of this material formed filaments of preferred diameter 1.6-1.8nm. Similar structures were found in ultrasonically disintegrated purified elastin from bovine ligamentum nuchae and aorta. The authors suggested that filaments of this diameter would be consistent with the β-spiral conformation proposed by Urry and his colleagues (Urry and Long, 1977) as a model for the folding of the repeating hydrophobic amino acid sequences found in tropoelastin, the monomeric precursor of elastin (see also section 1.4). In support of this contention, it has been shown that coacervates of both tropoelastin (Cox et al., 1974) and synthetic elastin penta- and hexa-polypeptides (Volpin et al., 1976) show fibrils composed of parallel-aligned filaments approximately 5nm in diameter.

Fibrils having a similar appearance have also been described by Gotte and Volpin (1974) and Gotte et al. (1976) in negatively stained preparations of highly purified elastin. These fibrils were made up of an array of 5nm filaments. They had a 'beaded' appearance, with an axial periodicity of about 4nm which varied according to the amount of longitudinal stretching apparent in the fibrils. A similar beaded periodicity has been noted by Serafini-Fracassini et al. (1978) and by Quintarelli et al. (1973) in low angle x-ray diffraction and electron microscopic studies of filaments derived from sonicated purified elastins. Gotte (1977) interpreted these findings as indicative of a helical arrangement of the elastin filaments, with cross-linking regions being present between filaments, bonding them into larger fibrillar aggregates. Serafini-Fracassini et al. (1978) have disputed this interpretation implying instead that cross-links are present only within the 'primary filament', with fibrils composed of such primary filaments being held together by non-covalent interactions.

Despite the apparent lack of consensus amongst different investigators concerning the interpretation of their findings, the remarkable resemblances between the filaments produced by such varied methods does imply a high probability that such structures are not merely artefactual.

Pasquali Ronchetti and coworkers (Pasquali Ronchetti et al., 1979; Fornieri et al., 1982) have used cryotechniques to examine elastic fibres from bovine ligamentum nuchae in order to compare appearances in intact native elastin with those in purified and disrupted preparations. No ordered structure was found in unstretched specimens in either longitudinal or transverse freeze-fracture replicas; however fibres stretched to 150-200% of their resting length showed a filamentous structure, oriented in the direction of stress. These filaments formed an interconnecting three-dimensional meshwork, with individual filaments being composed of globular subunits with a diameter of about 5nm. It was also found that negatively stained cryosections of native elastin had a very similar appearance to those described for negatively-stained, chemically-purified and sonicated elastin. These findings are of particular significance because the preparative technique employed obviates the possibility of structural

alterations in the specimen due to dehydration, chemical purification or mechanical disruption. In view of the evidence that the extensibility of elastin molecules depends on interaction with an aqueous environment (Weis-Fogh and Andersen, 1970; Gosline, 1978), maintenance of hydration appears an essential prerequisite for meaningful study of structure-function relationships within elastic fibres.

In summary, there is much ultrastructural evidence to suggest that elastin has an ordered supramolecular structure of a filamentous nature. However, the precise nature of such molecular organization has yet to be defined.

1.2.3 Elastin-like Materials: Oxytalan and Elaunin Fibres

Two other types of connective tissue fibres, which may be related to elastic fibres, have been described on the basis of their tinctorial affinities for elastic stains.

Oxytalan fibres, first described by Fullmer and Lillie in 1958, are fibres which stain with Gomori's Aldehyde Fuchsin only after oxidation with peracetic acid. Although first discovered in periodontal membranes, oxytalan fibres have since been described in a number of tissues, including skin, tendons, ligaments and blood vessels (Fullmer et al., 1974; Cotta-Pereira et al., 1977). Correlative light and electron microscopic studies of these fibres has shown that they consist of bundles of parallel microfibrils with apparently identical morphology to elastin-associated microfibrils; however, no amorphous material was seen within these arrays (Fullmer et al., 1974). Keith et al. (1977a) have shown that oxytalan stains can also identify developing elastic fibres in aorta, lung and ligamentum nuchae before it is possible to stain such fibres with conventional elastic stains. These authors suggested that oxytalan staining may be due primarily to the microfibrillar component of elastic fibres.

The second fibre type, first described by Gawlik in 1965, is known as the elaunin fibre. This fibre stains with some of the classical elastic stains, but not all, and at the ultrastructural level appears to consist of bundles of microfibrils interspersed with occasional patches of amorphous material (Cotta-Pereira et al., 1975). Gawlik (1965) observed that, during embryogenesis of human aorta, fibres having the tinctorial

properties of oxytalan fibres appeared first. These progressively acquired the staining properties of elaunin fibres, and finally appeared as elastic laminae, suggesting that such fibres may represent sequential stages in elastogenesis. Bradamante and Svajger (1977) have reported similar findings in developing rat auricular cartilage.

Cotta-Pereira et al. (1976; 1977) have reported that oxytalan, elaunin and elastic fibres form a continuous plexus of fibres throughout the dermis of the skin, with elaunin fibres occupying an intermediate position between fine oxytalan fibres in the papillary dermis and the thicker elastic fibres in the deeper layer of the reticular dermis. Electron microscopic examination showed that bundles of microfibrils, corresponding to oxytalan fibres, were extensions of elaunin fibres. These in turn were composed of identical microfibrils together with patches of amorphous material. Elaunin fibres were shown to be continuous with true elastic fibres, and the authors suggested, therefore, that the three types of fibres seen in light microscopy corresponded to consecutive stages of normal elastogenesis.

Kobayasi (1968) and Briggaman and Wheeler (1975) have described similar networks of microfibrillar bundles in the skin. These microfibrils were found to insert directly into the basal lamina, passing deep into the dermis perpendicularly from the epidermal-dermal junction. In later work Kobayasi (1977) was able to demonstrate that some of these microfibrillar bundles were continuous with morphologically distinct elastic fibres deeper in the dermis, thus implying a direct connection between the basal lamina and dermal elastic fibres via these structures. Tsuji (1980) confirmed these findings, and showed that microfibrillar bundles were preferentially distributed in the dermal papillae. The arrangement of this system of microfibrillar bundles in the skin has led Kobayasi (1977) and Cotta-Pereira et al. (1976; 1979) to postulate that they may serve to anchor the epidermis to the dermis.

Kobayasi et al. (1977) have also shown that dermal microfibrils are susceptible to reduction with dithiothreitol (DTT), but not to digestion by collagenase or elastase. The authors noted that similar properties have been reported for elastin-associated microfibrils (Ross and Bornstein, 1969), a finding which they interpreted as indicating a degree of compositional identity.

Streeten and Licari (1983) have recently demonstrated an interconnecting system of elastic fibres in the adult bovine ciliary body analogous to that described in skin. They showed that zonular fibres (which are ultrastructurally identical to elastin-associated microfibrils) had the staining characteristics of oxytalan fibres. These zonular fibres appeared to be in continuity with larger elastic fibres in the pars plana, via an intermediate network of elaunin-staining fibres extending through the ciliary processes. Regional variation of the architecture was thought to be due to differing vectors of tractional force in the anterior and posterior ciliary body. Ultrastructural examination of the elaunin-staining fibres showed them to be composed predominantly of microfibrillar bundles containing clumps of amorphous elastin, corresponding closely to those described in skin by Cotta-Pereira et al. (1975).

The presence of oxytalan- and elaunin-staining fibres in mature connective tissues has led Fullmer et al. (1974) and Streeten and Licari (1983) to propose that in certain situations bundles of microfibrils may be normal constituents of adult tissues, occurring independently of amorphous elastin. This implies that microfibrils may have a role in the physical functioning of some connective tissues, particularly those subjected to repetitive mechanical stresses, e.g. ocular zonules, skin, periodontium and tendon.

It should be noted that these differing concepts of the physiological role of microfibrils in 'pre-elastic' fibres are not necessarily mutually exclusive. Thus it may be postulated that the persistence of microfibrils into adult life is the result of a control mechanism which is able to modulate the process of elastogenesis so as to selectively prevent the deposition of elastin within these bundles. A much more precise understanding of the biochemical nature of microfibrils seems an essential prerequisite, however, before studies aimed at uncovering such a control mechanism could be designed.

1.3 ELASTIN-ASSOCIATED MICROFIBRILS

As ultrastructural appearances form the basis for the definition of microfibrils, it is inevitable that a major portion of the literature pertaining to them has involved electron microscopic experiments. Cleary and Gibson (1983), in a detailed review of elastin-associated microfibrils, have emphasized the need for caution in interpreting electron microscopic appearances in view of the known technical limitations of the instrument, and the potential for artefact production during specimen preparation. However, as no agreement has yet been reached regarding the biochemical identity of elastin-associated microfibrils (see section 1.3.3), ultrastructural observations will continue to be of importance in future studies of their structure.

1.3.1 Ultrastructural Studies

Fine filaments have been recognized in the extracellular matrix of many tissues, and have been described under a variety of names. These structures were first described as 'microfibrils' by Low (1961; 1962), who used this term to indicate all those filaments of less than 20nm diameter which did not possess the characteristic 64nm banding of collagen.

It has subsequently been shown that the smaller (diameter 3-5nm) filaments are proteoglycan in nature, as determined by their susceptibility to hyaluronidase digestion (Frederickson and Low, 1971; Mayer et al., 1981). Similarly, it has been found that some of the larger (diameter 18-20nm) unbanded matrix filaments which correspond to Low's original description are collagenous (Frederickson et al., 1977; Jones et al., 1980), or react immunohistochemically with labelled anti-fibronectin antibodies (Mayer et al., 1981). It has been suggested by Cleary and Gibson (1983) that such filaments should be excluded from the modern definition of microfibrils altogether, leaving the group of fibrils of mean diameter 11-14nm termed 'small microfibrils' by Low and his colleagues (Frederickson et al., 1977). This group of filaments are similar in ultrastructural appearance, have an affinity for cationic stains (e.g. uranyl acetate, and lead citrate) and have been described in a wide variety of tissues (see section 1.3.5). Those

microfibrils which are seen in association with elastic fibres form an identifiable subgroup, and were designated 'elastic fiber microfibrils' by Ross (1973). In this thesis, they will be referred to as elastin-associated microfibrils.

The typical appearance of elastin-associated microfibrils has been clearly described in a recent high resolution electron microscopic study of human, sheep, pig and bovine aortic elastic fibres (Fanning et al., 1981). In longitudinal sections, microfibrils were shown to have a lucent central axis with a linear array of dark-staining particles arranged along their surface, either as parallel lines, or in places appearing to spiral across the surface of the microfibril. At low resolution, an apparent 'beaded' periodicity consisting of alternating dark and light staining areas was observed. In transverse sections, the microfibrils were found to be nearly circular, consisting of a poorly stained or unstained central region between 4nm and 5nm in diameter, surrounded by 4 to 6 discrete, spherical, electron-dense subunits. No difference in morphology was apparent in different species, although a small, but significant, difference in microfibrillar diameter could be measured.

The different staining characteristics of elastin-associated microfibrils (in comparison to amorphous elastin) has stimulated interest in the possibility that their chemical composition may be distinct from that of elastin. Ross and Bornstein (1969; 1970) showed that elastin-associated microfibrils in foetal calf ligamentum nuchae were susceptible to digestion with trypsin and chymotrypsin, but were resistant to treatment with collagenase or elastase. Amorphous elastin however, was rapidly digested by elastase, but was unaffected by trypsin or chymotrypsin. Neither elastic fibre component was affected by treatment with hyaluronidase or β-glucuronidase. The same authors also claimed that microfibrils were able to be extracted selectively by 5M guanidinium chloride (GuHCl) only in the presence of the sulphydryl bond reducing agent, DTT. The resultant material was thought to be glycoprotein in nature, but this was not conclusively demonstrated. (These and subsequent studies are reviewed in detail in section 1.3.3.)

Yu and Lai (1970) demonstrated that elastin-associated microfibrils of developing rat aortic elastic fibres stained intensely with ruthenium red, a finding which they interpreted as confirming the presence of glycoprotein material in such microfibrils. However studies on the action of ruthenium red by Luft (1971) do not support such a restricted interpretation of this finding. He considered that glycosaminoglycans were the most likely matrix components to bind this stain, and noted that any polyanionic material (including acid glycoproteins) could also react with it.

Histochemical evidence of the presence of glycoprotein in elastin-associated microfibrils has come from a recent study by Birembaut et al. (1982). These authors showed that subendothelial microfibrils of rabbit aorta, exposed by prior collagenase digestion, would bind not only ruthenium red stain, but also the lectins Concavalin A and Ricinus Communis. Further digestion with chymotrypsin removed elastin-associated microfibrils completely and abolished the staining reactions described above, suggesting the presence of a glycoprotein component. It is possible, however, that the glycoprotein(s) responsible for this staining may have been derived from the plasma. Fanning and Cleary (1984) have shown that elastin-associated microfibrils of the aortic media react with alkaline bismuth subnitrate only after periodic acid oxidation (used according to the method of Ainsworth, 1972), indicating a glycoprotein moiety intimately associated with elastin-associated microfibrils.

However, neither of these studies has been able to indicate if the microfibrils themselves are composed of glycoprotein, or whether they simply have a surface coating of glycoprotein. The possibility that elastin-associated microfibrils may consist of two distinct components, i.e. a surface coating (perhaps of glycoprotein) and a dissimilar core, is discussed further in the following section 1.3.2.

1.3.2 Relationship of Elastin-associated Microfibrils to Elastogenesis

The temporal and morphological relationship of microfibrils to elastin in developing elastic fibres was firmly established by Fahrenbach et al. (1966) and Greenlee et al. (1966) in separate electron microscopic studies of elastogenesis in bovine ligamentum

nuchae. They showed that microfibrils appeared early in foetal life, preceding the development of amorphous elastin, as aggregates in parallel array. These aggregates were usually oriented parallel to the long axis of the ligament and were often found in close apposition to fibroblasts, sometimes occupying infoldings of the cell membrane. Definitive elastin appeared first as small clumps of amorphous material within bundles of microfibrils, which subsequently combined to form true elastic fibres. More recent work by Kewley et al. (1978) has served to confirm these earlier observations.

This sequence of elastogenesis seems to be consistent in a variety of tissues from different species, a similar process having been described in human aorta (Haust et al., 1965), rat flexor digital tendon (Greenlee and Ross, 1967), embryonic chick aorta (Takagi, 1969; Kadar et al., 1971), rat aorta (Albert, 1972), chick lung (Jones and Barson, 1971) and foetal lamb pulmonary alveolar septum (Fierer et al., 1977). Ultrastructural studies of cultured mesenchymal cells, including foetal bovine ligamentum nuchae fibroblasts (Jones et al., 1980) and smooth muscle cells derived from guinea pig (Ross, 1971), rabbit (Moskalewski et al., 1976; Toselli et al., 1981), rat (Hinek and Thyberg, 1977; Sandberg et al., 1981a) and pigeon (Wight et al., 1977) aortic tissues have indicated that a similar process also occurs during the in vitro formation of elastic fibres. The integral involvement of microfibrils in elastogenesis thus appears highly probable.

The mechanism by which elastic fibre formation occurs is, however, much less clear, and a good deal of speculation has appeared concerning the part played by microfibrils. Ross et al. (1977) have suggested that microfibrillar aggregates take the form, shape and orientation of presumptive elastic fibres, thus directing the morphogenesis of elastic fibres by acting as a 'scaffold' on which elastin is deposited. They further suggest that microfibrils may have a nett negative charge, thus tending to hold soluble elastin (positively charged) on to the microfibrillar aggregate until cross-linking and insolubilization has taken place. Support for this view has come from a study by Pasquali Ronchetti et al. (1981) on the ultrastructural alterations which occur in the elastic fibres of chicks rendered lathyritic by treatment with β-aminopropionitrile

(BAPN). They reported that new elastin is deposited on pre-existing elastic fibres as button-like appendices which lack normal microfibrils, despite the presence of microfibrils around apparently unaffected portions of the same fibre. They interpreted this finding as confirmation of the role of microfibrils in directing elastin morphogenesis, implying that deposition of elastin may continue in their absence, but in a disordered fashion. They also demonstrated increased amounts of material reacting with toluidine blue 0 or ruthenium red stains when these were added to the fixatives. They interpreted this as indicating increased amounts of proteoglycan-like material present both in the extracellular matrix, and surrounding (and sometimes within) the abnormal expansion of lathyritic elastin described above. The significance of this interesting observation is uncertain; it may be speculated that highly-charged proteoglycan molecules, in the absence of elastin-associated microfibrils, act as a nidus for ectopic elastin deposition, resulting in the anomalous elastin formations observed.

Another possibility, that microfibrils may serve as a 'registration peptide', has been raised by Sandberg (1976). In this model, microfibrils would serve to align tropoelastin molecules in precise register, so that cross-linking regions are juxtaposed prior to oxidation by lysyl oxidase, which may be bound to the microfibrils in a co-ordinate complex, thus giving rise to the observed periodicity (see section 1.3.1). The ultrastructural appearance of elastic fibres in experimental copper deficiency, in which super-abundant microfibrils are found to surround sparse amounts of abnormally electron-dense elastin (Waisman and Carnes, 1967; Fanning and Cleary, 1974), is consistent with such an hypothesis. In this case, although normal microfibrils are present, normal elastogenesis cannot proceed due to lysyl oxidase deficiency, leading to the pathologically immature, poorly cross-linked elastic fibres which have been described. It is of interest to note the similar morphology of elastic fibres found in Menkes' Syndrome (Oakes et al., 1976), a human genetic disease thought to be caused by defective intestinal absorption of copper, leading to a state of copper-deficiency (Danks, 1977; Kivirikko and Peltonen, 1982).

A third postulate, that microfibrils may actually coalesce directly to form amorphous elastin, has been raised recently by Fanning et al. (1981). They noted the affinity of elastin-associated microfibrils for ruthenium red, and suggested the possibility that such microfibrils consist of a glycoprotein 'coat' surrounding a central core, perhaps composed of tropoelastin. This packaging may facilitate the extracellular transport and aggregation of soluble elastin, after which the peripheral 'coat' may be stripped off the tropoelastin as it is cross-linked into amorphous elastin. Consistent with this contention, they found that in some areas in developing elastic fibres, microfibrils apparently lose their more dense staining peripheral coat to varying degrees, and assume a staining and consistency similar to amorphous elastin. This may possibly also explain the relatively greater uptake of cationic stains by newly-formed elastin (see section 1.2.2). The same authors have also suggested that variations in microfibrillar diameter may be due to differing degrees of cross-linking of tropoelastin chains contained within them, a theory which is compatible with the observation that microfibril diameters are increased in association with copper deficiency (Fanning and Cleary, 1974; Cleary and Gibson, 1983). Alternatively, species differences in the composition of the glycoprotein coat could account for the observed differences in microfibril diameter; this concept is able more readily to explain the observed increase in diameter of elastin-associated microfibrils during foetal and post-natal development (Cleary et al., 1981; Cleary and Gibson, 1983). It is of considerable interest that Fanning and Cleary (1984) have recently convincingly demonstrated by histochemical means that elastin-associated microfibrils contain, or are coated with, glycoprotein (see section 1.3.1).

Finally, cognizance must be taken of the possibility that microfibrils may not be involved in elastogenesis. This appears very unlikely, as the overwhelming amount of evidence has indicated their intimate association with elastic fibre deposition. The presence of morphologically-identical microfibrils in a wide variety of tissues which do not contain elastin does, however, suggest that microfibrils may not invariably be involved in elastogenesis, but could also have other functions independent of elastin. This possibility is discussed in section 1.3.5.

In conclusion, it can be stated that the association of microfibrils with elastic fibre formation is firmly established. At present, insufficient evidence exists to differentiate clearly between the varying theories regarding the mechanism of elastogenesis; however it is likely that each contains elements of the truth, and a final answer must await further investigation.

1.3.3 Attempts at Biochemical Characterization of Elastin-associated Microfibrils

Studies by Cleary et al. (1967), on the purification of elastin from bovine ligamentum nuchae, first indicated that elastin and elastin-associated microfibrils may be different proteins. They found that elastin isolated from foetal ligament contained significant amounts of a non-collagenous protein which had a high content of polar amino acids, and which could not be removed by subsequent alkaline hydrolysis. In adult tissues, which contain much smaller amounts of microfibrils, no such material was found, thus implying that the protein contamination of foetal preparations was due to the presence of microfibrils.

Since then a number of attempts have been made to isolate and characterize elastin-associated microfibrils, but as yet these have failed to yield homogeneous preparations which could be confidently assessed as being of microfibrillar origin. The study of Ross and Bornstein (1969) has served as the prototype for many later investigations. These authors extracted foetal bovine ligamentum nuchae with 5M GuHCl followed by bacterial collagenase in order to produce a relatively pure elastic fibre preparation, then further extracted this preparation with a sulphydryl-reducing agent, DTT, in 5M GuHCl solution. Dialysis of the resultant extract produced both soluble and insoluble components, which differed significantly from each other in amino acid analysis. Both components had amino acid compositions markedly different from that of elastin and collagen, being rich in polar amino acids, and lacking hydroxyproline, hydroxylysine and desmosines. The reported high cysteine content (70-80 residues/1000 residues) led to the postulate that the insolubility of microfibrils may be due, at least in part, to disulphide bonds. The relationship of this extract to microfibrils was inferred

from electron microscopic examination of pellets before and after extraction with DTT, which they showed led to a decrease in the amount of visible microfibrillar material. This 'microfibrillar' fraction was later found to contain sugar moieties, including hexose and hexosamine, and was thus claimed to contain one or more glycoproteins (Ross and Bornstein, 1970).

In this later study the preparative procedure was modified to include additional extractions with GuHCl, collagenase and sodium dodecyl sulphate (SDS) before the final extraction with DTT/GuHCl. In neither paper, however, was an electrophoretic analysis of the final product undertaken, nor were enzyme inhibitors used during extraction to prevent proteolysis of extracted macromolecules by released tissue proteases.

In later work from the same laboratory (Muir et al., 1976), the authors stated that further characterization of the 'microfibrillar' extract was not possible, as attempts at purification resulted in a heterogeneous preparation with a wide range of molecular weights.

Serafini-Fracassini et al. (1975a) have also reported the isolation of a glycoprotein from adult (3-year-old) bovine ligamentum nuchae, using a modification of the Ross and Bornstein (1970) protocol, again without the use of protease inhibitors. The extract obtained (which they named 'microfibrillin') had an apparent molecular weight of 14,000 (as determined by equilibrium sedimentation), contained some hexose, and had an amino acid composition substantially different from that reported by Ross and Bornstein (1969). Extreme insolubility hampered further characterization of the preparation, and no direct evidence was advanced to identify the origin of the extract. However, the low molecular weight, and the absence of protease inhibitors during the isolation procedures and subsequent manipulations, suggest that significant degradation may have occurred during extraction.

Recently, Serafini-Fracassini's group have repeated the above study with the addition of protease inhibitors to all buffers (Serafini-Fracassini et al., 1981a,b). The extract obtained, when analyzed by polyacrylamide gel electrophoresis, revealed the presence of a number of proteins, the fastest-moving of which was also PAS-positive.

This band was isolated in pure form by preparative electrophoresis, yielding a glycoprotein of apparent molecular weight 34,000 which possessed peptidyl-lysyl oxidase activity, as well as being able to react with free lysine as a substrate. Amino acid analysis of this glycoprotein differed significantly from the previously described 'microfibrillin'.

This glycoprotein showed a marked tendency to aggregate in the absence of GuHCl and mercaptoethanol, and could be induced to precipitate from solution by exposure to copper ions in 8M urea solution, followed by dialysis into distilled water. Electron microscopic examination of the resulting precipitate showed 'cylindrical tactoids' which had a diameter of 10-11nm. The authors suggested, on the basis of this morphological appearance, that the glycoprotein so characterized represented the microfibrillar component of elastic fibres. No further evidence for its relationship to microfibrils other than the electron microscopic appearances was advanced and it remains to be established if such extracts are derived from microfibrils. Immunohistochemical studies using an antibody to this glycoprotein may be of value in establishing its tissue origin.

Kawaguchi (1982) has also reported the isolation of two glycoproteins from the ligamentum nuchae of 2-year-old cattle. Protein extracts, which he called 'Fraction A' and 'Fraction B', were obtained by repetitively extracting ligament with 6M GuHCl solutions under non-reducing conditions and in the presence of 20mM DTT, respectively. Protease inhibitors were incorporated into all buffers. Purified glycoproteins A and B were then prepared from these heterogeneous fractions by a combination of DEAE-cellulose ion-exchange chromatography and gel filtration. Glycoproteins A and B were found to be similar in apparent molecular weight (about 35,000) on SDS-polyacrylamide gel electrophoresis, and in amino acid analysis. Both glycoproteins were resistant to digestion by bacterial collagenase. Kawaguchi noted that his glycoproteins were similar in size and amino acid analysis to the glycoprotein previously described by Serafini-Fracassini et al. (1981a,b). He suggested that glycoproteins A and B may therefore be derived from elastin-associated microfibrils, but no attempt was made to directly establish such a relationship. It should be noted, however, that elastic fibres in

ligamentum nuchae from 2-year-old cattle possess very few elastin-associated microfibrils (Kewley et al., 1978) and this tissue would appear to be an unsuitable starting material for the extraction of microfibrillar components.

In an effort to determine the composition of elastin-associated microfibrils, Kewley et al. (1977a) have attempted, by immunological means, to establish identity between components of the Ross and Bornstein 'microfibrillar protein' extract and these microfibrils. They modified the extraction procedure of Ross and Bornstein to include a more exhaustive pretreatment protocol, but did not use protease inhibitors. Foetal calf ligamentum nuchae was sequentially extracted with 6M GuHCl (extract G) followed by purified bacterial collagenase (extract C) repeated in the order GCGCG. 'Microfibrillar protein' was then solubilized by a final extraction with 6M GuHCl containing 0.1M 2-mercaptoethanol. The extract thus obtained proved to be heterogeneous on analysis by polyacrylamide gel electrophoresis, but the most prominent protein band observed had an apparent molecular weight of 135,000 and stained strongly with periodic acid/Schiff (PAS) reagent (Sear et al., 1977). Amino acid analyses of the crude extracts were similar to that reported by Ross and Bornstein (1969). An antiserum against this extract was raised in rabbits, and showed at least three precipitin lines on immunodiffusion. By a secondary immunization procedure, using the major precipitin complex as antigen, they produced an antiserum which showed only a single line on immunodiffusion against the original extract, and did not react against serum proteins, pepsin- or oxalic acid-solubilized elastins (α-elastin), or proteoglycans. Immunofluorescence studies revealed strong binding of antibody to partially-purified elastic fibres (residue GCGCG), but no reactivity could be observed to elastic fibres after treatment with 2-mercaptoethanol, or to polymeric collagen fibrils which had been pepsin-digested to remove contaminating glycoprotein. Electron microscopic localization, using a direct immunoperoxidase technique, revealed intense reaction with elastin-associated microfibrils and lesser, but significant, binding to collagen fibres.

Further immunofluorescent studies of the tissue localization of this anti-'microfibrillar protein' antibody (Kewley et al., 1977b) indicated that the antibody

localized widely in varied tissues, including bovine ligamentum nuchae, aorta, spleen, and human kidney, with localization to reticulin fibres and basement membranes as well as to elastic fibres. The authors interpreted these findings as establishing an 'immuno-logical relationship' between elastin-associated microfibrils and morphologically-similar microfibrils which have been observed in basement membranes and other non-elastic tissues (see section 1.3.5). In later work from this laboratory, Sear et al. (1978; 1981a,b) used the Kewley anti-'microfibrillar protein' antiserum to detect immunologically cross-reacting macromolecules elaborated by foetal calf ligamentum nuchae fibroblasts in tissue culture. Two newly-synthesized glycoproteins of apparent molecular weight 150,000 and 300,000 were found, the smaller of which appeared to be collagenous in nature, and was chemically unrelated to the larger species. Although this work is quite elegant, the apparent polyspecificity of the antiserum on which it is based emphasizes the need for cautious scepticism about the relationship of these glycoproteins to microfibrils. The rationale and results of this, and other experiments in which microfibrillar components have been sought in cell culture, are considered in greater detail in section 1.3.4.

Cleary and Gibson (1983), in a review of 'microfibrillar proteins', have warned against the use of amino acid analysis as a criterion for evaluating such preparations. They point out that the amino acid analysis reported by Ross and Bornstein (1969) for 'microfibrillar protein' is that which may be expected of a mixture of proteins (Reeck and Fisher, 1973). In addition, electron microscopic monitoring of residues from extraction experiments on foetal calf ligamentum nuchae in this laboratory (Cleary et al., 1981) have indicated that microfibrils are, in fact, progressively solubilized with successive extractions using 6M GuHCl. Similar observations have been reported in foetal calf ligamentum nuchae by Sear et al. (1981b), and in chick wing tendon by Gibson et al. (1982). These findings emphasize the need for caution in identifying such preparations as being 'microfibrillar' in origin, without confirming this by immunohistochemical means.

Gibson and Cleary (1982) have reported recently the isolation of a glycoprotein from foetal calf aorta and ligamentum nuchae, using a modification of the procedure of Kewley et al. (1977a). They included a cocktail of protease inhibitors in all buffers and used a highly purified collagenase preparation for the digestive steps. This glycoprotein was the principal PAS-positive species found in 6M GuHCl extracts (both with and without DTT), and had an apparent molecular weight of 140,000 on polyacrylamide gel electrophoresis when run under reducing conditions. This protein was further purified by ion exchange chromatography, followed by preparative polyacrylamide gel electrophoresis. The purified protein was found to contain significant quantities of collagen-like amino acids (hydroxyproline and hydroxylysine), and to be susceptible in the purified form to digestion with highly-purified collagenase. Antibodies to the aortic protein (which they called collagen-like, or CL glycoprotein) were raised in rabbits, and were shown by ELISA assay to cross-react strongly with CL glycoprotein prepared from ligamentum nuchae, but not with types I and III collagen, human and bovine fibronectin, bovine α-elastins or porcine tropoelastin. Immunofluorescent studies (Gibson and Cleary, 1983) showed antibody localization to the intercellular matrix of nuchal ligament and aorta, with some binding to the basement membranes of aortic smooth muscle cells. No localization to elastic fibres was observed, and it was noted that CL glycoprotein exhibited considerable overlap in distribution with the interstitial collagens.

CL glycoprotein is thus unlikely to be related to elastin-associated microfibrils. It does have distinct similarities to the 150,000 dalton collagenous glycoprotein isolated from cultures of foetal calf ligamentum nuchae fibroblasts by Sear et al. (1978; 1981a,b), suggesting that the latter protein is probably also unrelated to elastin-associated microfibrils (see section 1.3.4). However, this postulate has yet to be confirmed. CL glycoprotein has also been noted to have similar properties to the short-chain collagens derived from pepsin digests of aortic intima and placenta (Jander et al., 1983; Odermatt et al., 1983).

It must be concluded that the composition of elastin-associated microfibrils is still obscure. However, it is clear that the immunological approach, taken in the studies of Kewley and Sear (as described above) is a promising way to tackle this problem. Using such a method, antigen extracts can be directly studied, and the origins of the extracted species much more closely defined.

The studies reported in Chapters Two and Three of this thesis are directed along the general lines established by these workers, in an attempt to confirm and extend their observations. The results of these studies have been reported in papers by Cleary et al. (1981) and Prosser et al. (1984).

Mention must also be made of the large and heterogeneous group of connective tissue proteins, called 'structural glycoproteins', some of which may be related to microfibrillar components. These are briefly considered in section 1.5.1.

1.3.4 Elastin-associated Microfibrils in Tissue Culture

Attempts to purify matrix proteins which are possibly related to elastin-associated microfibrils (variously designated as 'microfibrillar protein' or 'structural glycoproteins') have produced a wide range of materials. The products are usually extracted with strong denaturing solvents, are extremely insoluble and thus difficult to purify and characterize, and have commonly been heterogeneous. These difficulties have prompted the investigation of cell culture techniques as an alternative source of microfibrillar materials. Cell cultures have particular advantages for the study of soluble precursors of extracellular matrix proteins which are insoluble in the mature forms, and they have provided a great deal of information about collagen precursors and tropoelastin (recently reviewed by Burke and Ross, 1979). It is possible that precursors of elastin-associated microfibrils could similarly be secreted into the medium, or be retrievable in a more soluble form from the extracellular matrix of cell cultures which are able to synthesize elastin. Several workers have used cultures of vascular smooth muscle cells and of nuchal ligament fibroblasts for this purpose.

a. Smooth muscle cells

It has been shown that vascular smooth muscle cells from a variety of species are capable of synthesizing both tropoelastin and insoluble elastin in culture (Ross, 1971; Abraham et al., 1974; 1977; Narayanan et al., 1976; Snider et al., 1981; Oakes et al., 1982). Burke and Ross (1979) and Chamley-Campbell et al. (1979) have reviewed the biosynthetic capabilities of cultured smooth muscle cells.

Ross (1971) first demonstrated that guinea pig aortic smooth muscle cells were capable of synthesizing both components of elastic fibres. Harvested cell layers were extracted with 5M GuHCl followed by collagenase digestion, according to the method described by Ross and Bornstein (1969), but no reducing step was used. The insoluble residue remaining after extraction was shown by ultrastructural examination to be rich in microfibrils. Amino acid analysis of this residue was similar to that of the 'microfibrillar protein' previously reported by Ross and Bornstein (1969; 1970). No further characterization of this material was carried out.

Prompted by 'numerous unsuccessful attempts' to obtain an homogeneous protein by applying the Ross and Bornstein (1969) technique to foetal calf ligamentum nuchae, Muir et al. (1976) attempted to isolate newly-synthesized 'microfibrillar protein' from the extracellular matrix laid down by monkey aortic smooth muscle cells in culture. Electron microscopic examination of the cell layer showed that the extracellular matrix of such cultures contained large quantities of elastin-associated microfibrils, elastic fibres, collagen and basement-membrane material.

Cultures were incubated with [^{3}H]-cystine in order to label newly-synthesized macromolecules, and the cell layer was then extracted with 1M NaCl containing 0.1% Triton X-100, followed by two extractions with 5M GuHCl, the second containing DTT. All buffers contained protease inhibitors. Polyacrylamide gel electrophoresis showed a major [^{3}H]-cystine labelled glycoprotein band to be present in the medium as well as in all three extracts of the cell layer. This glycoprotein aggregated under non-reducing conditions, was resistant to digestion with collagenase, and had an apparent molecular weight of 270,000 or 220,000, depending on whether gels were cross-linked with N,N^1-

diallyltartardiamide or methylene bisacrylamide. It was the only radioactively-labelled protein present in reductive extracts of the cell layer.

The authors suggested that the proteins found in the medium and in extracts of the cell layer may represent a soluble subunit of microfibrils. However, they pointed out that the glycoprotein found in labelled medium shared many features of 'fibroblast cell surface protein' (later shown to be fibronectin). Subsequent work from Ross's group has confirmed this association, and Burke and Ross (1979) state that the glycoprotein isolated was 'similar or identical to ... fibronectin'.

Schwartz and her co-workers have recently published studies of matrix proteins elaborated by calf aortic smooth muscle cells in culture (Schwartz et al., 1980; 1982). The extracellular matrix was examined electron microscopically, and the authors stated that, under scorbutic conditions, microfibrils formed the only identifiable matrix component. Cultures which were supplemented with ascorbate produced both collagen and microfibrils, but no amorphous elastin was recognized in either supplemented or unsupplemented cultures. Immunofluorescence studies using an anti-fibronectin antibody showed a 'web-like pattern of extracellular fibrils', indicating the presence of matrix fibronectin in ascorbate-supplemented cultures.

After extensive pretreatment, harvested cell layers from scorbutic cultures were finally extracted with a 1% SDS/0.33M mercaptoethanol buffer in the presence of protease inhibitors. In this heterogeneous extract two major protein bands could be identified on polyacylamide gel electrophoresis. The smaller of these proteins (apparent molecular weight 45,000) was identified as actin. The larger protein (apparent molecular weight 200,000) was found to contain significant carbohydrate, and reacted with anti-fibronectin antiserum on immunodiffusion assay. Although the authors have suggested that this latter material may be related to the 'soluble subunit of microfibrils' described by Muir et al. (1976), it seems certain that both of these glycoproteins were, in fact, fibronectin. There is no direct evidence that fibronectin is a component of elastic fibres or elastin-associated microfibrils (see section 1.5.2), and it is unlikely that either of these preparations is of microfibrillar origin.

Jones et al. (1979), in a study of the synthetic properties of cultured smooth muscle cells derived from rat heart, have described the preparation of a large fucosylated glycoprotein by prolonged extraction of the SDS-insoluble cell matrix with an 8M urea buffer containing 1% SDS and 1% mercaptoethanol. This protein had an apparent molecular weight of 250,000 and was said to be similar to both the 'microfibrillar protein' of Muir et al. (1976) and to fibronectin. However, no further effort was made to establish such a relationship, and there is no direct evidence to suggest this material was derived from elastin-associated microfibrils.

b. Bovine ligamentum nuchae fibroblasts

In a series of studies, Sear et al. (1978; 1981a,b) have used an antibody raised against 'microfibrillar protein' by Kewley et al. (1977a) to detect immunologically cross-reacting macromolecules secreted into the medium by foetal calf ligament fibroblasts in culture.

In initial studies (Sear et al., 1978) it was stated that cultures were propagated from a foetus of about 45 days gestation. As a foetus of this age would have a crown-rump measurement of only 3.1cm (Bogart, 1959), it is improbable that the ligamentum nuchae could be reliably identified or obtained from such a foetus in quantities sufficient to establish explant cultures. It is therefore likely that the stated gestational age is a typographical error. However, the authors published an electron micrograph of 240-day-old foetal calf ligamentum nuchae in their paper, and it seems probable that the donor age was nearer this figure, i.e. 245 days. This point is of some significance, as Mecham et al. (1984) have recently shown that donor age has a marked effect on the synthetic capabilities of cultured ligamentum nuchae fibroblasts (see section 1.6.2).

Cultures were maintained under scorbutic conditions in these initial experiments. Electron microscopic examination of the cell layer showed large numbers of microfibrils, but insoluble elastin was not observed, even after 5 weeks in culture. Cultures were incubated with [^{3}H]-fucose, and newly synthesized glycoproteins secreted into the medium were detected by polyacrylamide gel electrophoresis. Five major fucosylated species were found, two of which could be immunoprecipitated from labelled medium by

the anti-'microfibrillar protein' antiserum of Kewley et al. (1977a), These glycoproteins had apparent molecular weights of 150,000 and 300,000, and were called 'MFP I' and 'MFP II' respectively (Sear et al., 1981a,b).

As a control, 'microfibrillar protein' was prepared from foetal calf ligamentum nuchae by the method of Kewley et al. (1977a), with the addition of protease inhibitors to all buffers. The resulting extract showed a number of PAS-positive bands, one of which corresponded in electrophoretic mobility to MFP I (apparent molecular weight 150,000). A similar glycoprotein could also be extracted under non-reducing conditions from the cell layer of cultures maintained for 2 weeks post-confluence.

It was thus proposed that MFP I was related to elastin-associated microfibrils. MFP I and MFP II were thought to be either immunochemically related, or physically associated with one another.

Further characterization of these glycoproteins was undertaken in later work from this group (Sear et al., 1981a,b). The smaller, MFP I was found to contain 4-hydroxyproline and hydroxylysine, and was susceptible to digestion by highly purified collagenase, whereas MFP II (the larger glycoprotein) contained no hydroxyproline, and was resistant to collagenase digestion. MFP I and MFP II also differed in solubility in ammonium sulphate solutions. Further, ascorbate supplementation of cultures produced a doubling of the synthesis of MFP I, whilst that of MFP II was unaffected. Neither MFP I nor MFP II could be precipitated from labelled medium by anti-fibronectin antiserum, although significant amounts of fibronectin could be isolated in this way.

The authors concluded that MFP I was a novel collagenous glycoprotein, which, on the basis of the specificity of the Kewley anti-'microfibrillar protein' antibody, formed a component of elastin-associated microfibrils. MFP II (which appeared to be chemically unrelated to MFP I) was thought to be associated with MFP I in an unspecified manner, perhaps as a component or organizer of microfibrils.

Despite the elegance of this later work, the antibody preparations on which it is based have not been adequately characterized (see Chapter Two) and they exhibit apparent polyspecificity on immunohistochemical localization (Kewley et al., 1977a,b).

It is of interest to note the similarity of MFP I to CL glycoprotein isolated from foetal bovine ligamentum nuchae by Gibson and Cleary (1982), which has been discussed in section 1.3.3. CL glycoprotein has been shown in immunohistochemical studies (Gibson and Cleary, 1983) not to be associated with elastic fibres. It has a tissue distribution which is quite distinct from that reported for the anti-'microfibrillar protein' antibody preparation of Kewley and his co-workers (Kewley et al., 1977a,b).

Lamberg et al. (1980) have recently reported a study in which they attempted to isolate microfibrils from calf ligamentum nuchae fibroblast cultures, using saturated fluorescein mercuric acetate as an extractant. The resultant product was examined electron microscopically, and it was claimed that a microfibril-rich fraction had been obtained. Amino acid analysis of this material was similar to that reported for 'microfibrillar protein' by Ross and Bornstein (1969). Microfibrils obtained by this method were also shown to be susceptible to digestion with trypsin, but not with elastase, collagenase or hyaluronidase. However, the information presented by these authors is insufficient to identify positively their preparation as being of microfibrillar origin, and further characterization is needed.

In conclusion the question of identity of extracts purporting to be 'microfibrillar' is one which needs to be approached with considerable caution. So far, no specific property or biological activity can be ascribed to microfibrils which will allow their confident identification in biochemical preparations. The use of amino acid analysis, based on the data of Ross and Bornstein (1969; 1970) is suspect and insufficient adequately to identify extracted materials as being derived from microfibrils (Cleary and Gibson, 1983). The studies of Kewley et al. (1977a,b) and Sear et al. (1978; 1981a,b) have approached this problem by attempting immunochemical identification of microfibril-associated moieties; however the antibody preparation on which this work is based is clearly polyspecific (see Chapter Two). Despite this objection, it is the only approach so far in which proof of a relationship to microfibrils has been sought, and it seems capable of refinement and thus to be more likely to yield definite results than unmonitored extraction experiments.

1.3.5 Microfibrils in Non-elastic Tissues

It is now known that microfibrils which have the typical morphology described above, are not unique to developing elastic fibres, and apparently-identical structures have been demonstrated convincingly in tissues which are devoid of elastin. These include the ciliary zonule (Raviola, 1971), the mesangium of the kidney (Farquhar, 1978; Hsu and Churg, 1979) and a variety of tissues which contain oxytalan fibres (discussed in detail in section 1.2.3).

The ciliary zonule acts as a suspensory ligament for the ocular lens, binding it to the ciliary body, thus forming the boundary separating the anterior and posterior chambers of the eye. Raviola (1971) showed that ciliary zonules of monkey, cow and human eyes were composed of microfibrils of diameter 11-12nm, arranged in bundles or sheets. These microfibrils had an indistinct periodicity of 49nm in longitudinal section, but only when observed in tightly-packed bundles. Cross-sections showed a tubular appearance with heavily-staining periphery and lucent core, very similar to that described for elastin-associated microfibrils (see section 1.3.1). These microfibrils were not digested by collagenase, but disappeared after treatment with chymotrypsin, thus showing similar enzyme susceptibility to that described for elastin-associated microfibrils by Ross and Bornstein (1969).

Amino acid analysis of whole human and bovine ocular zonules revealed no hydroxyproline or hydroxylysine, a low content of glycine and relatively high levels of cysteine and acidic amino acids (Buddecke and Wollensak, 1966; Swann and Streeten, 1978; Streeten et al., 1983). Polyacrylamide gel electrophoresis of zonular material solubilized after sonication and reduction with DTT in 5M urea revealed at least five peptide bands, ranging in molecular weight from 35,000 to 170,000 daltons, as well as high molecular weight material which would not enter a 3% gel (Streeten et al., 1983).

Further analysis of this material suggested the presence of at least two different glycoconjugates, one a xylose-containing proteoglycan, the other a sialofucose-containing glycoprotein. Similarities with the compositional data of Ross and Bornstein (1969) led the authors to suggest that zonules were chemically similar to elastin-associated microfibrils.

Streeten et al. (1981) have shown recently that antibodies, raised against sonicated whole bovine ciliary zonules, localized immunohistochemically to elastin-associated microfibrils in bovine ligamentum nuchae and chick aorta, as well as to the ciliary zonules of both bovine and human eyes. Using an indirect immunoperoxidase technique, they demonstrated a periodic localization of reaction products at 35-45nm intervals on microfibrils in each of these tissues. Some reaction was also seen on collagen fibres, often coinciding with collagen banding sites. These appearances were similar to those reported by Kewley et al. (1977a), using an anti-'microfibrillar protein' antiserum prepared against an extract of foetal bovine ligamentum nuchae (see section 1.3.3). The authors considered that there was a 'structural similarity or identity between components of the zonules and microfibrils of elastic tissue', and pointed out the possibility that in systemic connective tissue diseases such as Marfan's syndrome and homocystinuria, where vascular elastic tissue abnormalities and lens dislocation co-exist, the primary defect may reside in the microfibrils rather than in collagen or elastin.

More recently, Streeten and Licari (1983) have shown that zonular microfibrils reacted strongly with an anti-'microfibrillar protein' antibody, prepared by these authors against an antigen extracted from foetal calf ligamentum nuchae using the method of Kewley et al. (1977a). Immunoperoxidase reaction product bound to microfibrils showed a 'macroperiodicity' of 40-45nm, similar to that observed in their earlier experiments with an anti-zonular antibody (Streeten et al., 1981). Some minor labelling of collagen fibres was again apparent. They concluded that zonular and elastin-associated microfibrils share antigenic determinants, and 'belong to the same family of fibrils'. They also noted that zonules display strong elasticity in situ during accommodation, a characteristic which appears to be unrelated to the presence of elastin.

Microfibrils, of typical appearance, were first observed in the renal mesangium by Farquhar and co-workers (Farquhar et al., 1961; Farquhar, 1978), and have recently been described in detail by Hsu and Churg (1979). These authors found morphologically-similar microfibrils in the intima of small renal arteries and in the mesangium, often in association with amorphous basement membrane-like material. These microfibrils were

measured to be 11-13nm in diameter and appeared tubular, with occasional evidence of 'beaded' periodicity in longitudinal sections. Cross-section showed a lucid core of 6-7nm diameter, surrounded by a darker peripheral coat consisting of some 8-10 densely-staining dots about 2-3nm in diameter. These appearances are very similar to those described for elastin-associated microfibrils, and it is of interest to note that Kewley and his co-workers showed immunofluorescent localization of their anti-'microfibrillar protein' antiserum to glomerular capillaries and tubular basement membranes in human kidneys (Kewley et al., 1977b). The immunohistochemical studies presented in Chapter Three of this thesis have also shown localization of an anti-(microfibrillar antigen) antiserum to the mesangium of the foetal bovine kidney. These studies have been reported in the paper by Prosser et al. (1984).

The evidence presented above suggests that, in addition to the observed morphological resemblance, immunochemical similarities may exist between elastin-associated microfibrils and those which have been described in non-elastic tissues. These findings raise the possibility that such microfibrils may play a role in maintaining the physical and functional integrity of the tissues in which they are found, independently of elastin. However, until the chemical identity of microfibrils has been established (see section 1.3.3) their role in non-elastic tissues must remain speculative.

1.4 MOLECULAR BIOLOGY OF ELASTIN

Whilst it must be concluded at present that little is known with certainty about the biochemical identity of elastin-associated microfibrils (see section 1.3), the chemical and physical structure of elastin has been described with much greater precision. Elastin has been shown to exist in two closely related forms, being synthesized initially as a soluble precursor molecule, tropoelastin, which subsequently becomes chemically cross-linked to form the insoluble, amorphous core of elastic fibres. Current knowledge of the molecular biology of elastin is briefly reviewed in this section.

1.4.1 Insoluble Elastin

Insoluble elastin is a chemically inert, highly elastomeric, fibrous protein which is extremely resistant to dissolution. Prior to the discovery of tropoelastin, it was possible to define elastin only in operational and qualitative terms as the insoluble protein residue remaining after all other tissue components had been solubilized. Conventional methods for isolating insoluble elastin have usually involved a combination of autoclaving of elastic tissues followed by extraction with hot dilute alkali, based on the early investigations of Lowry et al. (1941) and Lansing et al. (1952). The rationale and practical applications of these methods have been discussed in detail by Jackson and Cleary (1967).

Elastin prepared in this way has a characteristic amino acid composition, containing about 95% non-polar residues. The hydrophobic amino acids glycine, alanine, valine and leucine together comprise approximately 80% of the total amino acid residues. Glycine alone accounts for approximately 30% of total residues, and the content of valine is the highest of any known protein. Purified elastin contains a small amount (1-1.5%) of hydroxyproline, but no hydroxylysine, troptophan, cysteine, methionine or histidine (Jackson and Cleary, 1967; Sandberg, 1976; Rucker and Tinker, 1977; Sandberg et al., 1981b; Foster, 1982). This unusual amino acid composition is sufficiently distinctive to allow confident use of amino acid analysis as a means of identifying purified elastin.

The composition of insoluble elastins prepared from different tissues of a given species by hot alkali extraction have been shown to be remarkably similar (Gotte et al., 1963), and it has been suggested on this basis that elastin may exist as a single genetic type in all tissues. However, Sandberg (1976) has pointed out that alkaline extraction of elastic tissues may lead to degradation of some labile amino acids and partial hydrolysis of the purified elastin, which may therefore be reduced to a common composition.

More recently a number of less drastic methods have been described for the purification of insoluble elastin which utilize various combinations of treatments with enzymes, denaturants and mild hydrolytic agents (Jackson and Cleary, 1967; Steven and Jackson, 1968; Ross and Bornstein, 1969; Richmond, 1974; Rasmussen et al., 1975; Serafini-Fracassini et al., 1975b; Starcher and Galione, 1976; Paz et al., 1982). In general, the amino acid composition of elastins obtained by these methods closely resembles those obtained from similar tissues by hot-alkali extraction, with the exception that more polar amino acids and fewer NH_2-terminal amino acids are present.

Keith and his co-workers (Keith et al., 1977b; 1979; Paz et al., 1982) have found that elastin purified from elastic cartilage by the use of such a mild isolation technique differed significantly in levels of acidic and hydroxyl amino acids from that obtained from ligamentum nuchae. These findings led them to suggest that cartilage elastin may be of a different genetic type to that found in other tissues. Foster (1982) has, however, noted that although the use of mild extraction procedures may result in less degradation of purified elastin, the problem of contamination of such preparations with closely associated proteins may well be magnified. Therefore claims regarding the existence of different genetic types of insoluble elastin made on the basis of compositional differences should at present be regarded with some caution.

In its native form, insoluble elastin is a polymer of linear polypeptide (tropoelastin) chains, randomly cross-linked into a highly extensible, three-dimensional network by an unusual system of lysine-derived compounds (Gallop and Paz, 1975). These cross-links are derived from the side-chains of lysine residues found as closely-spaced pairs on tropoelastin molecules. Lysine pairs occur in areas of alanine enrichment, suggesting a

special type of secondary structure in cross-linking regions, possibly an α-helix (Gray et al., 1973; Anwar, 1977; Franzblau et al., 1977; Gray, 1977).

The biosynthesis of elastin cross-links is not entirely clear, but the initial step involves the oxidative deamination of lysine residues to allysine (e-aminoadipic acid δ-semialdehyde) by the copper-dependent metallo-enzyme lysyl oxidase (Pinnell and Martin, 1968; Siegel et al., 1970). This appears to be the only enzymatic step involved in cross-link formation (Siegel, 1979). A newly-formed allysine residue may then combine either with a lysine residue to form dehydrolysinonorleucine (which may itself be reduced to form lysinonorleucine), or may react with another allysine residue to form an 'aldol condensation product' (Foster et al., 1974). This aldol condensation product may itself condense with the side-chain of a lysine residue to form dehydromerodesmosine and its reduction product, merodesmosine (Paz et al., 1971a,b). Ultimately three allysine residues and one lysine residue undergo cyclization via a complex and largely unknown mechanism to form the tetrafunctional amino acid isomers demosine and isodesmosine (Thomas et al., 1963; Gallop and Paz, 1975).

Although a number of pathways for the formation of desmosines have been proposed (reviewed by Gallop and Paz, 1975; Sandberg, 1976 and Rucker and Tinker, 1977), the two which at present seem most likely are the reaction of the aldol condensation product with dehydrolysinonorleucine (Foster et al., 1974; Gray, 1977) or the reaction of dehydromerodesmosine with an allysine residue (Francis et al., 1973). It should be noted, however, that all of the lysine-derived compounds so far described (with the exception of allysine) can theoretically serve as cross-links (Gallop and Paz, 1975; Rucker and Tinker, 1977; Foster, 1982). This system of cross-links theoretically enables the formation of inter-molecular linkages between two, three or four tropoelastin molecules as well as intra-molecular linkages.

1.4.2 Soluble Elastin - Tropoelastin

The identification of elastin cross-links, and the recognition that they arise from lysine residues by the action of lysyl oxidase has provided the basis for the isolation of the

soluble elastin precursor, tropoelastin. As lysyl oxidase requires copper as a co-factor (Siegel et al., 1970), induction of dietary copper deficiency results in inhibition of inter-molecular cross-linking of tropoelastin. Soluble elastin then accumulates in developing elastic tissues in sufficient amounts to be successfully extracted and characterized. Tropoelastin has been isolated in this way from the aortae of copper-deficient pigs (Sandberg et al., 1969; Smith et al., 1972) and chicks (Rucker et al., 1973) and the ligamentum nuchae of copper-deficient calves (Whiting et al., 1974).

Elastin cross-linking can also be inhibited by the lathyrogens β-aminopropionitrile (BAPN) and α-aminoacetonitrile (AAN) which irreversibly inactivate lysyl oxidase, probably by covalently bonding to it (reviewed by Siegel, 1979). It has been shown that tropoelastin can be extracted from lathyritic chick aorta (Sykes and Partridge, 1974; Foster et al., 1975) and from the aorta and ear cartilage of lathyritic pigs (Foster et al, 1980a).

Tropoelastin obtained from both copper-deficient and lathyritic animals has a molecular weight of 72,000-74,000 daltons, and contains approximately 850 amino acid residues (Sandberg et al., 1977). Tropoelastin has been firmly established as a precursor of insoluble elastin by several criteria (Sandberg et al, 1981b): 1) it has an identical amino acid composition to insoluble elastin apart from a much higher content of lysine, apparently due to conversion of lysine residues into cross-linking compounds (Sandberg et al., 1969); 2) the addition of [^{14}C]-lysine-labelled tropoelastin to an unlabelled smooth muscle cell culture results in the appearance of the label in desmosine and isodesmosine (Narayanan and Page, 1976); 3) antibodies prepared to oxalic acid-solubilized elastin (α-elastin) cross-react with tropoelastin (Daynes et al., 1977; Mecham and Lange, 1982).

In recent years, some controversy has arisen as to whether tropoelastin is the initial gene product in elastin biosynthesis. Studies by Foster et al. (1977, 1978), Rucker et al. (1977) and Heng-Khoo et al. (1979) have indicated the existence of high molecular weight species of soluble elastins (90-140,000 daltons), claimed to be primary gene products and to represent 'pro' forms of tropoelastin analagous to the collagen

precursor, procollagen. Further investigations, including cell free translation studies of purified elastin mRNAs, have failed to substantiate these claims, however, and it now appears that elastin is first synthesized as a 70,000-75,000 dalton molecular weight protein (Burnett and Rosenbloom, 1979; Foster et al., 1980b; Rosenbloom et al., 1980; Davidson et al., 1981). Although the nature of the high molecular weight soluble elastins is unclear, the isolation by Abraham and Carnes (1978) of a cross-linked dimer of elastin, molecular weight 150,000 daltons, suggests that covalently linked dimers of soluble elastin (or fragments thereof) may explain the origin of such 'proelastins'. The sensitivity of tropoelastin to proteolytic degradation (Sandberg et al., 1975; Mecham and Foster, 1977) would tend to support this contention.

New evidence also suggests that there are two tropoelastin species present in cell free translation products from elastin mRNAs, which have been called tropoelastin **a** (molecular weight 73,000 daltons) and tropoelastin **b** (molecular weight 70,000 daltons). Tropoelastin **a** and **b** have been found to be produced by elastin mRNAs from a number of tissues, including chick aorta and lung (Cheah et al., 1981; Foster et al., 1980b, 1981a) and pig aorta and lung (Barrineau et al., 1981a). Although these proteins were reported to share antigenic determinants and were chemically very similar, significant differences were found in overall charge, differential isotope incorporation and peptide mapping. Tropoelastin **b** behaved identically to conventionally prepared soluble elastin, and Foster et al. (1981a) have proposed that tropoelastin **a** represents a novel protein. Barrineau et al. (1981b) have reported that the proportion of tropoelastin **a** and **b** appears to change in chick aorta during development, but not in chick lung, and they postulated that differing ratios of tropoelastin **a** and **b** may vary the molecular organization of insoluble elastin in different tissues. The possibility that these proteins are separate gene products is intriguing, but further evidence is necessary in order to show that they do not arise from genetic polymorphism. It is of interest to note that Rich and Foster (1984) have recently reported the isolation of a protein, with similar properties to tropoelastin **a**, from lathyritic chick aorta during the course of studies aimed at producing a 'microfibrillar' extract. This protein was purified from a reductive GuHCl

extract of aorta by DEAE-cellulose chromatography. The relationship of this protein to the 'microfibrillar protein' extracts of Ross and Bornstein (1969) and Kewley et al. (1977a,b) is not clear. However, the use of a radically different tissue source as well as a different pretreatment protocol and isolation method imply that these studies are not directly comparable.

1.4.3 Molecular Models of Elastin

The possible arrangement of tropoelastin monomers within insoluble elastin has also been a source of debate. Sequence data from tryptic peptides of tropoelastin (Foster et al., 1973; Gerber and Anwar, 1974; Sandberg et al., 1977) indicate the presence of large areas of hydrophobic amino acid residues within the molecule, in which repeating pentapeptide (Val-Pro-Gly-Val-Gly) and hexapeptide (Ala-Pro-Gly-Val-Gly-Val) sequences can be discerned. Both of these repeating sequences contain the β-turn as the dominant conformational feature, and it has been suggested that polymers of each repeat sequence may form a right-handed β-spiral structure (Urry and Long, 1977; Urry, 1978). Urry (1983) has proposed a 'fibrillar' model of elastin in which large areas of β-spiral structures form the extensible part of the molecule between small cross-linking regions of α-helical structures. This model is consistent with many of the ultra-structural observations of elastin-derived materials which were discussed in section 1.2.2.

A similar model which differs significantly in the conformation of the extensible coil segment of the molecule has also been proposed (Gray et al., 1973; Sandberg et al., 1981b). This 'oiled-coil' model views the extensible segment as a broad left-handed spiral of repeating tetrapeptide units (Gly-Val-Pro-Gly), interspersed with α-helical cross-linking regions. A common feature of both of these models is their reliance on hydrophobic interactions with an aqueous environment for the maintenance of elasticity.

A third model, originally proposed by Partridge (1966) and modified by Weis-Fogh and Anderson (1970), envisaged elastin as a two-phase system consisting of globules of

compact elastin cross-linked together, surrounded by free aqueous spaces. However this model is not consistent with NMR observations (Torchia and Piez, 1973), and does not allow for volume changes (Hoeve and Flory, 1974). The latter authors also pointed out that similar mechanical objections apply to the 'oiled-coil' model discussed above.

The fourth major model views elastin essentially as a random network of cross-linked tropoelastin chains similar to that found in polymeric rubbers (Hoeve and Flory, 1974). This model, and the 'fibrillar' model of Urry (1983), are perhaps best able to explain the elastomeric properties of elastin.

Whichever of these model systems is closest to the truth, the possibility should be recognized that the structure of elastins in different tissues may vary widely, as suggested by the comparative peptide sequences of Gerber and Anwar (1974). The different morphological organization of elastin found in vascular tissue, ligament and elastic cartilage (see section 1.2.2) is consistent with this possibility, and reinforces the need for caution in generalizations about the molecular ordering of elastin.

1.4.4 Biosynthesis of Elastin

It is likely that elastin is synthesized on membrane-bound ribosomes and secreted into the cisternae of the rough endoplasmic reticulum (Mecham, 1981a; Rosenbloom, 1982). Cell-free translation studies of elastin mRNAs indicate that nascent tropoelastin chains contain a leader or 'signal' peptide (Foster et al., 1980b; Foster et al., 1981a; Karr and Foster, 1981; Davidson et al., 1982a) which may serve to direct the growing protein into the endoplasmic reticulum (Blobel and Dobberstein, 1975).

Tropoelastin undergoes several post-translational modifications before export from the cell. The leader sequence is removed by proteolysis (Rosenbloom, 1982; Sandberg et al., 1982) and some proline residues are hydroxylated. The functional significance of the presence of hydroxyproline in tropoelastin is uncertain. In contrast to collagen, post-translational hydroxylation of proline residues in tropoelastin appears to be unimportant in fibrillogenesis. Lack of proline hydroxylation does not affect the secretion of tropoelastin (Uitto et al., 1976; Mecham et al., 1981), the oxidation of lysyl residues

(Narayanan et al., 1976; 1978) or the incorporation of tropoelastin into elastic fibres (Abraham et al., 1974) These findings have led to the suggestion that proline residues in elastin may be 'accidentally' hydroxylated by collagen prolyl hydroxylase, present in the endoplasmic reticulum as a consequence of the concomitant synthesis of procollagen by the cell (Rosenbloom, 1982).

An alternative view has been put forward by Sandberg and his co-workers (Sandberg et al., 1982). They have found collagen-like sequences in chick tropoelastin (Smith et al., 1981), implying that prolyl hydroxylase may specifically 'recognize' these sequences.

Transport of processed tropoelastin from the cell to the site of fibre formation is equally uncertain. Tropoelastin may be packaged in the Golgi complex, and subsequently secreted from the cell via 'acanthosomes' (Fahrenbach et al., 1966; Thyberg et al., 1979; Damiano et al., 1981). Electron microscopic studies suggest that elastic fibre formation may occur in the extracellular matrix quite separately from the cell. As microfibrils appear early in foetal life, preceding the development of amorphous elastin (Fahrenbach et al., 1966; Greenlee et al., 1966), it has been postulated that elastin-associated microfibrils may have a role in regulating the morphogenesis of elastic fibres, possibly by determining the initial alignment of elastin precursors (Ross et al., 1977). The possible involvement of microfibrils in elastogenesis has been discussed in detail in section 1.3.2.

Once fibre deposition has begun, further growth probably involves the interaction of tropoelastin monomers (Abraham et al., 1977) or multimers (Abraham and Carnes, 1978) with the nucleus of insoluble elastin. Subsequently, these small fibre units may themselves coalesce to form the larger elastic fibres observed in elastic tissues.

Cross-linking of tropoelastin chains is initiated by lysyl oxidase (see above sections 1.4.1 and 1.4.2), which has been shown to be associated with the developing elastic fibre extracellularly (Kagan et al., 1974). However, it is not known if this enzyme is secreted with tropoelastin, or in what way it is complexed with the developing elastic fibre. It has been suggested that microfibrils may serve as 'registration peptides' for aligning cross-linking regions in tropoelastin (Sandberg, 1976), or that they may themselves represent a tropoelastin-lysyl oxidase complex (Cleary and Gibson, 1983). In regard to

the latter proposal, it is of interest to note that a 'structural glycoprotein' (molecular weight about 34,000 daltons) has been isolated from bovine ligamentum nuchae which has peptidyl-lysine oxidase activity (Serafini-Fracassini et al., 1981a,b). It is possible that this species may be a component of elastin-associated microfibrils, perhaps (as suggested in the model proposed by Fanning et al., 1981) packed around a tropoelastin core. Electron microscopic localization of antibodies to this glycoprotein may be of value in investigating this possibility.

Developmental studies of elastin deposition in bovine ligamentum nuchae (Cleary et al., 1967) and sheep ligamentum nuchae (E.G. Cleary, personal communication) have indicated that an intense period of elastin biosynthesis begins during gestation near the onset of the last trimester and diminishes after the early post-natal period. Studies of elastin biosynthesis by cultures of bovine (Mecham et al., 1981; 1984) and sheep nuchal ligament fibroblasts (Davidson et al., 1981), sheep lung (Shibahara et al., 1981), and chick aorta (Eichner and Rosenbloom, 1979; Keeley, 1979) have also shown clear correlation between increasing accumulation of elastin in vivo and rates of synthesis in vitro. These studies suggest that elastin accumulation in many tissues is regulated at the level of protein synthesis. Cell-free translation studies of elastin mRNA's isolated from developing tissues including chick aorta (Burnett et al., 1980), sheep nuchal ligament (Davidson et al., 1981; 1982b) and sheep lung (Shibahara et al., 1981) have further indicated that elastin biosynthesis is largely controlled by the elastin mRNA content of these tissues. Quantification of elastin mRNA levels in developing chick aorta using an elastin cDNA probe (Burnett et al., 1982) has confirmed these observations.

The factors which affect the developmental control of elastin gene expression are, however, not as clear. The extracellular matrix has been shown to influence tissue development and cellular differentiation in most vertebrate tissues (Slavkin and Greulich, 1975; Bissell et al., 1982). Recent studies by Mecham et al. (1981; 1984) suggest that the extracellular environment may act as an inducer of elastin synthesis by bovine ligamentum nuchae fibroblasts. As matrix macromolecules are non-diffusible,

these authors suggest that the effects of the extracellular matrix in modulating the synthetic activities of the cell may be due to structural changes in the matrix surrounding the cell. Frisch and Werb (1983) have shown that changes in cell shape influence tropoelastin gene expression in rat aortic smooth muscle cells, an observation which is consistent with the above hypothesis. Alternatively a tissue-specific matrix 'inducer' molecule may be postulated which appears during development, and which may also function as a feed-back control (Mecham et al., 1984). It is possible that both of these mechanisms operate to a variable extent in differing elastic tissues, but it must be concluded that the mechanisms of elastin gene control by cell-matrix interactions are as yet largely unexplored.

Metabolic turnover of mature, cross-linked elastin appears to be extremely slow and the half-life of rat aortic elastin has been estimated to be approximately 40 years (Rucker and Tinker, 1977). However, Starcher and Goldstein (1979) have shown that some matrix elastin is continuously degraded and replaced by newly-synthesized protein. In addition, elastin degradation is markedly increased in a variety of pathological conditions, including the major human diseases of emphysema and atherosclerosis (reviewed by Ayer, 1964; Sandberg, 1976; Sandberg et al., 1981b). Elastin synthesis may be re-activated in mature tissues by destruction of elastin, as shown by Kuhn et al. (1976) in a study of elastase-induced emphysema in hamsters.

The factors which may affect elastolysis and turnover in the extracellular matrix are complex and have not yet been clearly defined. Full discussion of this aspect of elastin metabolism is outside the scope of this section, but recent reviews by Bieth (1978), Barrett and McDonald (1980) and Werb et al. (1982) provide an overview of the subject. In general terms, it has been postulated that cellular control may be achieved in a number of ways. Thus the amount, activity and substrate affinity of lysyl oxidase may be modulated by inhibitors or activators; the interactions between tropoelastin and the other components of the elastic tissue matrix may be subtly changed; or the activity of extracellular proteins that degrade elastin precursors or elastic fibre constituents may be modulated. As an example, Kagan et al. (1981) have recently shown

that synthetic or naturally occurring amphiphile ligands, including fatty acids and bile salts, can markedly stimulate or inhibit digestion of elastin by pancreatic elastase, whilst exerting a reciprocal effect on elastin oxidation by lysyl oxidase. These studies suggest the possibility of a multi-component control mechanism, in which different species of substrate ligands may modulate biosynthetic, reparative and degradative metabolism of elastin in a complementary fashion. A further example of a potential control mechanism is suggested by studies which have shown that tropoelastin and peptides derived from insoluble elastin by elastase digestion act as chemoattractants for fibroblasts (Senior et al., 1980; 1982). It is therefore possible that elastin degradation products participate in directing reparative elastic synthesis.

In conclusion, it has become evident over recent years that elastin biosynthesis and metabolism is a far more dynamic process than was previously believed. The interactions of elastin with the surrounding extracellular matrix and in turn with the cell surface are now receiving more attention than ever before. It is likely that these studies will, in the future, allow a much more complete understanding of the biology of elastogenesis and its pathological alterations.

1.5 ELASTIC TISSUE MATRIX GLYCOPROTEINS

Elastic connective tissues contain a variety of proteins, including the fibrous proteins, elastin and collagen, and two types of protein-carbohydrate complexes, namely proteoglycans and glycoproteins. Glycoproteins are components of many of the 'microfibrillar protein' extracts described in the preceding section, as well as of the rather ill-defined group of 'structural glycoproteins', tissue fibronectin, and other recently discovered basement membrane proteins such as laminin (Timpl et al., 1979) and entactin (Carlin et al., 1981). The relationships between these rather diverse proteins are not clear, but they are present to some extent in extracts of elastic tissues. They are thus of potential importance in any consideration of elastin-associated microfibrils.

1.5.1 Structural Glycoproteins

A significant amount of glycoprotein can be extracted relatively easily from elastic tissue (e.g. with physiological saline), and a large fraction of this appears to derive from the extravascular pool of serum proteins (Fricke, 1960; Enselme et al., 1961). Other glycoproteins, which are insoluble under physiological conditions, can only be isolated from connective tissues by harsh chemical extraction, or following enzymic digestion of collagen. The taxonomy and biochemical characteristics of this group of proteins are somewhat controversial, and have been reviewed in detail by Anderson (1976; 1981).

'Structural glycoproteins' (SGP) have been claimed to include those non-collagenous connective tissue glycoproteins which have been extracted using a denaturant (GuHCl, urea or SDS) with or without a reducing agent (2-mercaptoethanol, DTT or sodium borohydride), or by the use of strong acid or alkali solutions (Bach and Bentley, 1980). Proteins which have been claimed to belong to this group have a wide range of molecular weights. It is not clear whether this heterogeneity is due to aggregation or polymerization of subunits (Barnes and Partridge, 1968), degradation of extracted proteins (Moczar et al., 1977) or simply to the presence of a large number of proteins (Brown et al., 1977).

Characterization of such preparations largely rests on the amino acid composition of material solubilized by relatively harsh reagents. Thus preparations have been described which have been isolated from tissues following autoclaving (Keeley et al., 1969; Keeley and Labella, 1972), extraction with hot trichloroacetic acid (Moczar and Robert, 1970; McCullagh et al., 1973), digestion with collagenase of unspecified purity (Timpl et al., 1968; Wolff et al., 1971) or treatment with dilute sodium hydroxide (Barnes and Partridge, 1968; Timpl et al., 1969). All of these treatments may result in degradation of proteins and oligosaccharide side-chains. Also, in most studies no attempt was made to inhibit tissue proteases released during the extraction of whole tissue. This leads to the further possibility of enzymic degradation of solubilized proteins (Anderson, 1976).

'SGP' has been isolated from almost every connective tissue so far studied, such extracts being assumed to represent the same tissue fraction on the basis of extractibility, amino acid and carbohydrate composition. Aorta has been extensively studied, and very high yields of 'SGP' have been claimed, including 10% of pig aorta (Moczar and Robert, 1970), 17% of elephant thoracic aorta and 44% of human thoracic aorta (McCullagh et al., 1973). Although these preparations were probably contaminated with collagen, fibronectin and cellular protein, it is hard to account for the extraordinarily large amounts of material extracted.

Some studies have also suggested identity of 'SGP' and elastin-associated microfibrils on the basis of electron microscopic examination of extracted material. (Robert et al., 1971; McCullagh et al., 1973; Richmond, 1981). These claims are subject to the same problems of identity outlined in section 1.3.3, and should be treated with caution. In fact the very existence of structural glycoproteins as a discreet entity has been called into question by Bach and Bentley (1980). Their attempts to isolate 'SGP' from bovine aorta led them to the rather surprising conclusion that actin, myosin and collagen were the major identifiable components of extracts which, according to the literature, should have contained large amounts of 'SGP'. Although Bach and Bentley's studies may represent an extreme view, the work was carried out very carefully and their findings highlight the difficulties associated with extraction of glycoproteins from elastic tissues.

1.5.2 Fibronectin

Fibronectin is a large, non-collagenous glycoprotein which is present in a wide variety of mammalian tissues in both an insoluble form associated with cell surfaces and connective tissue matrices, and in a soluble form in plasma and other body fluids (Ruoslahti et al., 1981). Prior to the general acceptance of the term 'fibronectin' (Kuusela et al., 1976), different forms of the protein had been described under a variety of names including: large external transformation sensitive (LETS) protein, soluble fibroblast (SF) antigen, cell surface protein (CSP), cell adhesion factor (CAF), galactoprotein a, z protein, cold insoluble globulin (CIg), opsonic protein, cell-spreading factor and anti-gelatin factor (reviewed by Mosesson and Amrani, 1980 and Pearlstein et al., 1980).

Interest in this glycoprotein was initially stimulated by observations that fibronectin is lost from the surface of fibroblasts as a consequence of transformation in vitro by oncogenic viruses (Hynes, 1976; Vaheri and Mosher, 1978). It was subsequently recognized that the plasma protein known as cold insoluble globulin (CIg) was antigenically identical to cell- and tissue-associated fibronectin (Ruoslahti and Vaheri, 1975; Ruoslahti and Engvall, 1978). Both forms of fibronectin exhibit close chemical similarity, but differ in solubility (Yamada et al., 1977; Alexander et al., 1978) and biological activity (Hynes et al., 1978; Yamada and Kennedy, 1979). It is not yet certain if the observed differences between these two forms of fibronectin are due to post-translational modifications, or whether they represent separate gene products (Ruoslahti et al., 1981).

Fibronectin is synthesized by a wide range of connective tissue cells in vitro, usually as fibrillar matrices that are situated on cells, between cells, and between cells and the substratum (reviewed by Vaheri and Mosher, 1978, and Ruoslahti et al., 1981).

Cellular fibronectin, although membrane-bound, does not appear to be a conventional intrinsic membrane protein (Marciani and Bader, 1975; Hynes et al., 1976) and can be removed from cell surfaces in substantially pure form by treatment with low concentrations of urea (Yamada et al., 1978). In addition, much of the cellular fibronectin is secreted into the culture medium without being incorporated into an extracellular matrix (Yamada and Olden, 1978).

Fibronectin monomers have an apparent molecular weight of about 220,000 daltons, and are rich in cysteine residues which participate in both intra- and inter-chain disulphide bonds (Mosesson et al., 1975; Hynes et al., 1978; Wagner and Hynes, 1979). In plasma, cerebrospinal fluid and cell-culture medium, fibronectin occurs primarily as a disulphide-bonded dimer of approximately 450,000 daltons molecular weight (Chen et al., 1977; Keski-Oja et al., 1977; Jaffe and Mosher, 1978; Mosesson, 1978), whilst fibroblast cell surface fibronectin appears to exist largely as multimers, perhaps due to a higher degree of interchain disulphide-bonding than is found in the plasma forms (Hynes and Destree, 1978; McConnell et al., 1978).

Both forms of fibronectin contain about 5% carbohydrate linked to the polypeptide backbone, but there are slight differences in carbohydrate composition between the two forms (Carter et al., 1978; Wrann, 1978; Fukuda and Hakamori, 1979). However, amino acid compositions of both plasma and cellular types have been reported by a large number of laboratories, and in all cases are very similar. Peptide mapping techniques have also indicated a high degree of homology between plasma and cellular fibronectin (Vuento et al., 1977; Birdwell et al., 1980).

Small differences have been found in the electrophoretic mobility of the two forms. Cellular fibronectin migrates as a single band, whereas plasma fibronectin forms a closely spaced doublet, with apparent molecular weights of approximately 210,000 and 220,000 (Yamada and Kennedy, 1979; Birdwell et al., 1980). Ruoslahti et al. (1981) have suggested that the observed electrophoretic differences between fibronectins from different sources may be mostly due to variations in the glycosylation of the fibronectin molecule.

Immunofluorescence studies have shown that fibronectin is widely distributed in connective tissue in vivo, having been found in a variety of basement membranes, around smooth muscle cells, in the sinusoidal walls of the liver, in the stroma of lymphatic tissue and in the loose 'reticular' connective tissues of a number of organs (Linder et al., 1975; 1978; Stenman and Vaheri, 1978; Courtoy et al., 1982). Fibronectin also has an apparent co-distribution with type I and type III collagen both in foetal

dermis (Linder et al., 1978) and in fibroblast cultures (Bornstein and Ash, 1977; Vaheri et al., 1978), a finding which correlates with its affinity for collagen in vitro. Studies have indicated that fibronectin will bind to all known collagens, exhibiting a stronger affinity for denatured rather than native collagens, regardless of type (Engvall et al., 1978; Jilek and Hormann, 1978).

Electron microscopic immunohistochemical studies have demonstrated localization of anti-fibronectin antibodies to a variety of tissue components within cells, on cell surfaces and in the extracellular matrix (reviewed by Ruoslahti et al., 1981). Although binding of anti-fibronectin antibodies has been reported to 'primary microfibrils' of the notochord region of the developing chick (Mayer et al., 1981) and to thin filaments (less than 5nm in diameter) in the extracellular matrix of cultured chick embryo fibroblasts (Chen et al., 1978), no interaction with elastin-associated microfibrils has yet been documented (Cleary and Gibson, 1983).

Immunohistochemical studies in human vascular tissues have demonstrated strong localization of antibodies to the luminal side of the internal elastic lamina, in the region of the basement membrane of the endothelial surface, in both elastic and muscular arteries. Additional localization of antibodies to the adventitial layer and around smooth muscle cells in the medial layer was found in muscular arteries (Linder et al., 1978; Stenman and Vaheri, 1978). A similar distribution of fibronectin in foetal calf thoracic aorta has been described by Gibson and Cleary (1983). These authors demonstrated clearly that fibronectin was not related to elastic fibres in this tissue by the use of antibodies to both tropoelastin and to elastin-associated microfibrils. These studies are consistent with the biochemical observations of Ruoslahti and Engvall (1978), who found that fibronectin did not interact with soluble elastin.

It should be noted, however, that Natali et al. (1981) have described a topographical association of fibronectin with vascular elastic fibres. These authors claimed that anti-fibronectin antibodies localized to both sides of the internal elastic lamina of the aorta, renal artery and renal vein in the rat, but there was no evidence of localization to the other elastic lamellae in the aortic medial layer. In view of the rather inconsistent

nature of the antibody binding observed, one is reluctant to accept their interpretation that fibronectin is co-distributed with elastic fibres in blood vessels. The significance of their findings, which are at variance with the immunohistochemical data reported by other workers, is uncertain.

The postulated functions of fibronectin are manifold, and relate to the observed interactions of fibronectin with collagen, fibrin, glycosaminoglycans and cell surfaces (Ruoslahti et al., 1981). Studies of cellular fibronectin in vitro have implied a role for fibronectin in cell adhesion (Yamada et al., 1978; Pearlstein and Gold, 1978) and cell motility (Ali and Hynes, 1978). In addition, studies of fibronectin localization in embryonic tissues suggest that cellular expression of fibronectin may be linked with cell differentiation and organogenesis (Linder et al., 1975; Wartiovaara et al., 1976; 1978; Kurkinen et al., 1979), both in the positioning and migration of cells and in organization of the extracellular matrix.

It has been suggested that plasma fibronectin may be of importance in wound healing, due to its affinity for fibrin(ogen) and collagen (Engvall, 1978). The binding of fibronectin to these proteins during clot formation at a site of injury may provide a scaffold for cellular infiltration of the wound whilst giving greater tensile strength to the organizing thrombus (Ruoslahti et al., 1981). Fibronectin may also act as an opsonin (Saba and Jaffe, 1980), thus facilitating phagocytosis of cellular debris in these areas.

1.5.3 Basement Membrane Proteins

The term 'basement membrane' is used to denote the specialized extracellular matrix layer which separates an epithelial surface from the underlying connective tissue. Basement membranes are found in a diverse range of tissues, including the renal glomeruli and tubules, pulmonary alveoli, cardiac and skeletal muscle, around vascular smooth muscle cells, and underlying the endothelium of blood vessels. The epithelial surfaces of the urogenital and gastrointestinal systems, and the epidermis are also separated from the subjacent connective tissue by a continuous basement membrane.

Basement membranes can be demonstrated histologically by PAS or silver impregnation stains, and are seen by light microscopy as a thin layer closely applied to the base of the epithelium.

The ultrastructure of basement membranes varies between tissues, but (when stained with uranyl and lead salts) usually consists of the electron-lucent 'lamina rara' adjacent to the plasmalemma of the epithelial cell and an outer electron-opaque lamina densa, which in some tissues appears to possess a filamentous substructure. Underlying the basement membrane a 'reticular layer' composed of fine collagen fibrils and microfibrils (which resemble those associated with elastic fibres) is often seen. This layer is probably mostly responsible for the light microscopic appearance of basement membranes. The morphology and structure of basement membranes has been reviewed by Kefalides et al. (1979), Grant et al. (1981), Briggaman (1982) and Martinez-Hernandez and Amenta (1983).

The major functions of basement membranes appear to fall into four broad categories:

1) They act as differential permeability barriers to the passage of macromolecules across epithelial surfaces, a property which is of most importance in capillaries, especially those of the renal glomerulus (Farquhar, 1978);

2) They provide flexible physical support for epithelial lining cells, for example, in such structures as the renal tubules, blood vessels and ocular lens capsule. Type IV collagen may be of particular importance to this function.

3) Some basement membrane components may facilitate cell attachment, particularly type IV collagen (Stanley et al., 1980) and laminin (Terranova et al., 1980).

4) Laminin and type IV collagen may also play a role in directing morphogenesis in developing tissues (Ekblom et al., 1980; Leivo et al., 1980).

The chemical composition of basement membranes is not yet completely clear, owing partly to the difficulty in isolating basement membrane material free of contaminants, and partly to their relative insolubility under mild conditions (Grant et al., 1981). However in recent years these problems have been overcome to some extent by

the use of cell lines which secrete large amounts of basement membrane-like material in culture. Cell-lines derived from murine neoplasms such as the EHS-tumour (Orkin et al., 1977) and embryonal carcinoma (Chung et al., 1979) have proved of particular importance. Biosynthetic experiments using these cell lines have enabled characterization of a number of discrete glycoproteins associated with basement membranes.

The macromolecular components of basement membranes can be classified as either collagenous (type IV and V collagens) or glycoprotein (fibronectin, laminin, entactin, heparan sulphate proteoglycan and bullous pemphigoid antigen) in nature. Discussion of the basement membrane collagens is beyond the scope of this section, but recent reviews by Kefalides et al. (1979), Bornstein and Sage (1980), Grant et al. (1981), Sage (1982) and Martinez-Hernandez and Amenta (1983) give details of their structure and metabolism. However, in view of the association of microfibrils with basement membrane structures and their possible glycoprotein composition (see section 1.3), a brief discussion of some of the known glycoprotein components of basement membranes is necessary.

Fibronectin. Although immunofluorescence studies have suggested that fibronectin is a component of basement membranes (Linder et al., 1975; Stenman and Vaheri, 1978; Wartiovaara et al., 1979), the immunohistochemical experiments of Martinez-Hernandez and his colleagues have failed to confirm this association (Boselli et al., 1981; Martinez-Hernandez et al., 1981). These workers found that anti-fibronectin antibodies localized to the connective tissue stroma adjacent to (but not within) the basement membrane in most tissues. Minimal binding was, however, observed to basement membranes with an important filtration function, such as Reichert's membrane and glomerular basement membrane. It has been shown that plasma fibronectin can become insolubilized in extracellular matrices both in vitro (Hayman and Ruoslahti, 1979) and in vivo (Oh et al., 1981) and it is thus possible that plasma fibronectin becomes trapped in these membranes during filtration and is not an intrinsic component. The presence of fibronectin within such basement membranes may nonetheless be of functional significance.

Laminin. This glycoprotein was first isolated from the matrix of cultured EHS-tumour cells by Timpl et al. (1979), and from embryonal carcinoma-derived cells (under the name GP-2) by Chung et al. (1979). Laminin has a molecular weight of about 900,000 daltons, and consists of three A chains (molecular weight about 200,000 daltons) and one B chain (molecular weight about 400,000 daltons) which are linked by disulphide bonds (Timpl et al., 1982). Both epithelial and endothelial cells in culture have been shown to produce laminin, but mesenchymal cells do not appear to secrete this glycoprotein (Foidart et al., 1980). Antibodies to laminin localize exclusively to basement membranes (Foidart et al., 1980; Madri et al., 1980) and it has been suggested that laminin may play a role in the attachment of epithelial cells to the basement membrane (Terranova et al., 1980). Laminin, like fibronectin, has been shown to be lost from the surface of transformed kidney cells (Hayman et al., 1981), and is produced only transiently in developing tissues (Ekblom et al., 1980). These observations have been interpreted as indicating involvement of laminin in directing tissue morphogenesis.

Entactin. This highly-sulphated glycoprotein was isolated from the matrix elaborated in cell culture by a mouse teratocarcinoma-derived cell line (Bender et al., 1981; Carlin et al., 1981). It has an apparent molecular weight of about 150,000 and is immunologically and chemically distinct from laminin and fibronectin. Entactin has been shown to be present in mouse and rat glomeruli (Carlin et al., 1981) and murine parietal yolk sac membrane (Hogan et al., 1980). Antibodies to entactin react with a variety of basement membranes, and immunoelectron microscopic studies have shown that localization is principally to the region of basement membrane closest to the cell plasmalemma (Bender et al., 1981; Carlin et al., 1981), suggesting a possible function in cellular adhesion.

Heparan Sulphate Proteoglycan. This proteoglycan has been isolated from glomerular basement membrane (Kanwar and Farquhar, 1979; Kanwar et al., 1981) and EHS-tumour cell culture matrix (Hassell et al., 1980). The reported molecular weights of heparan sulphate proteoglycans vary widely, ranging from 160,000 to 750,000 daltons for the proteoglycan and 14,000 to 70,000 daltons for the glycosaminoglycan. Antibodies to

heparan sulphate proteoglycan have been localized to several basement membranes by immunofluorescence (Hassell et al., 1980) and to glomerular basement membrane by ultrastructural studies (Mynderse et al., 1983). It has been suggested that heparan sulphate proteoglycan plays a major role in the filtration function of glomerular basement membrane (Kanwar and Farquhar, 1979; Kanwar et al., 1981; Mynderse et al., 1983) due to its polyanionic nature.

Bullous Pemphigoid Antigen. Pemphigoid, a bullous skin disease which affects the elderly, has been shown to be associated with a circulating IgG-class antibody which binds in vivo to the basal lamina of the epidermis (Beutner et al., 1968; Schaumberg-Lever et al., 1975). Use of this circulating antibody has enabled isolation of a glycoprotein antigen, called 'bullous pemphigoid antigen', which is synthesized by epidermal cells in culture (Woodley et al., 1980; Stanley et al., 1981). This glycoprotein has a molecular weight of about 220,000 daltons, and is immunologically distinct from other basement membrane glycoproteins. The function of bullous pemphigoid antigen in normal tissues is not known, but it has been suggested that it may play a role in epidermal cell adhesion (Stanley et al., 1982).

1.6 BIOSYNTHESIS OF ELASTIN IN CELL CULTURE

Extensive use has been made of connective tissue-derived cells, cultured in vitro, as a method for investigation of the biosynthesis and metabolism of connective tissue matrix proteins. These techniques are important for the study of elastin biosynthesis as there are few in vivo model systems in which to test the effects of environmental or physiological stimuli on elastin metabolism. Although elastin is synthesized in vivo as the soluble precursor molecule, tropoelastin, soluble elastin chains are quickly cross-linked into insoluble polymeric elastin, which cannot be disassociated into its component subunits (Rucker and Tinker, 1977). The constraints imposed by this process make the in vivo study of elastin biosynthesis and chemical evaluation of precursor molecules difficult. Therefore, many investigators have turned their attention to the study of elastogenesis in cell culture.

1.6.1 General Considerations

A number of cell types have been shown to synthesize either tropoelastin or insoluble elastin in culture. These include vascular smooth muscle cells (Ross, 1971; Abraham et al., 1974; Narayanan et al., 1976; Burke and Ross, 1979; Snider et al., 1981; Oakes et al., 1982), endothelial cells from human umbilical cord veins (Jaffe et al., 1976), cloned rat lung endothelial cells (Cantor et al., 1980), calf pulmonary artery endothelial cells (Mecham et al., 1983), auricular chondrocytes (Quintarelli et al., 1979; Moskalewski, 1981; Mecham et al., 1983) and ligamentum nuchae fibroblasts (Jones et al., 1980; Mecham et al., 1981; 1984).

Although many cell types are able to synthesize elastin, major differences are apparent in both the matrix which they secrete and the morphological organization of the elastin networks they produce in vivo. For example, in large arteries elastin is deposited as fenestrated sheets or lamellae, whereas in ligamentum nuchae, lung and skin it forms rope-like fibres. These differences imply that different cell types are able to direct the spatial organization of elastin in the matrix of elastic tissue. The factors which regulate these genetic expressions of elastin synthesis are still largely unknown.

However, recent advances in methodology for the identification and characterization of tropoelastin enable the investigation of elastogenesis at a molecular level in vitro as well as in vivo (Foster et al., 1981b). The ability to quantitate accurately minute amounts of tropoelastin, by the use of immunoassay (Mecham and Lange, 1980; Starcher and Mecham, 1981) and immunoprecipitation (Foster et al., 1980b; Davidson et al., 1981), has allowed evaluation of the effect of culture conditions on elastin biosynthesis.

It has become evident that the ability of cells in organ or tissue cultures to synthesize elastin varies considerably. Thus rabbit auricular chondrocytes synthesize mature cross-linked elastin in culture very quickly (Quintarelli et al., 1979; Starcher and Mecham, 1981; Moskalewski, 1981), as do rabbit aortic smooth muscle cells (Snider et al., 1981). Conversely, foetal calf ligamentum nuchae fibroblasts (Mecham et al., 1981; 1984) and calf smooth muscle cells (Schwartz, 1980) produce tropoelastin, but no insoluble elastin, under the culture conditions usually employed in these studies.

The ability of different cell types to maintain differentiation in culture also varies widely. For example, rabbit auricular chondrocytes rapidly lose the ability to synthesize mature elastin (Moskalewski, 1981) or cartilage specific proteoglycans (Madsen and Lohmander, 1979) if subcultured. On the other hand Abraham et al. (1977) have reported that a line of pig aortic smooth muscle cells maintained the ability to synthesize elastin over some 65 subcultures. Species differences (Sandberg et al., 1981a) and degree of development of the donor tissue also appear to be important determinants of elastogenic capabilities (Mecham et al., 1981; 1984; Starcher and Mecham, 1981; Madsen et al., 1983).

Culture conditions have a major effect on elastogenesis. Ascorbate supplementation has been reported to suppress the deposition of insoluble elastin in cultures of rat heart smooth muscle cells (Scott-Burden et al., 1979; De Clerck and Jones, 1980) and rabbit pulmonary artery smooth muscle cells (Dunn and Franzblau, 1982), whilst promoting the synthesis of collagen. Faris et al. (1984) have recently shown that ascorbate levels as low as 2μg/ml were sufficient to substantially reduce insoluble elastin synthesis and lysyl oxidase activity in cultures of rabbit aortic smooth muscle cells. Collagen syn-

thesis was maximally stimulated at this level, which is far below that normally used (50μg/ml) in tissue culture experiments. Reduction of ascorbate concentration to 0.5μg/ml allowed synthesis of insoluble elastin to proceed at control levels.

Mecham et al. (1981) have, however, reported that supplementation of bovine ligamentum nuchae fibroblast cultures with 50μg/ml of ascorbate had no effect on the synthesis of tropoelastin. As ligamentum nuchae fibroblasts do not appear to synthesize insoluble elastin under 'normal' culture conditions (see section 1.6.2), these findings raise the possibility that ascorbate supplementation may exert different effects on the biosynthesis of each of the components of the elastic fibre (microfibrils, tropoelastin and cross-linked insoluble elastin).

The effect of other pharmacological agents is as yet incompletely investigated, but may be of importance. The irreversible lysyl oxidase inhibitor, BAPN, has been demonstrated to inhibit cross-linking of newly-synthesized tropoelastin in cultures of primate arterial smooth muscle cells which normally form insoluble elastin (Narayanan et al., 1976). BAPN was used in these cultures at a concentration of 50μg/ml. However, Mecham et al. (1981) have shown that another lathyrogen, AAN (50μg/ml), had no effect on total tropoelastin synthesis by cultures of foetal calf ligamentum nuchae fibroblasts which do not usually produce insoluble elastin. In other studies (Sandberg et al., 1981a), BAPN has been reported to be toxic to rat aortic smooth muscle cells, decreasing soluble elastin synthesis at low levels (10μg/ml) and total protein synthesis at higher levels (200μg/ml).

In contrast, dexamethasone has been observed to stimulate elastin biosynthesis in ligamentum nuchae fibroblast cell cultures (Mecham et al., 1981) and embryonic chick aorta organ cultures (Burnett et al., 1982).

It is therefore apparent that careful selection of cell type with regard to species and donor age, as well as adequate specification of culture conditions, is necessary in any study of elastin biosynthesis by cell cultures, and that some caution should be exercized in the general interpretation of the results obtained from such model systems. If attention is given to these factors, the culture of elastin-synthesizing cells is likely to

prove useful in elucidating the physiological and biochemical mechanisms that regulate elastin gene expression. In the studies reported in this thesis, use has been made of foetal calf ligamentum nuchae fibroblasts (Chapter Four) and auricular chondrocytes (Chapter Five) to study the synthesis of elastin-associated microfibrils in vitro. A brief review of studies that have examined the synthetic capabilities of these cell types in culture is presented in section 1.6.2 and 1.7 respectively. The use of smooth muscle cell and bovine ligamentum nuchae fibroblast cultures in the study of elastin-associated microfibrils has been discussed in section 1.3.4.

1.6.2 Elastin Biosynthesis in Cultures of Bovine Ligamentum Nuchae Fibroblasts

Bovine ligamentum nuchae has proved to be a useful model for studies of elastin metabolism; it has a very high elastin content (Cleary et al., 1967), contains a single fibroblast cell type (Fahrenbach et al., 1966; Greenlee et al., 1966; Kewley et al., 1978), and is readily propagated in culture (Sear et al., 1978; Mecham et al., 1981; 1984). Explant cultures of calf ligamentum nuchae fibroblasts have been the subject of a number of studies in recent years.

Sear et al. (1978) have studied the ultrastructural characteristics of cultures said to have been obtained from a foetal calf of approximately 45 days gestation. Cells were grown to confluence in Dulbecco's modification of Eagle's medium (DMEM) containing 20% foetal calf serum (FCS), and were maintained thereafter in serum-free Waymouth's medium MAB 87/3 under scorbutic conditions. Examination of the cell layer revealed an extensive extracellular matrix containing collagen fibrils and bundles of 13nm microfibrils which were morphologically identical with elastin-associated microfibrils. No amorphous elastin was observed, even after 5 weeks in culture. A similar finding has been reported by Lamberg et al. (1980), who used cultures derived from neonatal calves and foetal calves of 3-6 months gestation. Cultures were grown in DMEM containing 10% FCS and 10% calf serum under scorbutic conditions. Microfibrils of typical appearance were seen in the extracellular matrix after 5 days in culture, but no amorphous elastin was observed up to 3 weeks after plating in either primary or subcultures.

The authors found that after about 3 weeks in culture, the cell layer tended to separate from the dish and aggregate.

Jones et al. (1980) have systematically studied the effects of ascorbic acid supplementation on ligamentum nuchae fibroblast subcultures derived from a foetal calf of 100 days gestation, making electron microscopic observations at a number of different time points after cell confluency was reached. Cultures were grown to confluence in DMEM containing 20% FCS, changing to 10% FCS thereafter. In unsupplemented cultures, detachment of the cell layer occurred after about 15 days, forming 'cell knots' in the culture flasks, similar to those observed by Lamberg et al. (1980). The cultures were maintained in this state for a further 6 weeks, during which time cell outgrowth from the 'cell knots' occurred, with the accumulation of an extensive extracellular matrix at the periphery of these aggregates. Elastic fibre formation was observed in these areas and appeared to follow the general pattern of elastogenesis previously described in intact ligamentum nuchae (Fahrenbach et al., 1966; Greenlee et al., 1966). Cultures supplemented with ascorbic acid formed 'cell knots' even more quickly than unsupplemented cultures, such aggregations appearing at about 5 days post-confluence. Elastic fibre formation was almost completely suppressed in these cultures, only scattered microfibrils being seen after 57 days post-confluence, while collagen synthesis was markedly enhanced. Cultures which had been supplemented with the lathyrogen, BAPN, in the presence of ascorbic acid, showed similar findings.

Mecham (1981b) has reported that ligamentum nuchae fibroblasts derived from a foetal calf of 210 days gestation, when cultured under normal conditions (using DMEM containing 5% FCS) will produce tropoelastin but no measurable insoluble elastin. However, if these same cells were grown on a substrate of 'killed' ligament, formation of insoluble elastin was stimulated. Ligament 'minces' cultured in vitro also retained the capacity to synthesize cross-linked elastin, suggesting that elastic fibre formation might be dependent on (as yet undefined) matrix components which are missing in cultures of enzymically-isolated cells. This hypothesis is consistent with the promotion of elastic fibre deposition by cell layer aggregates (see above) observed by Jones et al. (1980).

In a later study, Mecham et al. (1981) further reported that culture conditions have a profound effect on tropoelastin synthesis by ligament cells. Tropoelastin synthesis was maximal in cultures which had just reached confluence and was dependent upon high (greater than 5%) concentrations of foetal calf serum in the medium. Soluble elastin production was found to decrease with serial subculture, falling 50-60% by the sixth trypsinization. Increasing age in culture also decreased tropoelastin biosynthesis, twelve-week-old cultures being found to produce 20 times less tropoelastin than at one week in culture.

This study indicated that donor age was an important factor regulating the capacity for elastin biosynthesis, as cells derived from foetal calves in the third trimester produced much more tropoelastin in culture than those from younger foetuses. These findings corresponded to the observations on chemical composition of developing bovine ligamentum nuchae made by Cleary et al. (1967), who showed that an abrupt increase occurred in the rate of elastin deposition starting at approximately 180 days of gestation and was sustained until the end of the first post-natal month. Similar observations on the influence of donor age have also been made in explant cultures of sheep nuchal ligament (Davidson et al., 1981; 1982b) and sheep lung (Shibahara et al., 1981).

More recently, Mecham and his co-workers have studied in detail the mechanisms that regulate elastin gene expression during development of foetal bovine ligamentum nuchae (Mecham et al., 1984). They showed that non-elastin producing cells derived from early gestation foetal calves could be induced to synthesize elastin when grown on an extracellular matrix substratum produced by cells from a later stage of differentiation. This did not occur when extracellular matrix from foetuses of an age younger than that at which elastin production occurs normally in vivo (ie approximately 180 days gestation) was used. Induction of elastin synthesis could be prevented by pretreatment of cells with bromodeoxyuridine to block new DNA synthesis, but once elastin biosynthesis had been induced, this continued even after removal of cells from the substratum.

In conclusion, elastogenesis in cultures of bovine ligamentum nuchae appears to be affected by a number of factors, many of which are not yet well understood. Elastin biosynthesis in culture is favoured by the use of cells obtained from donor animals in which rapid elastin production has commenced, by the use of early subcultures, and by the presence of extracellular matrix material as a substratum for cell growth. It is also apparent from the above observations that careful specification of culture conditions is necessary, especially with respect to medium additives. However, it has been shown that elastic fibre formation appears to involve the participation of microfibrils in vitro in a similar manner to that observed in vivo. This gives greater relevance to the results obtained from experiments using ligamentum nuchae fibroblast cultures to study elastin-associated microfibrils (Sear et al., 1978; 1981 a,b; Lamberg, 1980). These studies were discussed in section 1.3.4.

1.7 ELASTIN STRUCTURE AND BIOSYNTHESIS IN AURICULAR CARTILAGE

The principal feature which distinguishes cartilages conventionally described as 'elastic' from other types of cartilage is the presence of elastin as a major component of the extracellular matrix. In contrast, articular cartilage contains no biochemically or ultrastructurally demonstrable elastin, while fibro-cartilage contains only minute quantities of this protein (Peters and Smillie, 1971).

Sanzone and Reith (1976), in a study of the murine pinna, have pointed out that elastic cartilages differ from non-elastic cartilages in several other important ways. Elastic cartilages are readily deformable, do not mineralize, and are predominantly cellular tissues, whereas non-elastic cartilages resist deformation, often mineralize during endochondral bone formation or with age, and are largely composed of extracellular matrix. In addition, the cytology of chondrocytes in elastic cartilages changes markedly during development of the tissue, the adult auricular chondrocyte being significantly different from that found in non-elastic cartilages. These authors also noted that the elastic components of elastic cartilages are in a relatively unstressed state when compared to elastic fibres found in other tissues.

In the mammalian body, 'elastic' cartilage is present in the auricle of the external ear, the epiglottis, and several of the skeletal elements of the larynx (Serafini-Fracassini and Smith, 1974). Biochemical analysis has shown that elastin comprises 20%-30% of the dry weight of auricular and epiglottic cartilage and about 10% of laryngeal cartilage (Peters and Smillie, 1971; Keith et al., 1977b). The other components of the extracellular matrix are found in similar proportion to that in other non-elastic cartilages, collagen (type II) comprising approximately 50% and glycosaminoglycans about 12% of the dry tissue mass (Peters and Smillie, 1971).

It has recently been suggested that elastogenesis in auricular cartilage may proceed without the participation of microfibrils (Quintarelli et al., 1979). For this reason a study of auricular cartilage was undertaken to determine whether microfibrils are associated with elastogenesis in cartilage, both in vivo and in vitro. The results of this study are reported in Chapter Five of this thesis. In the following section, a brief

review of the literature concerning elastic fibre morphology, structure and biosynthesis in auricular cartilage is presented in order to provide background to this study.

1.7.1 Morphology of Auricular Cartilage

Ear cartilage has long been considered 'elastic' due to its staining properties with the classical 'elastic tissue stains' (see section 1.2.2). The histology of adult auricular cartilage shows little variation between species.

The perichondrium consists primarily of dense bundles of collagen interlaced with elongated fibroblasts. In cartilage from young animals three zones can be distinguished (see figure 5.1). The outermost zone, immediately subjacent to the perichondrium, consists of flattened chondrocytes with their long axes parallel to the surface. Elastic fibres are not readily evident in this layer or in the perichondrium; it is possible, however, to demonstrate oxytalan-staining fibres in this zone (Bradamante et al., 1975; Bradamante and Svajger, 1977; Keith et al., 1977b; Kostovic-Knezevic et al., 1981). There is a relatively distinct boundary with the intermediate zone, in which the cells are larger and more rounded. The cells and lacunae in this zone are completely surrounded by fine elastic fibres which enclose them in a three-dimensional meshwork, leaving a 'clear' area adjacent to the cells (Sheldon and Robinson, 1958). In the central part of the cartilage there is much more elastic tissue present to the extent that it obliterates the collagen-staining matrix in many regions. Tinctorial elastica appears in foetal life. It has been observed in the rabbit ear cartilage at 26 days of foetal age (Cox and Peacock, 1977) and toward the end of the first trimester in the foetal calf (Keith et al., 1977b).

The arrangement of collagen bundles in the central region of auricular cartilage has been shown to be preferentially oriented at right angles to the perichondrial surface, merging in the intermediate zone into curving 'gothic arches', the fibres of which terminate at the perichondrium, parallel to the cartilage surface (Zambrano et al., 1982). This histologic pattern was found to be similar in a variety of species, and corresponds closely to that found in other non-elastic cartilages which have perichondrium on both surfaces.

Chondrocytes in growing elastic cartilage are approximately round or oval in shape and have an extremely irregular surface, with numerous fine, branching cytoplasmic processes extending into the extracellular matrix. This appears to be a typical feature of young chondrocytes, having been reported in both elastic (Holm Neilsen, 1976; Quintarelli et al., 1979) and hyaline cartilage (Dearden et al., 1974; Quintarelli et al., 1975). The ultrastructural alterations which occur in auricular chondrocytes during development are quite complex, and discussion of the potential significance of such changes is beyond the scope of this review. This subject has been considered in detail by Sanzone and Reith (1976) and Cox and Peacock (1977).

The extracellular matrix in ear cartilage contains a number of distinct fibre types. The whole matrix is traversed by a network of fine straight collagen fibrils of (at this level) seemingly random orientation. Only the largest of these fibres show distinct cross-banding, with an apparent periodicity of approximately 60nm (Serafini-Fracassini and Smith, 1974; Sanzone and Reith, 1976; Holm Neilsen, 1976). Collagen fibrils in both hyaline and auricular cartilages are very similar in ultrastructural appearance, and it has been shown (Eyre and Muir, 1975) that both tissues contain only type II collagen. The reason for the relative paucity of typical cross-striated collagen fibres in both types of cartilage is not known. It has been suggested that this may be due to the high proteoglycan content of the tissues, or to a structural peculiarity of type II collagen.

Zambrano et al. (1982) have recently measured the diameters of collagen fibrils in a variety of cartilages from a number of different species. They reported that collagen fibrils in elastic cartilages were relatively smaller and more uniform in diameter (ranging from 15 to 45nm) than those observed in articular cartilage. In the latter tissue, fibril diameters were highly variable, ranging from 15 to 120nm. The authors postulated that such differences may be related to the much greater physiological stresses to which articular cartilage is exposed. In both types of cartilage, however, the diameter of collagen fibrils was also found to increase with increasing distance from the cells, the largest fibres usually being found equidistant between adjacent cells.

In aldehyde-fixed auricular cartilage, most of the interfibrillar space is occupied by a network of rounded or polygonal particles ranging in diameter from about 10 to 80nm. Thin filaments often project from these granules, connecting them with collagen fibrils and other granules, thus forming a loose reticulum (Thyberg et al., 1973). These stellate particles are thought to represent a fixation artefact, and probably contain both proteoglycan and non-collagenous matrix proteins (Smith, 1970). A similar 'stellate' reticulum has been demonstrated in elastic cartilages from a number of species, including the rat (Holm Nielsen, 1976; Kostovic-Knezevic et al., 1981), mouse (Geyer and Tews, 1971; Sanzone and Reith, 1976), rabbit (Cox and Peacock, 1977; Quintarelli et al., 1979) and bovine (Serafini-Fracassini and Smith, 1974). Although the stellate reticulum is a ubiquitous feature of the extracellular matrix, it has been reported that around the large elastic fibres and in the immediate neighbourhood of the chondrocytes, the stellate particles appear larger than elsewhere. This was also true of the interstices within the reticulum in these regions (Serafini-Fracassini and Smith, 1974). Proteoglycan classes found in auricular cartilage do not appear to differ significantly from those of hyaline cartilage (Madsen and Lohmander, 1979; Madsen et al., 1983).

In all species so far studied, amorphous elastin first appears in auricular cartilage during foetal life, in close apposition to the chondrocyte membrane, some developing fragments being partly surrounded by cytoplasmic processes. With increasing maturity the elastic fibres become much larger and tend to lie further from the cells, eventually becoming situated about midway between adjacent chondrocytes. In mature cartilage, elastin forms a network of branching fibres 1 to 2 microns in diameter, extending throughout the matrix to form the three-dimensional meshwork described above (Sheldon, 1964; Anderson, 1964; Serafini-Fracassini and Smith, 1974; Sanzone and Reith, 1976; Cox and Peacock, 1977). This elastic fibre meshwork is best developed in the central zone of the cartilage and becomes attenuated toward the perichondrial surfaces, so that in the boundary zone only isolated clumps of elastic fibres are evident. The perichondrium also contains a few elastic fibres running between thick bundles of collagen fibres interspersed with fibroblastic perichondrial cells (Kostovic-Knezevic et

al., 1981). It is of interest to note that Holm Nielsen and Bytzer (1979) were unable to identify elastic fibres in a high resolution scanning electron microscope study of rat epiglottis, despite having previously reported their presence as seen with the transmission electron microscope (Holm Nielsen, 1976). They could offer no explanation for this discrepancy.

The intensity of staining of cartilage amorphous elastin with cationic heavy metal stains appears to decrease with age (Cox and Peacock, 1977), and in mature cartilage, elastic tissue stains very lightly (Serafini-Fracassini and Smith, 1974; Kostovic-Knezevic et al., 1981). Similar observations have been made in foetal calf ligamentum nuchae (Fahrenbach et al., 1966) and vascular elastic tissue (Thyberg et al., 1979). The significance of this phenomenon is not clear. Fahrenbach et al. (1966) have suggested that increased cross-linking of insoluble elastin with age may be responsible.

Some controversy exists concerning the role of microfibrils in elastin fibrillogenesis in ear cartilage. Microfibrils of 10nm diameter have been observed in association with immature elastic fibres during the post-natal development of the mouse pinna (Sanzone and Reith, 1976). They were also observed, but with lesser clarity, in the rabbit ear (Cox and Peacock, 1977). However, in the discussion of their results neither group of authors felt able to conclude unreservedly that the relationship between the microfibrillar and amorphous components of these elastic fibres was similar to that observed in elastogenesis in developing nuchal ligament (Fahrenbach et al., 1966; Greenlee et al., 1966). The interpretation of these results appears to have been influenced by a failure to demonstrate microfibrils in association with amorphous elastin in adult elastic cartilage.

Many other studies of adult elastic cartilages have failed to demonstrate elastin-associated microfibrils. This has been true of rabbit ear (Sheldon and Robinson, 1958; Quintarelli et al., 1979), rat ear (Anderson, 1964; Serafini-Fracassini and Smith, 1974), bovine ear (Serafini-Fracassini and Smith, 1974) and rat epiglottis (Akisaka and Yamamoto, 1979). It should be noted that Serafini-Fracassini and Smith reported demonstrating a filamentous substructure at the edges of elastic fibres in the ear

cartilage of young rats, but these fibrils were able to be followed for only short distances before they ran out of the plane of the section, and were not seen as discrete microfibrils.

Holm Neilsen (1976) has claimed identification of microfibrils at the periphery of elastic fibres in adult rat epiglottic cartilage, but the published micrograph is of insufficient resolution to allow objective confirmation of this fact.

Takagi et al. (1983), using specific histochemical stains for proteoglycans and glycoproteins, have recently demonstrated what appear to be typical elastin-associated microfibrils in the perichondrium of young rabbit ear cartilage, but not in association with amorphous elastin in the extracellular matrix of the cartilage itself.

Kostovic-Knezevic et al. (1981) also found that microfibrils could not be demonstrated in mature elastic fibres in the central zone of adult rat ear cartilage, but found bundles of 11nm fibres near the surface of modified chondrocytes in the boundary zone of the cartilage. They interpreted these findings as being consistent with the generally accepted view of elastogenesis, and suggested that the observed zonal differences reflected differing synthetic activity of the cells in each region. Thus they envisaged active synthesis of elastic fibres occurring predominantly in the boundary zone of the cartilage.

Quintarelli et al. (1979), however, take a very different view of elastin fibrillogenesis. These authors studied both neonatal (2-day-old) and young (16-day-old) rabbit ear cartilage, and were unable to demonstrate peripheral elastin-associated microfibrils in either tissue. Instead, they proposed that slender 'primary fibrils', found at an early stage of tissue development, might initiate elastogenesis by direct condensation to form electron dense amorphous elastin, without interposition of a microfibrillar moiety. Although the diameter of these 'primary fibrils' was not stated, they are equated with 3-4nm filaments which were demonstrated within purified elastin obtained from cultures of isolated auricular chondrocytes (see also section 1.7.3).

Recent evidence suggests that cartilage elastin is biochemically extremely similar, if not identical, to that found in other tissues (Foster et al., 1980a; Heeger and

Rosenbloom, 1980). Acceptance of such a radically different concept of elastin fibrillogenesis as that proposed by Quintarelli et al would call into question the generally accepted view of elastic fibre development in other tissues (see section 1.3.2), in which microfibrils are ascribed a central role. For this reason clarification of the involvement (or otherwise) of microfibrils in elastogenesis in auricular cartilage is of major importance. This question has been addressed as a major portion of this research project (see Chapter Five).

1.7.2 Properties of Cartilage Elastin

Conventional elastin purification procedures, such as autoclaving and treatment with hot alkali, have largely failed to produce acceptably pure preparations of elastin from cartilaginous tissues. Gotte et al. (1963) found that alkaline treatment of bovine ear cartilage resulted in a preparation relatively rich in polar amino acids, the concentration of which could not be lowered to the level typical of ligamentum nuchae elastin even by prolonged exposure to alkali. A similar result was reported by Anwar (1966) in rabbit ear cartilage. These findings were interpreted by the authors as indicative of the presence of a strongly bound contaminating polar protein.

Recently, Keith et al. (1977b) isolated elastin from a variety of bovine elastic cartilages using a mild isolation technique which avoided the harsh hydrolytic conditions previously employed. They found that ear, epiglottic and laryngeal cartilage elastins contain elevated levels of acidic and hydroxyl amino acids relative to ligamentum nuchae elastin. In a later study, the same authors further reported that bovine ear cartilage also contained significantly less valine than ligament elastin, and had a four-fold difference in the number of valyl-proline sequences (Keith et al., 1979). On the basis of this data they suggested that cartilage elastin may possibly represent a different genetic type of elastin. Field et al. (1978), using an isolation procedure similar to that employed by Keith and his colleagues, found that bovine auricular cartilage and ligament elastins different in amounts of polar amino acids, valine content and in peptide fingerprints generated by elastase digestion. They too postulated that elastin may exhibit tissue-related compositional variability.

Although these reports are suggestive of differences in cartilage elastins, the problem of establishing rigorous criteria of purity for insoluble elastins makes assessment of possible polar protein contamination of these preparations very difficult. In an attempt to resolve this difficulty, several recent studies have reported the isolation of the soluble precursor of elastin, tropoelastin, from cartilage tissues. Tropoelastin can be precisely characterized by biochemical techniques, thus enabling direct comparison between preparations obtained from different tissues. Foster et al. (1980a) have compared tropoelastin isolated from lathyritic pig aorta and ear cartilage, showing that cartilage and aortic tropoelastins are extremely similar in molecular weight, amino acid composition, immunological determinants and primary structure as examined by limited trypsin proteolysis. This result was corroborated by the study by Heeger and Rosenbloom (1980), who compared tropoelastin synthesized by hamster aorta and ear cartilage in organ culture. These findings indicate a high degree of homology between tropoelastins isolated from cartilage and aorta, but final confirmation must await complete sequencing of the tropoelastins from both tissues.

It is of interest to note that Foster et al. (1980a) found that purification of cartilage tropoelastin required an extra coacervation step, which suggested the presence of a closely-bound contaminating protein. This species could become associated (perhaps covalently) with insoluble elastin during cross-linking, resulting in differences in purified auricular cartilage elastins compared to those obtained from other tissues.

The functional significance of the presence of elastin in cartilage is not clear. It may contribute to the flexibility and elasticity of ear cartilage, especially in animals which are able to direct the pinna voluntarily toward the source of sound (Keith et al., 1977b). Elastin is a low modulus, elastic element which can undergo extensive deformations and which on the release of load will restore the tissue to its initial unstrained dimensions (Gosline, 1976), properties which would suit it admirably to this role. Another hypothesis has been advanced by Serafini-Fracassini and Smith (1974). They noted that the environmental factor common to all elastic cartilages is that they are found in the sound generating, modifying and receiving systems of the body, and

suggested that the presence of elastic fibres might be connected with desirable vibration properties of these tissues.

Whilst both these views are intuitively attractive, a more precise understanding of the physiological role of elastin in cartilages must await further investigation.

1.7.3 Biosynthesis of Elastin by Auricular Chondrocytes in Culture

Cell and tissue culture techniques have been used extensively in studies of chondrocyte differentiation and of the factors involved in the expression of the cartilage phenotype. However, most attention has been focused on hyaline cartilage, and relatively few investigations on cells derived from elastic cartilage have appeared.

It has been shown that chondrocytes, enzymatically isolated from rabbit ear cartilage are capable of reconstituting elastic cartilage in auto- or allo-geneic transplants (Kawiak et al., 1970; Moskalewski and Rybicka, 1977; Thyberg and Hinek, 1977) and in monolayer and suspension cultures (Moskalewski, 1976; Thyberg and Hinek, 1977). Elastogenesis appears to occur very rapidly in cultures of auricular chondrocytes. Moskalewski (1981) has reported that orcein-stainable fibres and granules could be observed after 6 to 8 days in aggregated cultures. Electron microscopic examination of these cultures revealed the presence of typical elastic fibres.

Biochemical evidence of elastin synthesis by rabbit auricular chondrocytes in primary culture has been presented by Quintarelli et al. (1979). They showed that radioactively-labelled lysine added to the culture became incorporated into desmosine and isodesmosine cross-links in elastin, which was isolated from the cell layer by autoclaving and hot alkali treatment. The amino acid composition of this elastin preparation showed some differences in basic and acidic residues in comparison to ligamentum elastin, but it is possible that such differences may be due to the presence of a closely-bound contaminating species (Foster et al., 1980a - see section 1.7.2). Starcher and Mecham (1981) have also demonstrated (by desmosine radioimmunoassy) that young rabbit auricular chondrocytes rapidly form insoluble elastin in culture, but they were unable to detect any soluble elastin in these cultures.

Maintenance of the chondrogenic phenotype in culture is essential if auricular chondrocytes are to provide a valid model system for the study of elastogenesis in vitro. Primary cultures of rabbit auricular chondrocytes were shown to maintain a normal appearance throughout a 5-week period of observation, depositing a large amount of extracellular matrix containing collagen and elastin fibrils and proteoglycan granules (Thyberg and Hinek, 1977). However, Madsen and Lohmander (1979) found that cells plated at a low initial density after enzymic dissociation had a decreased ability to synthesize cartilage-type proteoglycans relative to high-density cultures, despite apparently normal morphology. Subculture of chondrocytes resulted in loss of phenotypic expression, as determined by proteoglycan synthesis. Moskalewski et al. (1979) reported that subcultured chondrocytes also rapidly lost their ability to form stainable elastic fibres in culture. These studies indicate the importance of plating density and the use of primary culture in order to maintain the normal synthetic potential of auricular chondrocytes.

The tissue culture system employed may also significantly affect the production of extracellular matrix. In hyaline cartilage, cells which were aggregated by suspension culture in spinner bottles deposited more matrix than comparable monolayer cultures. Larger aggregates also accreted more matrix than smaller ones (Moskalewski et al., 1975; Lohmander et al., 1976). Although the effect on elastogenesis of aggregate versus monolayer cultures has not been studied in auricular chondrocytes, Madsen and Lohmander (1979) found that transferring monolayer subcultures of auricular chondrocytes to spinner bottle culture partly restored their ability to produce aggregated proteoglycans. The mechanism of this effect is not understood. Conceivably, the aggregation of chondrocytes or the lowering of oxygen tension in suspension may favour chondrogenic expression (Nevo et al., 1972).

A number of other factors have also been shown to affect the capacity of auricular chondrocytes to form elastin in culture. Cells from older donor animals have a greatly decreased ability to form elastin compared to those from young animals. This has been shown both in transplantation (Moskalewski and Rybicka, 1977) and in cell culture experiments (Moskalewski et al., 1979; Madsen et al., 1983).

The presence or absence of ascorbic acid in the culture medium is important in elastogenesis (see section 1.6). Cultures grown under scorbutic conditions produce elastin in normal proportion (expressed as a percentage of dry tissue mass) to that of the donor cartilage, with decreased production of collagen and proteoglycan (Quintarelli et al., 1979). This correlates with the ultrastructural findings of Moskalewski (1976), who described thick elastic fibres (with peripheral microfibrils) in the matrix of scorbutic cultures in which almost no other formed elements were visible. No comparative study of scorbutic and ascorbate-supplemented cultures has so far been reported; however, Thyberg and Hinek (1977) have examined ascorbate-supplemented cultures of auricular chondrocytes from young rabbits. These cultures formed thick cartilaginous sheets containing large amounts of extracellular matrix, including collagen fibrils and proteoglycan granules, but which had a reduced number of elastic fibres compared to native cartilage. This is consistent with reports that ascorbate reduces elastin formation whilst stimulating deposition of collagen in other mesenchymal culture systems, such as ligamentum nuchae fibroblasts (Jones et al., 1980), rat heart smooth muscle cells (Scott-Burden et al., 1979; De Clerck and Jones, 1980), rabbit pulmonary artery smooth muscle cells (Dunn and Franzblau, 1982) and rat aortic smooth muscle cells (Faris et al., 1984). The absence of ascorbate does not appear to have any effect on the proliferation of auricular chondrocytes, and it would seem that scorbutic culture conditions may be desirable in studies of elastin deposition in culture. The only apparent chemical difference in elastin produced under such conditions seems to be underhydroxylation of proline residues. Neither cross-linking nor ultrastructure appears to be affected (Jones et al., 1980; De Clerck and Jones, 1980).

Moskalewski and co-workers (Moskalewski, 1981; Moskalewski et al., 1983) have reported that addition of BAPN (an irreversible inhibitor of lysyl oxidase) at a concentration of 10μg/ml to cultures of enzymically-dissociated rabbit auricular chondrocytes completely prevented the formation of elastic fibres, but did not appear to harm the cells.

The ultrastructure of auricular chondrocyte cultures derived from young rabbits has been described by Hinek and Thyberg (1977). The cells grew in overlapping layers, and in older cultures some degenerating cells were noted, especially in the deeper parts of the cell layer. The appearance of these cells was very similar to that described by Cox and Peacock (1977) in developing rabbit ear cartilage. However the elastin synthesized by these cultures (which were supplemented with ascorbate) appeared to be very electron-dense and the microfibrillar component could not easily be discerned. This is very similar to the elastin described by Quintarelli et al. (1979) in young rabbit ear cartilage. In both cases particles resembling proteoglycan granules can be seen accreted to the surface of the elastic fibres. A common factor was the extensive proteoglycan component observed in the matrix, and it seems possible that highly-charged proteoglycan molecules might bind to those sites on the microfibrils which normally bind cationic heavy metal stains, thus obscuring the underlying structure of the fibre.

In conclusion, auricular chondrocyte cultures appear to be an attractive model system in which to study elastogenesis in vitro. They offer a number of advantages: cultures are technically easy to establish, grow readily and produce elastin quickly. They require only simple media and, if high-density primary cultures are used, maintain an essentially normal phenotype for considerable periods of time. In addition they avoid the need for subculture and its attendant risk of contamination and loss of phenotypic expression. Much work remains, however, to delineate the factors which regulate elastin synthesis by auricular chondrocytes. The literature at present available indicates the need for careful specification of experimental conditions if interpretable results are to be obtained.

1.8 PLAN OF INVESTIGATION

The problem of the biochemical identity and structural function of elastin-associated microfibrils remains one of the most difficult in connective tissue research. Despite many claims in the literature to the contrary, review of the available evidence concerning elastin-associated microfibrils leads inescapably to the conclusion that very little is known about their macromolecular constituents.

The major aims of the studies described in this thesis were, therefore, to re-evaluate critically the evidence relating to the extractability of elastin-associated microfibrils and to describe more precisely the immunochemical properties of an antiserum raised to a Ross and Bornstein type 'microfibrillar protein' extract. It was envisaged that the antibody preparation thus obtained would be used as a probe to aid the isolation and characterization of microfibrillar subunits. The experimental approach to these objectives is outlined as follows:

i) A 'microfibrillar antigen' (MAg) extract was prepared from foetal bovine ligamentum nuchae by a modification of the procedure of Kewley et al. (1977a). Extraction residues were monitored by electron microscopy. The final MAg extract was used to produce an antiserum, which was characterized immunochemically by a combination of immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay. Contaminating antibody activity present in the crude antiserum was removed by immunoabsorption.

ii) The tissue localization of anti-MAg antibodies was determined in a number of foetal calf tissues by light and electron microscopic immunohistochemical techniques. The distribution of anti-MAg antibodies was also compared with that of anti-(CL glycoprotein) antibodies.

iii) As MAg extract proved difficult to analyze biochemically due to its extreme insolubility, several tissue culture systems were examined in order to assess their potential as a source of soluble microfibrillar components. Cultures of foetal bovine ligamentum nuchae fibroblasts were found to produce microfibrils but not amorphous elastin. However, no species which immunologically cross-reacted with anti-MAg antibody was detected in the medium of such cultures.

iv) A comparative ultrastructural study of the involvement of microfibrils in elastogenesis was performed in elastic (auricular) cartilage both in vivo and in vitro in foetal calf and rabbit ear cartilages. Immunohistochemical studies demonstrated the presence of material antigenically related to microfibrils in association with elastic fibres in native ear cartilage and perichondrium, as well as in the cell layer of auricular chondrocyte cultures.

The results and interpretation of these experiments are presented in Chapters Two to Five. Chapter Six consists of a concluding discussion and proposals for future research.

CHAPTER TWO

EXTRACTION AND IMMUNOCHEMICAL CHARACTERIZATION OF A MICROFIBRIL-ASSOCIATED COMPONENT OF ELASTIC FIBRES

2.1 INTRODUCTION

Despite a number of attempts to extract and characterize macromolecules derived from elastic-fibre microfibrils, the origin and identity of such extracts remains obscure (see section 1.3). Kewley et al. (1977a,b) have raised antibodies to a putative 'microfibrillar protein' extract prepared by a modification of the Ross and Bornstein (1969) procedure, and have shown this antibody to localize to elastic fibres, as well as to collagen and basement membranes. However their antibody preparation was assessed only by the relatively insensitive and solubility-dependent technique of Ouchterlony double radial immunodiffusion, which is unlikely to be reliable for the immunochemical characterization of the highly insoluble antigens obtained in such an extract.

The objective of the study described in this chapter was to prepare an antibody to a microfibrillar antigen extract from foetal bovine ligamentum nuchae, and to define this extract immunochemically using more sophisticated means of antigenic analysis. Microfibrillar antigen was extracted by a method based on that of Kewley et al. (1977a). Several improvements were made in order to minimize contamination and proteolytic degradation. Thus extensive use was made of protease inhibitors in all buffers, and the bacterial collagenase used to digest matrix collagen was chromatographically purified to eliminate non-specific proteases. Particular attention was paid to tissue preparation, and a preliminary saline extraction step was added to remove salt-soluble and serum proteins.

Foetal calf ligamentum nuchae was chosen as a suitable tissue for a number of reasons; it has a very high content of elastin, contains no basement membrane structures, and has been well documented as a model of elastogenesis. Cleary et al. (1967) have shown that elastin deposition is increasing rapidly at approximately 200 days of gestation and it seems likely that ligaments obtained from foetuses around this age would be most suitable for the extraction of microfibrillar components. Therefore all foetuses used in this study were of 200 to 220 days gestation.

Microfibrillar antigen (MAg) was used to immunize rabbits, and relatively high titres of precipitating antibody were obtained against this antigen. Use was made of an

enzyme-linked immunosorbent assay (ELISA) to characterize this preparation immunochemically. Some non-specific activity was detected, but could be successfully absorbed out from the antiserum without significant diminution of titres against MAg. Biochemical analysis of MAg has been hampered by its extreme insolubility in conventional buffers.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Phenylmethylsulphonyl fluoride (PMSF), epsilon-amino-n-caproic acid (EACA), benzamidine, dithiothreitol, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, Tris (hydroxymethyl) aminomethane (Tris), acrylamide (purified for electrophoresis) and polyoxyethylenesorbitan monolaurate (Tween 20) were all purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

The enzymes, alkaline phosphatase (type VII from calf intestine; EC 3.1.3.1) and bacterial collagenase (type I from clostridium histolyticum; EC 3.4.24.3) and the protein standards, bovine serum albumin (BSA), ovalbumin (OA; from chicken egg), phosphorylase-a (from rabbit muscle; EC 2.4.1.1) and bovine gamma globulin (Cohn fraction II) were also purchased from Sigma Chemical Company. Human plasma fibronectin was obtained from Collaborative Research, Massachusetts, USA. N-ethyl-maleimide (NEM) was supplied by Koch-Light Laboratories, Colnbrook, Buckinghamshire, England.

All chemicals used in this study were of analytical reagent grade unless otherwise stated.

The electron microscopic prerequisites, sodium cacodylate (EM grade), glutaraldehyde (EM grade; supplied as a 25% solution), osmium tetroxide (EM grade), uranyl acetate (EM grade), BEEM capsules and Spurr's epoxy resin kits were supplied by TAAB Laboratories, Emmer Green, England; electron microscope specimen grids were supplied by Graticules Limited, Tonbridge, England.

Freund's adjuvant solutions (complete and incomplete) and Bacto agar were made by Difco Laboratories, Detroit, Michigan, USA, and were supplied by Flow Laboratories (Australia Proprietary Limited), Stanmore, NSW. Linbro Titertek microtitration 96-well plates for haemagglutination assays were also supplied by Flow Laboratories. Cooke polystyrene microtiter plates, M220-29ART (96 well, flat-bottomed) were obtained from CA Greiner und Sohne, Nurtingen, West Germany. Foetal calf serum and sodium heparin came from Commonwealth Serum Laboratories, Parkville, Victoria.

Sepharose CL-4B, Staphylococcal Protein A linked to Sepharose CL-4B and Cynanogen Bromide-Activated Sepharose 4B, were supplied by Pharmacia (South Seas) Proprietary Limited, North Ryde, NSW.

Guanidinium chloride was obtained from E Merck, Darmstadt, West Germany, and solutions were clarified before use with activated charcoal. After filtering, the molarity of the GuHCl solution was determined by measuring the refractive index, and adjusted as necessary.

Myosin was prepared from rabbit skeletal muscle by a method based on that of Weeds and Hartley (1968). Porcine tropoelastin was isolated from the aortae of copper deficient pigs as described by Sandberg et al. (1975). Preparation of α-elastin from hot-alkali purified bovine ligamentum nuchae elastin was performed according to the method of Partridge et al. (1955).

2.2.2 Extraction of Microfibrillar Antigen

Foetal calves were obtained from the South Australian Meat Corporation at Gepps Cross, South Australia. Gestational age was estimated from crown-rump measurement (Bogart, 1959) and only 200 to 220 day foetuses were taken. Nuchal ligaments were dissected out within a half-hour of maternal death, and transported to the laboratory on ice in a solution of 0.9% (w/v) NaCl containing a cocktail of protease inhibitors (Saline Buffer A; see table 2.1). Adhering muscle and adipose tissue were carefully removed before extraction was begun. The experiments described below were repeated twice on fresh tissues from animals of the same age.

The extraction procedure used was essentially that described by Kewley et al. (1977a,b), with two important modifications:

1. Protease inhibitors were used in all buffers in order the minimize proteolytic degradation of extracted macromolecules. PMSF, NEM, EACA and EDTA were used to inhibit serine-, cysteine-, trypsin-like and metal-containing proteases respectively. EACA and EDTA were omitted from the buffers used in collagenase digestion steps. The exact constituents of each buffer are given in Table 2.1.

2. The bacterial collagenase (Sigma Type I), employed in the enzyme digestion steps, was chromatographically purified by the method of Lee-Own and Anderson (1975) in order to free it from contaminating non-specific proteases. A summary of the extraction procedure is shown in Figure. 2.1.

The ligament was frozen in liquid nitrogen and crushed using a stainless steel mortar and pestle. This procedure was repeated, usually three to five times, until the ligament was reduced to fine particles. The wet weight of the ligament was recorded, and 10 volumes of each extracting buffer were used relative to this weight. The tissue was suspended in cold Saline Buffer A (see Table 2.1), briefly homogenized with a Sorvall Omni-Mixer, and stirred for 24 hours at 4°C. The suspension was then centrifuged at 20,000g for 1 hour at 4°C in a Sorvall RC-2B refrigerated centrifuge (GSA head). The supernatant (extract S) was saved, the residue washed twice with fresh saline solution and re-centrifuged, the supernatants being added together. The residue was then suspended in 6M GuHCl/Tris Buffer B and extracted for 24 hours at 4°C with stirring, before centrifugation as above. The supernatant (extract SG) was saved, and the residue was washed twice with Tris Buffer C. Digestion of the residue with highly-purified bacterial collagenase was then carried out. The residue was resuspended in Tris Buffer C, and approximately 25 units of enzyme per gram of initial wet weight added. The mixture was then incubated for 24 hours at 37°C with stirring. The pH was checked and adjusted as necessary at half-hourly intervals for the first two hours after beginning digestion, and then three, eight and sixteen hours later. After digestion the suspension was centrifuged and the supernatant saved (extract SGC). The residue was washed with 6M GuHCl/Tris Buffer B, and extraction was repeated sequentially, to the point SGCGCG. At this stage the residue was extracted for 24 hours with 6M GuHCl/Tris Buffer B containing 50mM DTT. The resulting extract was designated 'microfibrillar antigen' (MAg) for descriptive purposes.

All extracts obtained were concentrated in an Amicon ultrafiltration cell with a PM-10 membrane, dialyzed extensively against Tris Buffer D at 4°C, then stored frozen at -20°C. Aliquots of each extract were then dialyzed exhaustively against distilled

Table 2.1.

Composition of Buffers used in the Extraction of Microfibrillar Antigen

Constituents	Saline Buffer A pH 7.4	GuHCL/Tris Buffer B pH 7.4	Tris Buffer C pH 7.4	Tris Buffer D pH 6.8
NaCL	150mM	-	-	150mM
Tris	50mM	50mM	50mM	62.5mM
GuHCl	-	6.00M	-	62.5
$CaCl_2$	-	-	5mM	-
EACA	10mM	-	-	10mM
EDTA	10mM	-	-	10mM
PMSF	1mM	1mM	1mM	1mM
NEM	2mM	2mM	2mM	2mM
Benzamidine	5mM	5mM	5mM	5mM
Na_3N	0.1% (w/v)	0.1% (w/v)	-	0.1% (w/v)

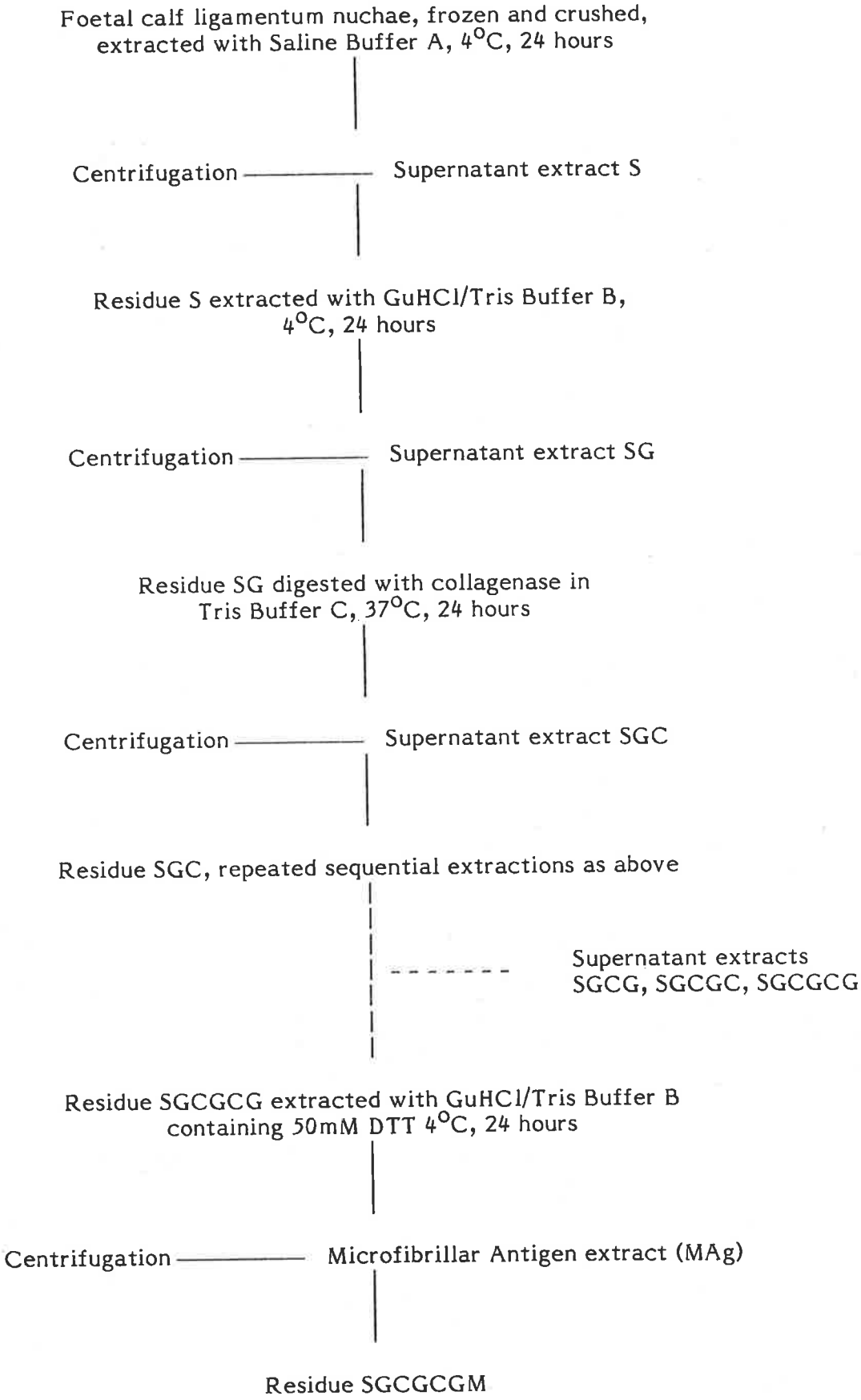


Figure 2.1
Flow Diagram of Extraction Procedure for Microfibrillar Antigen

water containing protease inhibitors and lyophilized. Protein concentration of each of the extracts was estimated by the method of Lowry et al. (1951) using crystalline BSA as a standard.

2.2.3 Transmission Electron Microscopy of Extraction Residues

A small amount of the residue was taken for electron microscopic examination at the completion of each stage of the extraction procedure. Residue samples were washed three times with distilled water to remove remaining buffer, then fixed with 2.5% glutaraldehyde in 0.15M sodium cacodylate buffer, pH 7.3, overnight at 4°C. Specimens were rinsed thoroughly with the same buffer, and post-fixed with 1% (w/v) osmium tetroxide (OsO_4), also in cacodylate buffer. Specimens were then dehydrated in graded ethanols, and embedded in Spurr's epoxy resin (Spurr, 1969) in BEEM capsules. A summary of this procedure is shown in Figure 2.2. The formulation of Spurr's resin employed was as follows:

ERL 4206	10.0g
DER 736	5.5g
NSA	26.0g
S-1	0.4g

The resin components were weighed out on a top-loading balance, and the mixture stored in a dessicator over phosphorus pentoxide (P_2O_5) during use. Blocks were polymerized overnight at 70°C, and ultrathin sections cut from them with glass or diamond knives on a Reichert Om-U2 ultramicrotome. Sections were picked up on rhodium plated copper grids, and stained for successive 15 minute periods with saturated uranyl acetate in 70% ethanol and lead citrate (Reynolds, 1963), before examination in a Philips EM 300 electron microscope operated at an accelerating voltage of 80kV.

2.2.4 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of sodium dodecyl sulphate (SDS) using the discontinuous buffer system of Laemmli (1970). Gels

Aldehyde-fixed specimens rinsed with 0.15M Na cacodylate buffer, pH 7.3

↓

Post-fixed with 1% (w/v) OsO_4 in cacodylate buffer, one hour

↓

70% (v/v) ethanol, 15 minutes*

↓

95% (v/v) ethanol, 15 minutes*

↓

2 changes of 100% ethanol (dried with anhydrous Cu_2SO_4), 20 minutes each*

↓

Specimens in dry ethanol stood in a vacuum dessicator over P_2O_5, 20 minutes

↓

Infiltrated with 50:50 mixture of Spurr's resin and 100% ethanol, 1 hour*

↓

Infiltrated overnight with undiluted Spurr's resin*

↓

Spurr's resin changed, infiltrated for further 3 hours*

↓

Embedded in BEEM capsules and polymerized overnight at 70°C

* These steps were carried out with specimens in capped bottles, being rotated at approximately 2 revolutions per minute. All manipulations were at room temperature.

Figure 2.2
Protocol for Embedding Specimens in Spurr's Epoxy Resin

were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bis-acrylamide, and polymerized by the addition of N,N,N',N'-tetramethyl ethylenediamine (0.045% w/v) and ammonium persulphate (0.075% w/v) to give final acrylamide concentrations of 6.5% for separating gels and 3% for stacking gels.

Vertical slab gels consisted of a separating gel of dimensions 14cm x 14cm x 0.2cm and a stacking gel of height 2cm. Cylinder gels were cast in glass tubes of internal diameter 0.6cm and consisted of a separating gel 9cm long and a stacking gel 1cm in height.

The electrophoresis buffers were identical to those described by Laemmli (1970) with the exception of the sample preparation buffer (SPB) which contained either 2M or 6M urea. Urea solutions were clarified by absorption with activated charcoal, filtered and deionized by passage over an ion-exchange column before use. PMSF (2mM) was also added to the SPB to inhibit proteolysis. Buffer compositions are given in Table 2.2.

Proteins were suspended in SPB at a concentration of approximately 1mg per ml, incubated in a boiling water bath for 2 minutes and then cooled quickly. This procedure was repeated until no more solubilization of protein occurred, after which any insoluble material was sedimented by centrifugation for 5 mins at 10,000g in a Beckman Microfuge. Samples were then applied to the gel either directly, or following dialysis against SPB (2 x 50mls) to remove excess salt as necessary.

Electrophoresis was carried out at room temperature using a constant current of 2mA per tube for cylindrical gels, and 25mA for slab gels which were cooled by passing water through a cooling jacket applied to the slab.

Gels were stained for protein with Coomassie Brilliant Blue R-250 using the method of Fairbanks et al. (1971) or for glycoproteins with periodic acid/Schiff reagent (PAS) by the method of Glossman and Neville (1971).

Mobilities of macromolecules were expressed relative to that of Bromophenol Blue marker dye, measurements being taken from the leading edge of the bands. Purified protein standards used for gels run under reducing conditions were ovalbumin (molecular

Table 2.2

Composition of Buffers used for SDS-Polyacrylamide Gel Electrophoresis

Constituents	Sample Preparation Buffer pH 6.6	Stacking Gel Buffer pH 6.8	Separating Gel Buffer pH 8.8	Electrode Chamber Buffer pH 8.3
Tris-HCl	62.5mM	125mM	375mM	250mM
SDS	2% (w/v)	0.1% (w/v)	0.1% (w/v)	0.1% (w/v)
Glycine	-	-	-	192mM
2-Mercapto ethanol	2% (v/v)	-	-	-
Glycerol	10% (v/v)	-	-	-
PMSF	2mM	-	-	-
Bromophenol Blue	0.001% (w/v)	-	-	-
Urea	2M or 6M	-	-	-

weight 43,000 daltons), bovine serum albumin (molecular weight 68,000 daltons), phosphorylase a (molecular weight 96,000 daltons), myosin (molecular weight 200,000 daltons) and fibronectin (molecular weight 220,000 daltons). Bovine γ-globulin (molecular weight 150,000 daltons) was also used as a standard in gels run under non-reducing conditions. Calibration curves of mobility versus molecular weight were drawn as described by Weber and Osborne (1969).

2.2.5 Development of Anti-MAg Antiserum

New Zealand White rabbits (which had been pre-bled) were injected subcutaneously with MAg emulsified in complete Freund's adjuvant and physiological saline. MAg prepared by the method detailed in section 2.2.2 was highly insoluble in buffers of low ionic strength, and some of the material injected was particulate. Two injections of 1mg of MAg contained in 1ml of emulsion were given at monthly intervals. Subsequent injections were made in incomplete Freund's adjuvant, also at monthly intervals. Rabbits were bled from the marginal ear vein approximately ten days after each injection, and the serum obtained assayed for activity against MAg by immunodiffusion or ELISA assay as described below. Good titres of precipitating antibodies were obtained in all three rabbits after the fourth injection. Thereafter, animals were injected with booster doses of MAg at monthly intervals and bled of relatively large amounts (usually 50mls). Sera were checked after each bleeding, and only those of high titre were used in the experiments which followed.

An intermediate extract, extract SGCG, was similarly used to raise antibodies in rabbits in order to check for cross-reactivity between MAg and the earlier extracts. Low titres of precipitating antibody were obtained with this antigen.

2.2.6 Immunochemical Characterization of Anti-MAg Antiserum

Several methods were used to determine the specificity and titres of anti-MAg antiserum. In early experiments, Ouchterlony double radial immunodiffusion and passive haemagglutination techniques were employed. However, both techniques have some deficiencies for analysis of the antigen determinants of MAg (see section 2.4).

These difficulties were overcome by the use of an indirect microplate enzyme-linked immunosorbent assay (ELISA) which has allowed better characterization of cross-reactivity with other macromolecules.

Immunodiffusion. Micro-immunodiffusion plates were prepared by coating 3cm x 7cm microscope slides with 5ml of 0.85% (w/v) Bacto agar (Difco) dissolved in saline-borate buffer, pH 8.0. Sodium azide, 0.1% (w/v), was added to inhibit bacterial growth. In order to accelerate and enhance the formation of precipitin lines, 2.5% (w/v) polyethylene glycol (molecular weight 6,000 daltons) was also incorporated in the gel (Harrington et al., 1971). Diffusion wells were spaced 1cm apart, the central well being 0.5cm and the outside wells 0.2cm in diameter. Antigen solution, approximately 1mg/ml, was placed in the central well, and doubling dilutions of antiserum were loaded in the peripheral wells. Plates were developed in a moist chamber at room temperature, and were inspected at 24 hour intervals. Due to the insoluble nature of the antigens, refilling of wells two or three times was necessary before precipitin lines became apparent. Plates were washed and dried before being stained with Coomassie Brilliant Blue R-250 by the method described by Weeke (1973).

Cross-reactivity of anti-MAg antiserum was tested by immunodiffusion against foetal calf serum (FCS), collagen types I and III (prepared in this laboratory from foetal calf skin), fibronectin (prepared from human serum, as described below), tropoelastin (prepared in this laboratory from copper-deficient pig aorta), and bovine ligamentum nuchae elastin solubilized by oxalic acid hydrolysis (α-elastin; prepared according to the method of Partridge et al., 1955). Reactivity of anti-MAg antiserum against extract GCG, and vice versa was also assessed by this method.

Passive Haemagglutination. Sheep blood was collected by jugular puncture, using heparin as an anticoagulant. Red blood cells were washed and formalinized by the method of Csizmas (1960), made up to a 50% (v/v) suspension with physiological saline, and stored at -20^{o}C in 5ml aliquots after snap-freezing in liquid nitrogen. Before use, cells were quickly thawed at 37^{o}C, and checked for agglutination by examination under a phase contrast microscope.

Cells were tanned and coated with antigen as described by Herbert (1973). Coated cells were made up to 1% (v/v) suspension in phosphate buffered saline (PBS; 0.02M phosphate, 0.13M NaCl, pH 7.4) and stabilized by the addition of 1% (v/v) inactivated normal rabbit serum (NRS), twice absorbed with packed cells from the same batch. Cell concentrations were determined by measuring the haematocrit of suspensions with a Hawksley micro-haematocrit apparatus.

Haemagglutination tests were carried out in the wells of plastic haemagglutination plates (Linbro Titertek), by adding 0.05ml volumes of tanned, coated cells to a doubling dilution series of the serum to be examined in 0.05ml volumes of PBS. Controls to exclude the presence of heterophile agglutinins were provided by incubating uncoated cells with the initial dilution of antiserum used for each titration. Controls with coated and uncoated cells in PBS alone were also prepared for each group of tests. Negative controls were performed using pre-immune rabbit serum, and a positive control was incorporated into each experiment by coating cells from the same batch with BSA and reacting these cells with a mouse anti-BSA immunoglobulin G (IgG) preparation of known titre (kindly provided by Dr P Ey of the Department of Microbiology, University of Adelaide). Cells were coated with MAg, FCS, tropoelastin and α-elastin in order to determine the reactivity of these antigens with anti-MAg antiserum.

Enzyme-linked Immunosorbent Assay (ELISA). Indirect microplate ELISA was performed by the method of Voller et al. (1976). Goat anti-(rabbit IgG) gamma globulin (see section 2.2.7) was conjugated to Sigma type VII alkaline phosphatase using glutaraldehyde as coupling reagent. Conjugate was stored at -20°C in the dark as 1ml aliquots, in 0.05M Tris buffer, pH 8.0, containing 1% (w/v) BSA and 0.02% (w/v) sodium azide. Optimal working strength of the conjugate was determined by checkerboard titration against rabbit IgG, as described by Voller et al. (1976).

Antigens were dissolved in 0.05M carbonate buffer, pH 9.6, and 0.2ml of solution was added to the wells of a polystyrene microtiter plate (Cooke M220-29-ART) which was then incubated overnight in a humid chamber at 4°C, to allow passive absorption to the plate surface. Antigen solutions were stored at -20°C and used repeatedly until

exhausted. After coating, the plates were washed three times with PBS, pH 7.4, which contained 0.05% (v/v) Tween 20 in order to prevent non-specific binding of serum proteins to the wells. Serial doubling dilutions of each antibody preparation under test were made in PBS-Tween buffer, and 0.2ml aliquots added to the wells. Controls containing buffer only or antigen only were incubated in each test in addition to known positive and negative sera. The plates were incubated for 2 hours at room temperature, then washed three times with PBS-Tween. Enzyme-labelled conjugate, diluted to working strength with PBS-Tween, was added to each well (0.2ml) and the plate incubated for a further 2 hours at room temperature. After washing and shaking dry as before, 0.2ml of enzyme substrate solution (1mg/ml p-nitrophenyl phosphate dissolved in 10% (v/v) diethanolamine buffer, pH 9.8) was added to each well, and colour development was allowed to proceed for 30 minutes at room temperature. The reaction was stopped by the addition of 0.05ml of 3M sodium hydroxide to all wells. The plate was read with a scanning densitometer set at a wavelength of 400nm, and the resulting titre determined by comparing the absorbance of test wells with a blank well (buffer only) and wells containing heat-inactivated pre-immune rabbit serum of comparable dilution. Samples were considered positive if their absorbance, relative to the blank, was at least double that of the corresponding negative.

A positive control row of MAg against anti-MAg IgG (see section 2.2.7) was incorporated in each plate so that any plate-to-plate variation could be taken into account when comparing different antigens. A wide range of antigens was assessed for cross-reactivity with anti-MAg IgG by ELISA.

2.2.7 Preparation of Goat Anti-(Rabbit IgG) Antibodies

A mixed breed goat was inoculated subcutaneously with 2mg of rabbit IgG (prepared as described below), emulsified in physiological saline and complete Freund's adjuvant. Four weeks later a second injection was given in incomplete Freund's adjuvant. After a further four-week period, the goat was bled of approximately 250ml of blood by jugular puncture. Serum was tested for activity against rabbit IgG by immunodiffusion and haemagglutination techniques as described above.

Gamma globulin was isolated from serum by the method of Gray et al. (1969). Briefly, a crude globulin fraction was prepared from serum by precipitation with 1.0M sodium sulphate at 4°C. This fraction was dissolved in 0.01M sodium phosphate buffer, pH 8.0, and applied to a diethylaminoethyl (DEAE)-cellulose (DE-52; Whatman) column equilibrated with the same buffer. Elution with a linear salt gradient was then performed, the limit buffer being 0.033M sodium phosphate, pH 8.0. The chromatogram showed three major peaks; the first to elute contained only gamma globulin, as shown by SDS-PAGE. Fractions from this peak were pooled, and the solution concentrated in an Amicon ultrafiltration cell using a PM-10 membrane. Protein concentration was estimated by assuming the extinction coefficient $E^{0.1\%}_{1cm}$ of goat IgG to be 1.38 at a wavelength of 280nm (Micusan and Borduas, 1977). Specific activity against rabbit IgG was assessed as before, and purity of the final preparation checked by SDS-PAGE under non-reducing conditions. Goat anti-(rabbit IgG) gamma globulin was stored at -20°C as a 20mg/ml solution in PBS, pH 7.4, containing 0.1% (w/v) sodium azide.

2.2.8 Preparation of Rabbit IgG

Rabbit IgG was prepared from pooled antisera in initial experiments by ammonium sulphate precipitation, followed by DEAE-cellulose chromatography as described by Fahey and Terry (1973). This proved to be time-consuming and impractical for multiple serum samples, and was supplanted by the use of staphylococcal protein A, linked to Sepharose 4B. The technique employed was that of Goding (1976). Protein A-Sepharose gel (5ml) was equilibrated with PBS, pH 7.4, and packed into a column made from a 10ml disposable plastic syringe. IgG was extracted by passing whole rabbit serum over the column, washing thoroughly with PBS, and eluting with 0.85% (v/v) glacial acetic acid in 0.15M sodium chloride. The IgG so prepared was then dialyzed against three changes of PBS, pH 7.4, concentrated by ultrafiltration, and 0.1% (w/v) sodium azide added before storage at 4°C. Protein concentration was assessed by assuming the extinction coefficient $E^{0.1\%}_{1cm}$ of rabbit IgG to be 1.43 at a wavelength of 280nm (Hardie and Regenmortel, 1977).

Purity of the rabbit IgG prepared by both methods was checked by SDS-PAGE. Specific activity of anti-MAg IgG was titrated by ELISA and haemagglutination assays.

2.2.9 Adsorption of Non-specific Activity from Anti-MAg IgG

Significant cross-reactivity of anti-MAg IgG was demonstrated against both human plasma fibronectin and a number of foetal calf serum proteins (principally BSA) by ELISA and by haemagglutination (see section 2.3.4 for details). In order to render the anti-MAg preparation monospecific, immunoadsorption of this unwanted activity was undertaken utilizing affinity chromatography.

Commercially available fibronectin was too expensive to be used in the quantities needed for coupling to an affinity support, hence purification of fibronectin from plasma was necessary before immunoadsorption experiments could be performed. The method of Vuento and Vaheri (1979) was followed for the purification of fibronectin. Fresh, citrated human plasma was generously provided by the Red Cross Blood Transfusion Service (South Australian Division).

Benzamidine was added to a concentration of 5mM, and 200ml of plasma were then passed through a column (3.5cm x 3.5cm) of Sepharose 4B equilibrated with 0.05M Tris/HCl, pH 7.5, containing 5mM benzamidine and 0.02% (w/v) sodium azide. Gelatine (Sigma type I) was coupled to cynanogen bromide-activated Sepharose 4B according to the manufacturer's instructions (Affinity Chromatography. Principles and Methods; Pharmacia Fine Chemicals, Uppsala, Sweden, 1979) and plasma proteins which did not bind to 'plain' Sepharose were applied to a column (1.6cm x 15cm) of gelatine-Sepharose equilibrated with the same buffer as above. After thorough washing of the column, crude fibronectin was eluted by 1M arginine in equilibrating buffer. Crude fibronectin solution was dialyzed extensively against Tris buffer, then applied to a column (1.6cm x 7cm) of arginine coupled to Sepharose 4B. Pure fibronectin which bound to this column was eluted with 1M NaCl in equilibrating buffer. Fibronectin so obtained was dialyzed against equilibrating buffer and stored at $4^{o}C$. An aliquot was dialyzed against distilled water, and lyophilized before analysis by SDS-polyacrylamide gel electrophoresis.

The results of this procedure are shown in Figure 2.3. Purified fibronectin migrated as a single PAS-positive band of apparent molecular weight 220,000, whilst crude fibronectin (after gelatine-Sepharose chromatography) also showed some low molecular weight contaminants. On the basis of this appearance, it was considered that highly purified fibronectin had been obtained. This preparation was used in subsequent affinity chromatography experiments described below.

Antigenicity of fibronectin prepared in this way was assessed by ELISA. Concentrations of solutions of purified fibronectin were determined by assuming $E^{0.1\%}_{1cm}$ to be 12.8 at a wavelength of 280nm (Mosesson et al., 1975).

Affinity Chromatography of Anti-MAg IgG. Immunosorbent columns were prepared by coupling FCS and purified fibronectin separately to cyanogen bromide-activated Sepharose 4B. Approximately 75mg of FCS was coupled to 15ml of gel, and 7mg of purified fibronectin was coupled with 7ml of gel. Both lots of gel were equilibrated with PBS (pH 7.4) after coupling and packed into small columns.

Anti-MAg IgG, 80mg in 6ml of PBS, was then pumped through both columns connected in series, at a flow rate of 5ml per hour. Protein passing straight through was collected, reconcentrated to approximately 15mg/ml, and checked for activity by ELISA. Affinity columns were regenerated by eluting with 0.85% acetic acid in 0.15M NaCl to remove IgG which was bound by the gel. The procedure was repeated until ELISA showed no residual non-specific activity in the resulting anti-MAg IgG preparation. Usually three to five cycles were necessary before this was achieved, the affinity columns being regenerated before each cycle. This highly-specific IgG preparation will be referred to as 'anti-MAg (ADS) IgG' in this thesis.

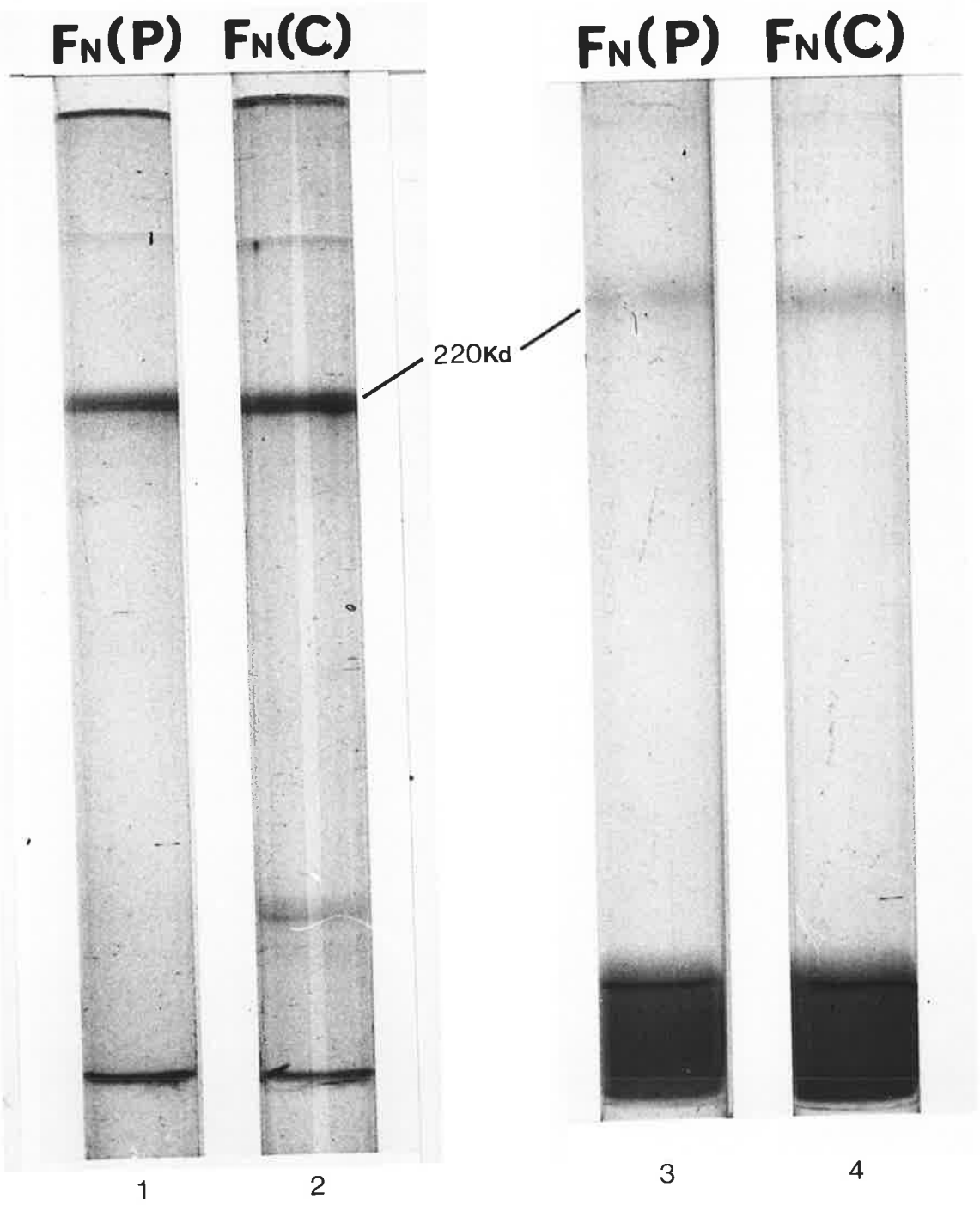
Figure 2.3. SDS-polyacrylamide gel electrophoresis of human plasma fibronectin

Fibronectin was isolated from human plasma by affinity chromatography (as described in section 2.2.9) and analysed on 6.5% cylinder gels under reducing conditions. Samples of 25µg protein in 25µl SPB were applied to each gel. The gels were stained with Coomassie Blue (gels **1** and **2**) and PAS stain for carbohydrate (gels **3** and **4**). The composition of the samples applied was as follows:

i) Gels **1** and **3**, labelled **FN(P)**, purified fibronectin following both gelatine-sepharose and arginine-sepharose affinity chromatography.

ii) Gels **2** and **4**, labelled **FN(C)**, crude fibronectin following gelatine-sepharose chromatography alone.

The major band seen in all four gels migrated with an apparent molecular weight of 22,000 (labelled **220Kd**).


FN(P) FN(C)
220Kd
FN(P) FN(C)
1
2
3
4

2.3 RESULTS

2.3.1 Extraction of Microfibrillar Antigen

Foetal calf ligamentum nuchae was extracted as described in section 2.2.2. After concentration (by ultrafiltration) and dialysis as described, each of the seven extracts obtained was assayed for protein content. The results for a typical extraction are given in Table 2.3. The bulk of the extracted protein appeared in the first three extracts (typically more than 90%). Although the total amount of protein extracted appears small, it should be noted that the water content of ligament at this age is of the order of 85%, and that polymeric collagen and alkali-insoluble elastin represent approximately 40% of the dry weight (Cleary et al., 1967). As the collagen is digested to dialyzable peptides during the extraction procedure, and insoluble elastin remains in the final residue this will account for the relatively small yields. It should be noted that although extract SGCGCG contained only 1.1% of the total extractable protein, subsequent extraction in the presence of DTT (extract MAg) resulted in a four-fold increase in the amount of protein solubilized.

Macroscopically, the last two guanidine extracts (SGCG and SGCGCG) and MAg extract displayed an increasing tendency to self-aggregation after dialysis into saline buffer, forming a finely dispersed white precipitate. Part of the precipitate formed by MAg was pelleted, fixed and processed for electron microscopy as described in section 2.2.3. Dissolution of precipitate could be achieved by dialyzing extracts back into 6M GuHCl buffer; however lyophilized material became almost irreversibly insoluble in all conventional biochemical buffers. In subsequent extractions, MAg was reduced and maleoylated according to the method described by Wieslander and Heinegard (1979), in an attempt to improve its solubility. This was partially successful, and allowed limited analysis of MAg by SDS-polyacrylamide gel electrophoresis (see section 2.3.3).

2.3.2 Electron Microscopy of Extraction Residues

A portion of the residue obtained at each stage of the extraction procedure was processed for electron microscopic examination as described in section 2.2.3. The electron micrographs obtained are shown in Figures 2.4 to 2.11.

Table 2.3

Appearance and Protein Content of Extracts Obtained from Foetal Calf Ligamentum Nuchae

Ligamentum nuchae from a foetus of 220 days (14.5g wet weight) was extracted as summarized in Figure 2.1 and the protein content of each extract was assayed by the method of Lowry et al. (1951).

Extract	Appearance*	Extract* Volume (ml)	Protein* Concentration (mg/ml)	Protein Extracted (mg)	% of Total Extracted Protein
S	Clear, viscous, slightly pink	40	5.10	204	43.6
SG	Opalescent, very viscous, no precipitate	30	5.75	173	37.0
SGC	Clear	22	2.15	47	10.0
SGCG	Opalescent, fine white precipitate	20	0.80	16	3.4
SGCGC	Clear	11.5	0.20	2	0.4
SGCGCG	Slight opalescence, fine white precipitate	12	0.40	5	1.1
MAg	Opalescent, fine white precipitate	14	1.50	21	4.5

* After concentration and dialysis as described in section 2.2.2.

The appearance of intact ligament corresponded closely to that described by Fahrenbach et al. (1966) and Greenlee et al. (1966). At this age the ligament is relatively cellular, containing longitudinally elongated fibroblasts of typical appearance. Collagen fibrils are the dominant feature of the extracellular matrix and often appear interconnected by fine, rather heterogeneous filaments which may represent matrix glycoproteins or proteoglycans (Myers et al., 1973). Developing elastic fibres with large numbers of associated microfibrils were seen; these microfibrils showed a beaded periodicity when cut longitudinally, and appeared to have a lucent core when seen in cross-section. Small aggregates of microfibrils were observed in embayments formed by the cell membrane; these aggregates were often seen to have a developing core of amorphous elastin.

Disruption of cells and loss of normal tissue architecture were apparent after saline extraction, with copious amounts of cell debris spread through the matrix (Figure 2.4). No extraction of collagen, elastin or microfibrils had apparently occurred. However, after extraction with 6M GuHCl (residue SG), almost all cellular structures had disappeared and only occasional clumps of debris were seen (Figure 2.5). Collagen fibrils were markedly swollen, and had a fibrillar, unravelled appearance. No other matrix components were identifiable apart from amorphous elastin, which appeared slightly darker-staining than normal. Most striking was the large decrease in the amount of microfibrillar material which could be seen around the periphery of elastic fibres. This indicates that GuHCl actually extracts microfibrillar components in the absence of sulphydryl bond-reducing agents, contrary to the findings of Ross and Bornstein (1969). Subsequent extraction with GuHCl (residues GCG and GCGCG) showed a much smaller, but discernible, further loss of peripheral microfibrils (Figures 2.7 and 2.8).

The residue after collagenase digestion (SGC) was devoid of any recognizable collagen, and contained only elastic fibres and a little debris (Figure 2.6). This appearance suggests that a single collagenase digestion step may be sufficient to solubilize matrix collagen if attention is paid to tissue preparation. Microfibrils appeared unaffected by collagenase digestion.

Little change appeared to occur in subsequent extractions, and residue SGCGCG was composed entirely of isolated elastic fibres (Figure 2.8). However, further extraction with 6M GuHCl containing 50mM DTT produced a marked change in the appearance of elastic fibres in the residue SGCGCGM (Figures 2.9 and 2.10). Fibres were fragmented and appeared 'frayed' with considerable marginal irregularity. The interstices of such fibres contained fibrillar material with no visible substructure, and no definite microfibrillar structures could be identified. The remaining amorphous elastin appeared to stain more densely than before. However, residue SGCGCGM proved difficult to section, and some of the differences in appearance may be artefactual due to increased section thickness.

Further extraction of MAg residue with 6M GuHCl containing dithiothreitol produced no apparent change in the appearance of the residue, and less than 2mg of protein was extracted. This suggests that nearly all of the extractable MAg is solubilized in a single step. No antigenic material which cross-reacted with anti-MAg antiserum was detected in this further extract.

Examination of the precipitate formed by dialysis of MAg extract into PBS showed aggregates of fine granular material, having a rather amorphous appearance in positively-stained preparations (Figure 2.11). At high power, darker-stained particles arranged in circles were occasionally seen, and short parallel rows of such particles, with a similar diameter to those in circular array, were also observed. Although of approximately the same diameter as elastin-associated microfibrils, such structures are difficult to interpret, and possibly may simply represent random arrangements of stained particles. Further studies, using negatively stained preparations, could be of value in elucidating this point.

2.3.3 Analysis of Extracts by SDS-PAGE

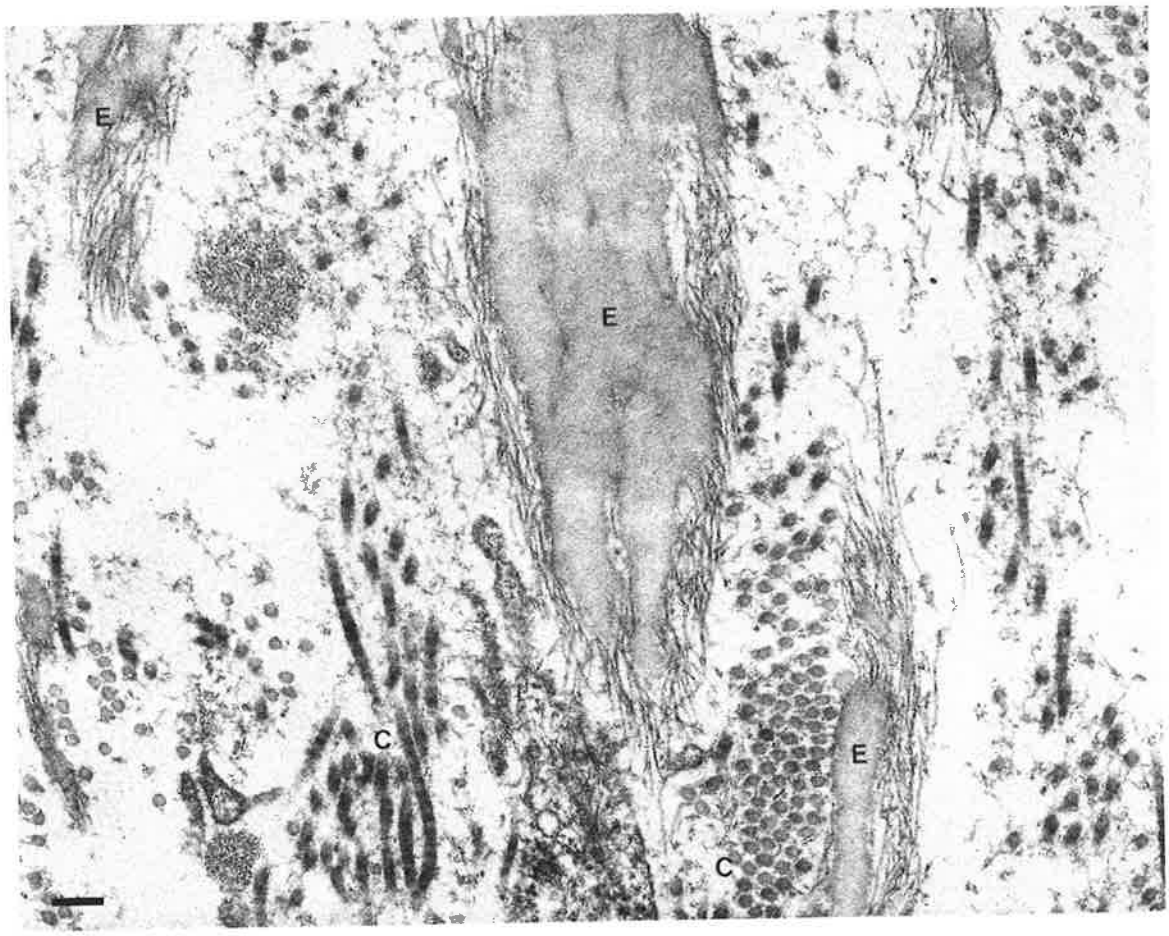
Ligament extracts were analyzed by PAGE in SDS, as described in section 2.2.4. Figure 2.13 shows the Coomassie Blue stained cylinder gels incorporating samples of the saline and guanidine extracts.

Figure 2.4. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue S

Several longitudinally-sectioned elastic fibres (E) with peripheral microfibrils are shown. Collagen fibrils (C) and cellular debris are also present in the matrix. X34,000. Scale = 200nm.

Figure 2.5. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SG

Portions of two elastic fibres (E) are shown, adjacent to which are greatly swollen collagen fibrils (C). Note the large decrease in the numbers of microfibrils (↑) which can be seen. X42,000. Scale = 200nm.


E
E
C
E
C

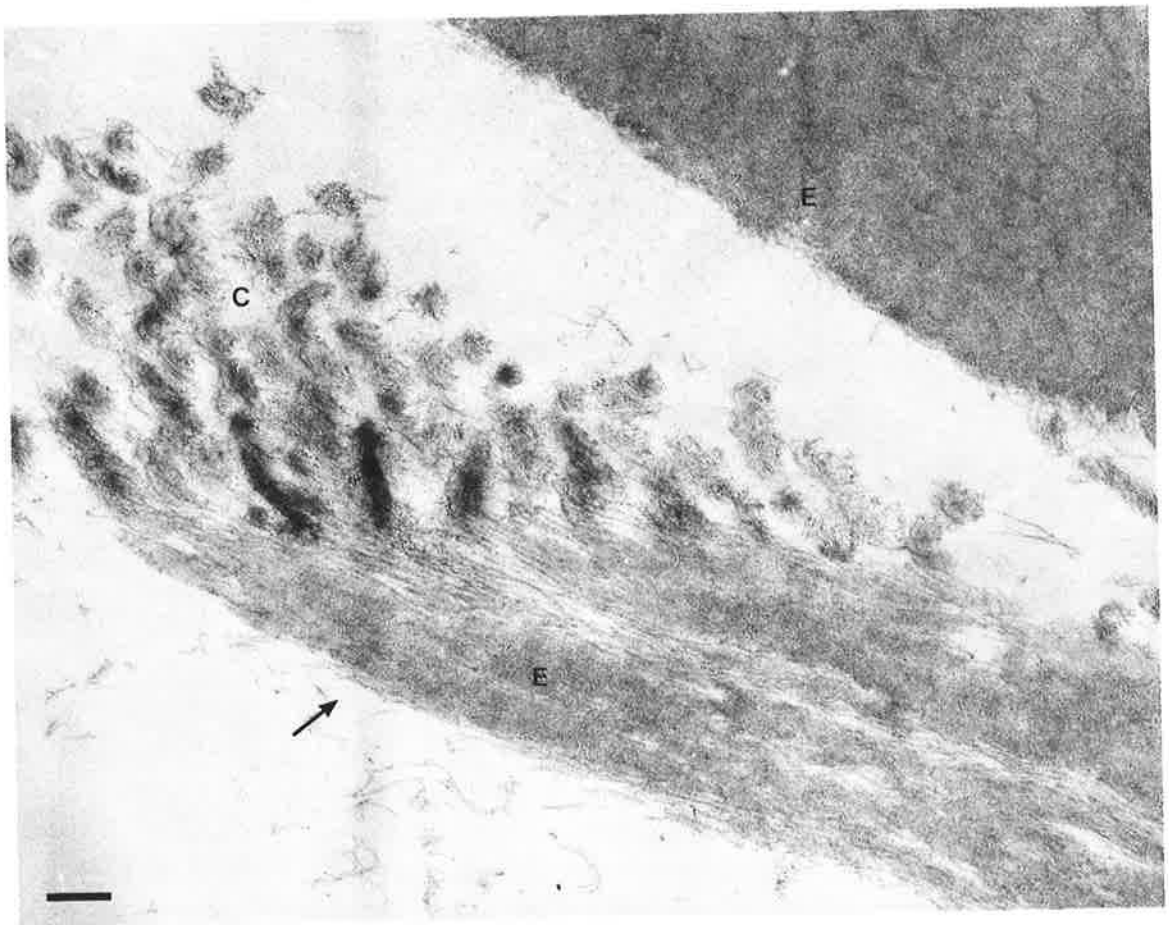
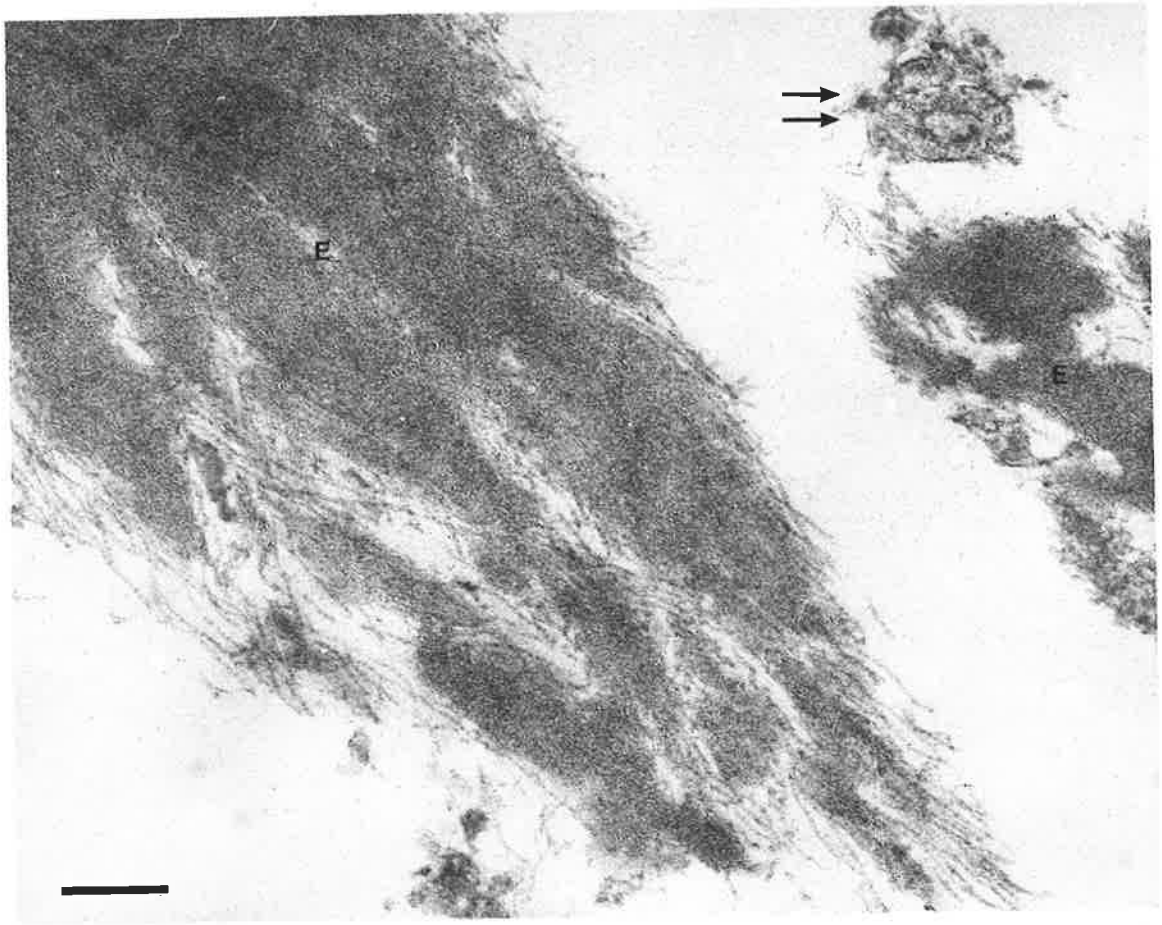

E
C
E

Figure 2.6. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SGC

Several longitudinally-sectioned elastic fibres (**E**) are shown which contain microfibrillar material within the interstices of the fibre. A small amount of cellular debris (**++**) is still present, but collagen fibres were not observed at this stage of the extraction procedure. X70,000. Scale = 200nm.

Figure 2.7. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SGCG

A tangentially sectioned elastic fibre (**E**) is shown which has an attenuated covering of microfibrils. These appear considerably shortened in comparison to previous extracts. No other matrix or cellular components remained after the second GuHCl extraction. X55,000. Scale = 200nm.


E
E

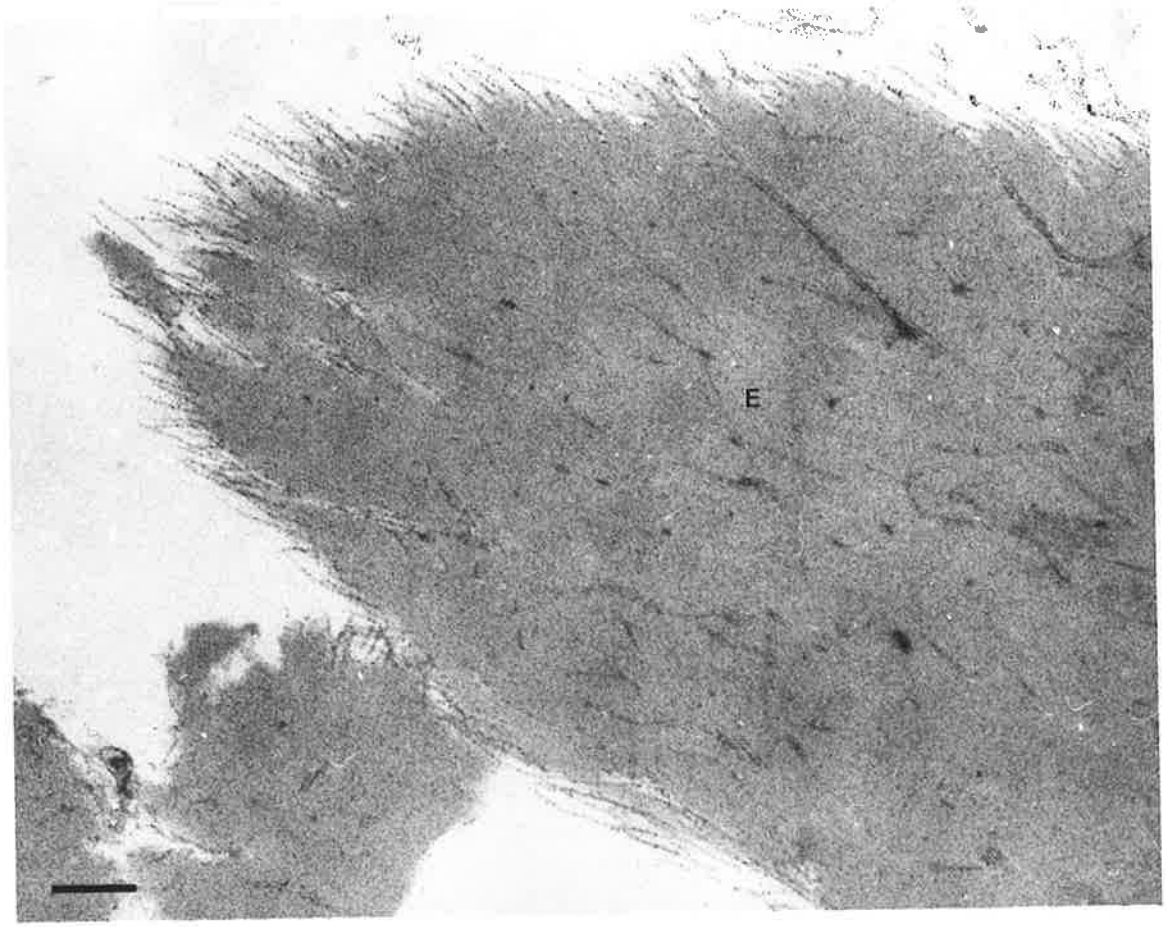
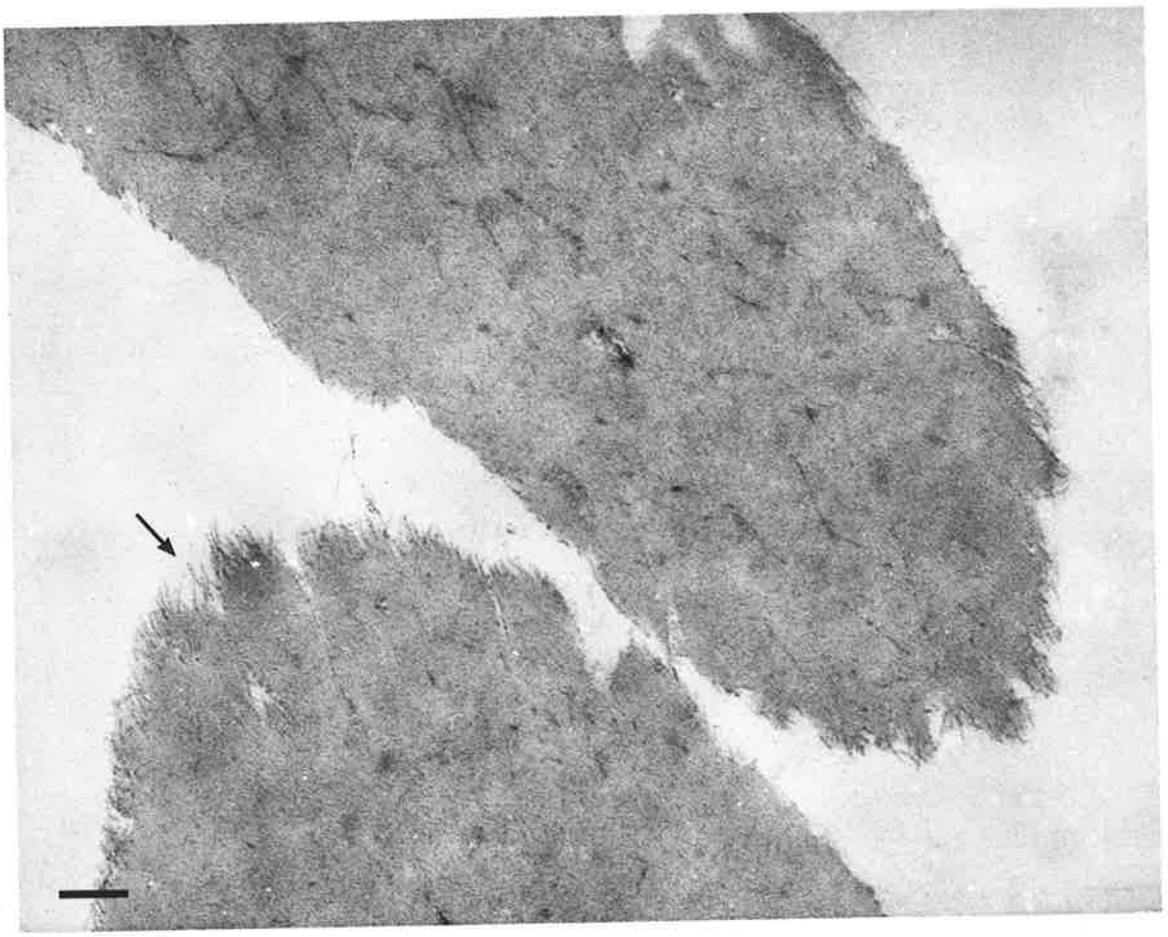

E

Figure 2.8. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SGCGCG

Portions of two longitudinally-sectioned elastic fibres can be seen. Note the continued decrease in numbers of elastin-associated microfibrils (↑), which are virtually absent from some areas of the fibre. Remaining microfibrils appear to have undergone further shortening. X45,000. Scale = 200nm.

Figure 2.9. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SGCGCGM

A number of elastic fibres are present in both longitudinal- and cross-sections. These are more electron-dense than before and appear to be fragmented and irregular. Amorphous granular material can be seen within the interstices of these fibres. Microfibrils could not be identified. X85,000. Scale = 200nm.



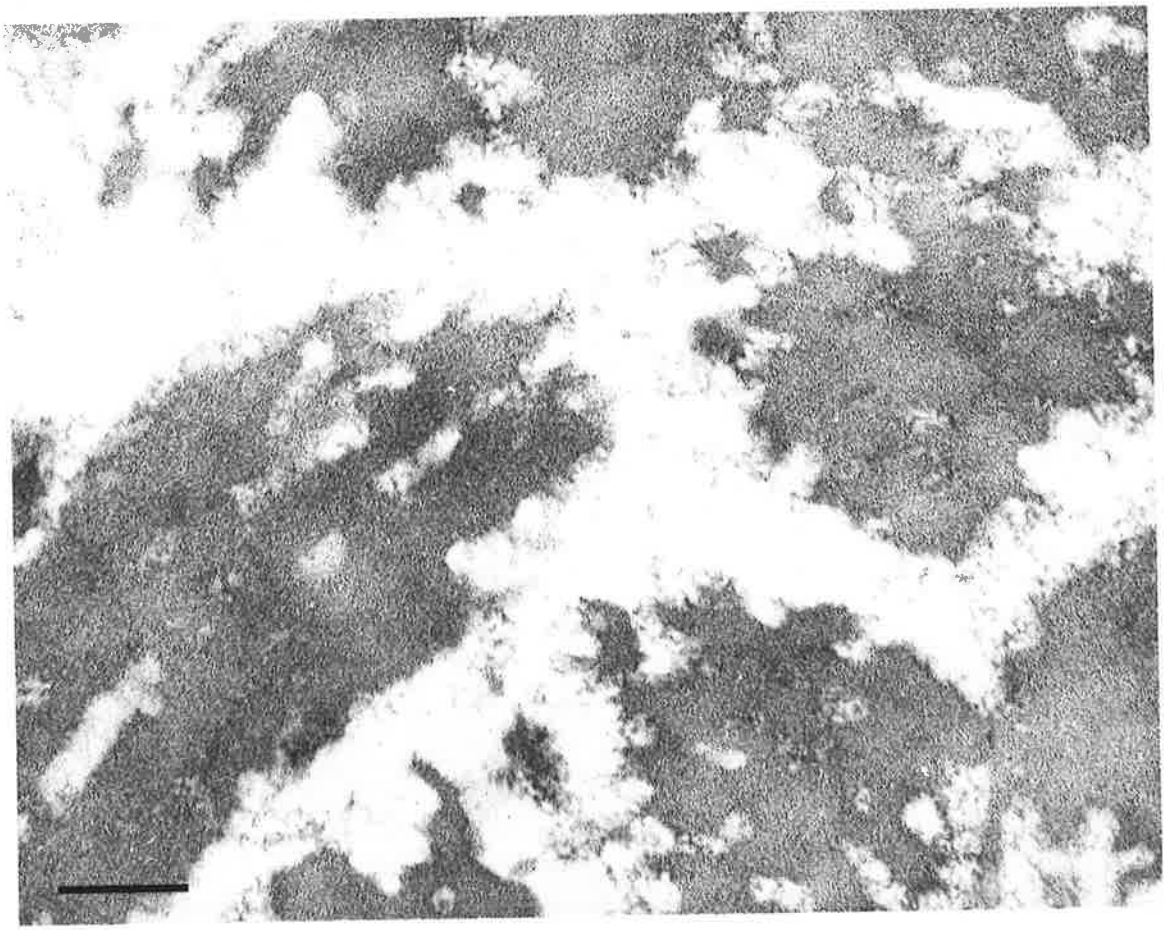
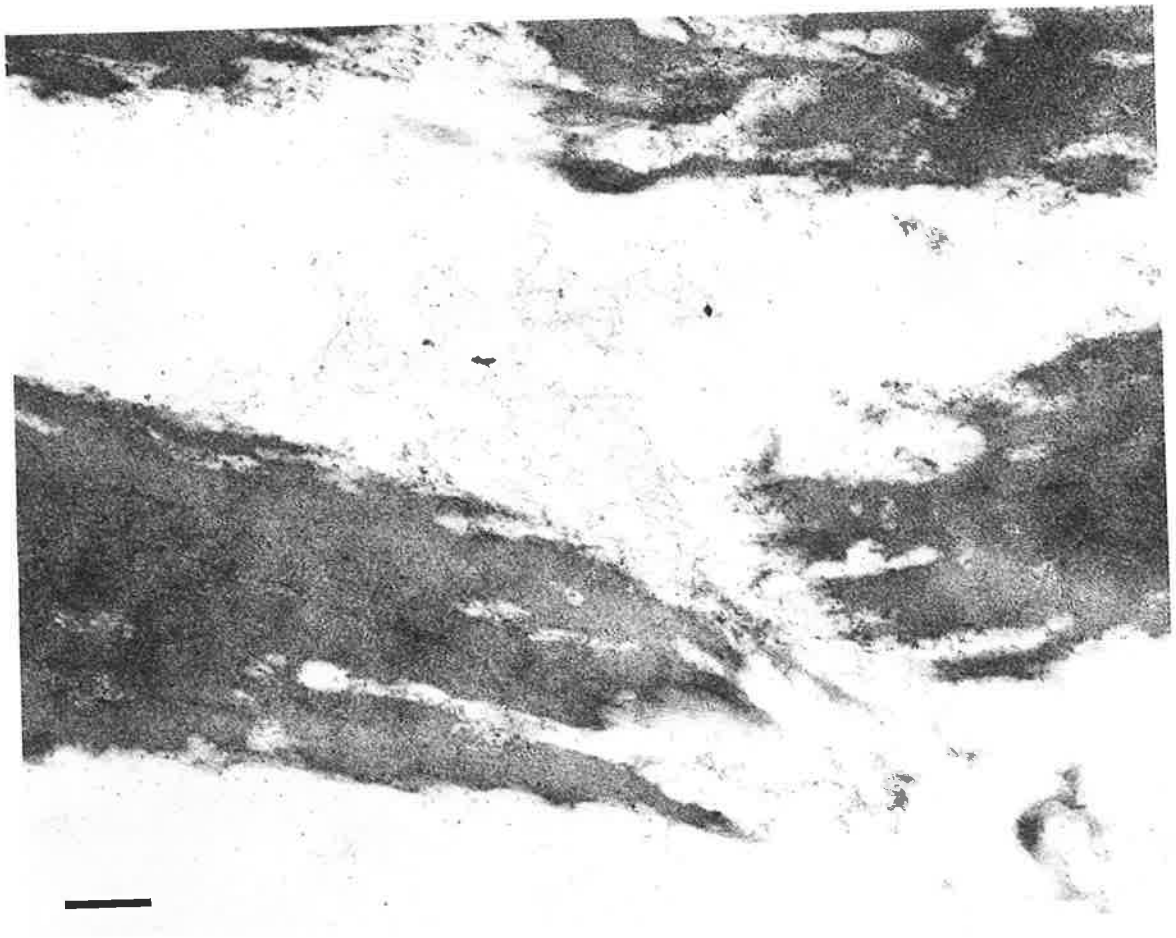


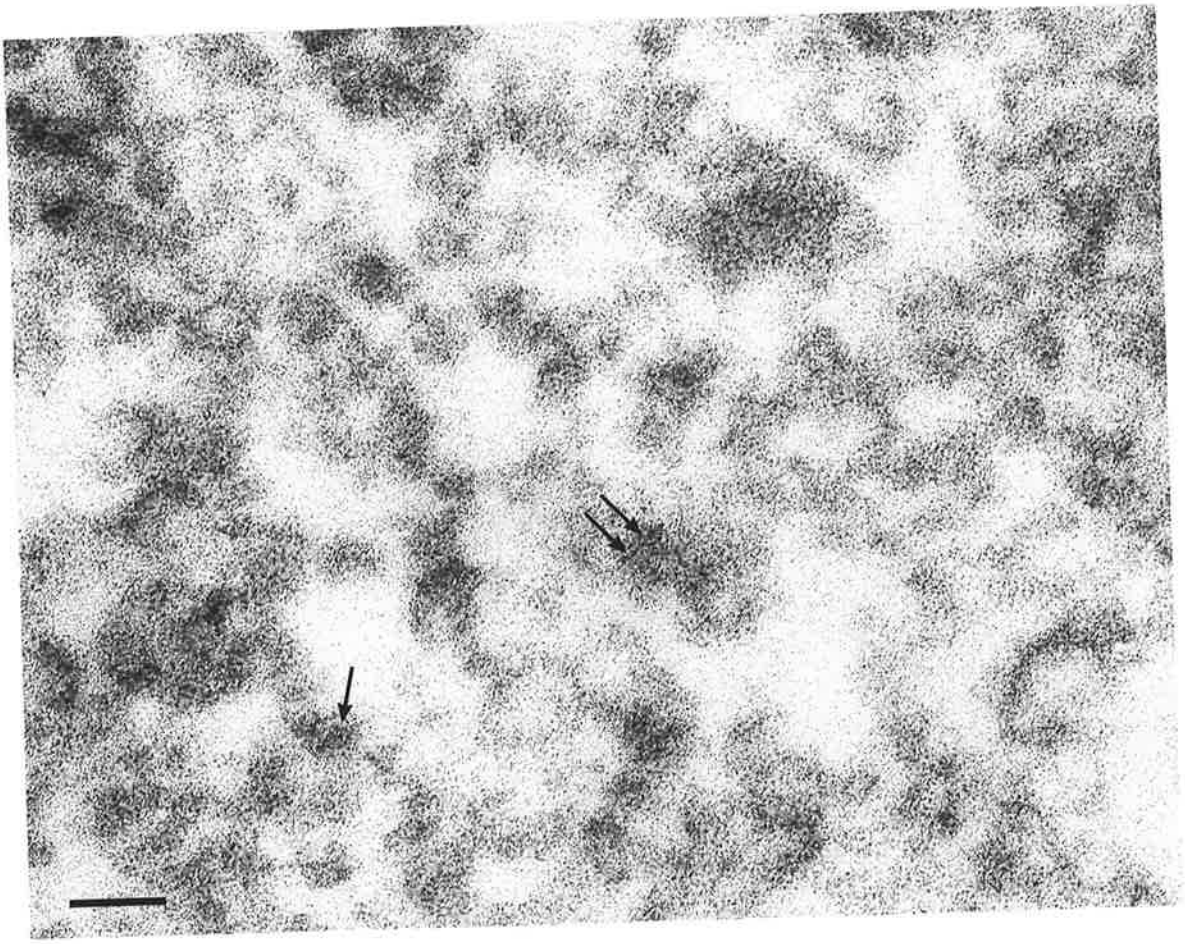
Figure 2.10. Transmission electron micrograph of foetal calf ligamentum nuchae extraction residue SGCGCGM

Similar section to Figure 2.9. Note the fine fibrillar material preent between elastic fibres. X55,000. Scale = 200nm.

Figure 2.11. Transmission electron micrograph of precipitate formed by MAg upon dialysis into PBS

Note the indistinct circular (†) and parallel (††) arrangements of darker-staining particles which can be discerned within areas of granular precipitate. X250,000. Scale = 50nm.





Extract S contained numerous polypeptides, the majority of which are probably serum-derived proteins or salt-soluble proteins released from disrupted cells.

The three succeeding GuHCl extracts, SG, SGCG, and SGCGCG showed increasing insolubility, similar amounts of sample having been applied to each gel. Several major bands present in SG extract co-migrated with a calf-skin collagen type I polypeptide standard, indicating that a significant amount of collagen had been extracted prior to collagenase digestion. Extracts SGCG and SGCGCG contained no bands corresponding to collagen, confirming the impression given by electron microscopic monitoring of extracts, which showed that no identifiable collagen could be found after extract SGC. In both of these extracts, a good deal of material was aggregated under standard running conditions, and heavy staining could be seen at the top of the stacking gel, and at the interface of the stacking and separating gels.

Many attempts were made to solubilize SGCGCG and MAg extracts so that adequate analysis of samples by SDS-PAGE could be carried out. Partial success was achieved by incorporating 5M urea in all buffers, and using samples which had been reduced and maleoylated whilst still in 6M GuHCl buffer (before dialysis and lyophilization). The result obtained by this technique is shown in Figure 2.12. Despite centrifugation of samples before application (as detailed in section 2.2.4), a considerable amount of material still failed to enter the separating gel, and remained at the interface with the stacking gel. Very large samples (300µl) were applied to the gels, and consequently SGCGCG and MAg extracts ran somewhat slower than the protein standards, despite adjustment of the volume of such standards by the addition of SPB. Gels electrophorezed in 5M urea took approximately twice as long to run as those using standard buffers.

A large number of polypeptide bands were seen in extract SGCGCG. In contrast, only two major bands were visible in MAg extract, having apparent molecular weights of 210,000 and 160,000, as calculated from the relative mobilities of the protein standards electrophorezed concurrently. Insufficient amounts of sample entered the gels for PAS staining to be successful, and no observations could be made on whether

Figure 2.12. SDS-Polyacrylamide gel electrophoresis of foetal calf ligamentum nuchae extracts SGCGCG and MAg

Ligamentum nuchae extracts SGCGCG and MAg (300µg in 300µl SPB) and protein standards were analyzed on 6.5% cylinder gels under reducing conditions, as described in section 2.2.9. 5M urea was incorporated into all buffers. The gels were stained with Coomassie Blue. The samples applied, from left to right, are listed as follows:

i) Protein Standards (**STAN**). The mobilities of myosin (**MYOSIN**), phosphorylase a (**PHOS a**), bovine serum albumin (**BSA**) and ovalbumin (**OA**) are marked.

ii) Type I collagen (**COLL I**) from foetal calf skin.

iii) Human plasma fibronectin (**F**).

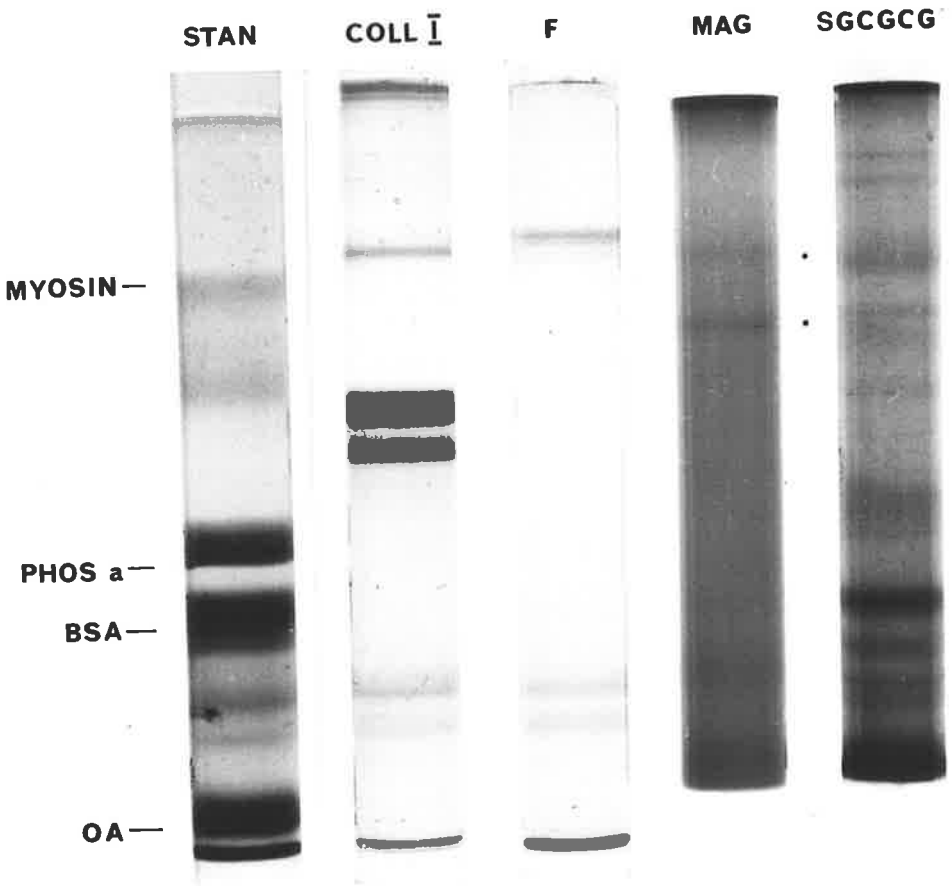
iv) Microfibrillar antigen (**MAG**).

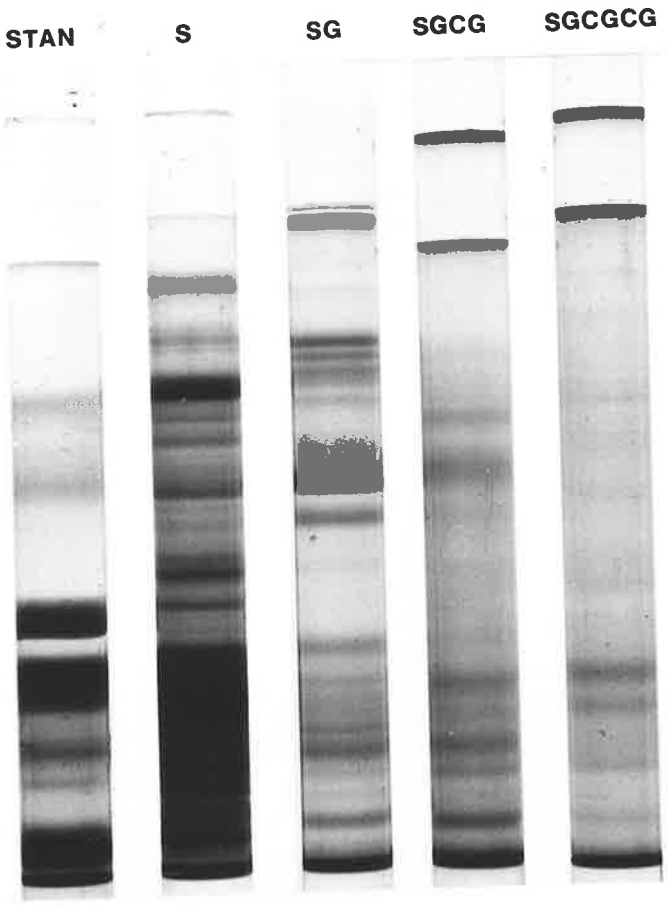
v) Extract **SGCGCG**.

Note the very high background staining of the gels incorporating tissue extracts MAg and SGCGCG. A large amount of material can also be seen at the interface of the stacking and resolving gels. The major protein species detected in MAg extract are marked with dots (molecular weight about 210,000 and 160,000 daltons).

Figure 2.13. SDS-Polyacrylamide gel electrophoresis of preliminary extracts of foetal calf ligamentum nuchae

Samples of ligamentum nuchae extracts **S**, **SG**, **SGCG** and **SGCGCG** (300µg in 300µl SPB) were analyzed on cylinder gels by an identical technique to that in Figure 5.12. Co-electrophorezed protein standards (**STAN**) were as above. Note the apparent increasing insolubility of extracts. A large amount of material in extracts **SGCG** and **SGCGCG** failed to enter either the stacking gel or the resolving gel.


STAN
COLL I
F
MAG
SGCGCG
MYOSIN
PHOS a
BSA
OA


STAN
S
SG
SGCG
SGCGCG

either of the major species detected contained sugar components. In view of the marked insolubility of MAg (causing loss of sample on centrifugation), the large amount of material which failed to enter the gel, and the high overall background staining of such gels, the presence of other polypeptide bands could not be excluded. Thus the two components observed on SDS-PAGE represent only the soluble portion of MAg, and the extract must be regarded as heterogeneous. Later experiments (see section 2.3.4) showed that an antiserum raised against MAg had significant activity toward plasma fibronectin. In view of this finding the 210,000 dalton polypeptide band seen on SDS-PAGE of MAg may represent matrix fibronectin, which is immunologically identical to plasma fibronectin (Ruoslahti and Vaheri, 1975).

2.3.4 Immunochemical Characterization of Anti-MAg Antiserum

As described in section 2.2.6, a number of different methods were used to examine the antigenicity of MAg extracts.

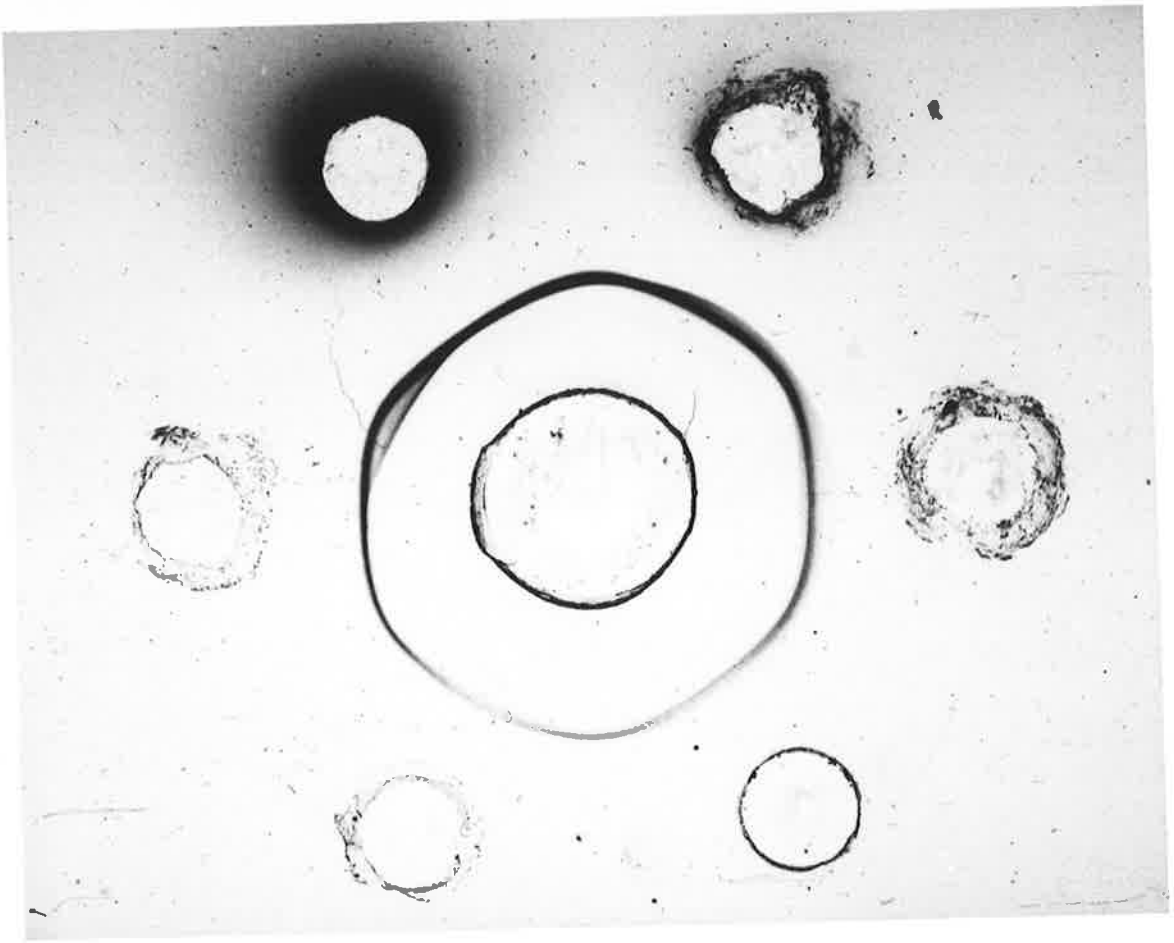
Immunodiffusion. Immunodiffusion of MAg and SGCG extracts against their respective antisera was carried out, and the results are shown in Figures 2.14 and 2.15. MAg showed only a single precipitin band which reacted against anti-MAg antisera from all three rabbits; both early and later bleedings showed this typical pattern. The titre of precipitating antibody as judged by immunodiffusion was 1/64 (shown in Figure 2.14). This pattern suggests that there is only a single soluble antigenic species within the MAg extract which is capable of forming precipitins in agar gels, but the possibility of other activity against insoluble material contained in the extract could not be excluded. Of the other extracts, only extract SG produced a precipitin line when reacted against anti-MAg antiserum, showing a 'reaction of identity' with the precipitin band formed by MAg extract. Reaction of anti-MAg antiserum against plasma fibronectin, FCS, collagen types I and III, tropoelastin and α-elastin did not produce any precipitin bands. Similarly, no reaction between anti-tropoelastin and anti-(α-elastin) antisera (produced in this laboratory by Dr E G Cleary) and MAg extract could be shown. In subsequent experiments, freshly prepared extracts of MAg behaved in identical fashion to the original preparation.

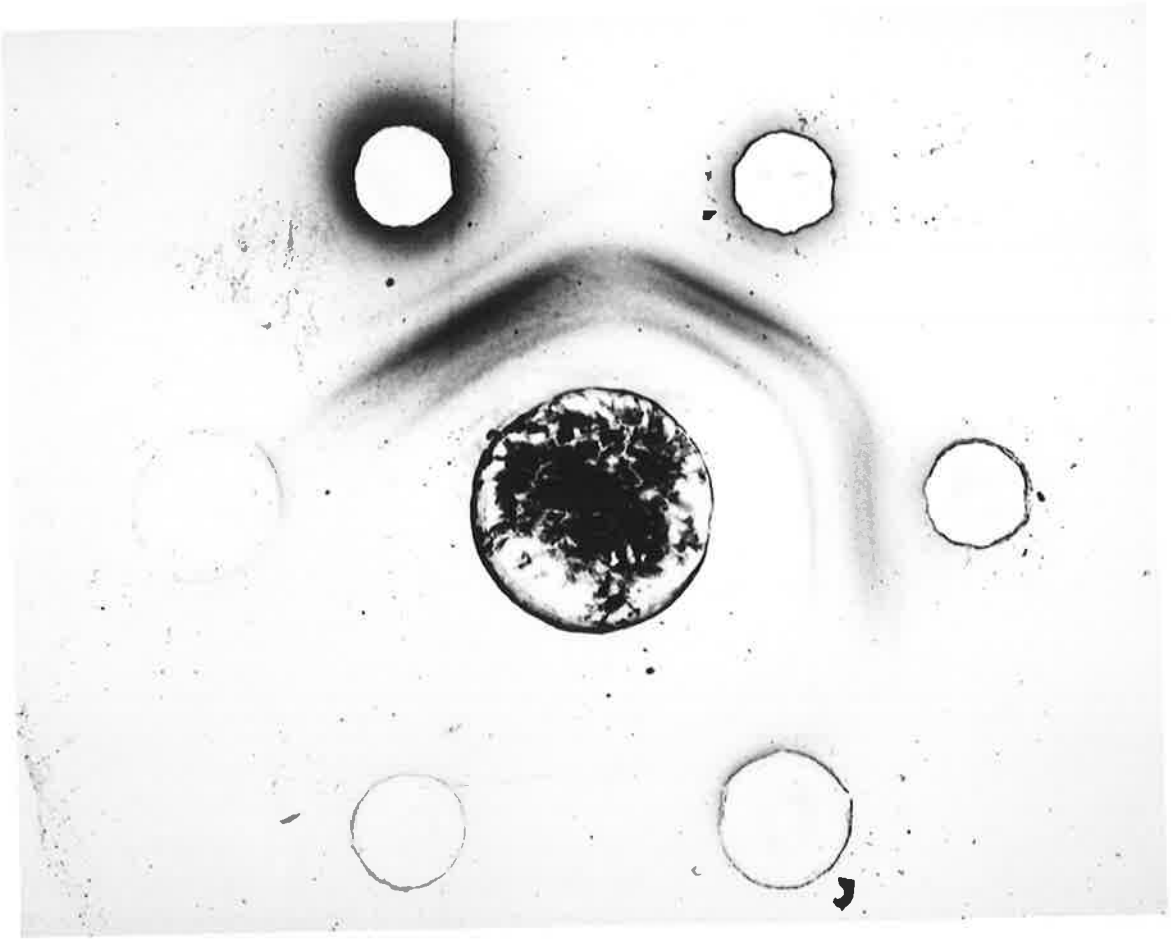
Figure 2.14. Double radial immunodiffusion of MAg extract against anti-MAg antiserum

The central well contained MAg extract (1 mg/ml) suspended in PBS. Dilutions of anti-MAg antiserum were arranged clockwise around the central well beginning at the 11 o'clock position with 1:2 dilution, then serially 1:4, 1:8, 1:16, 1:32 and 1:64. The gel was dried and stained with Coomassie Blue as described by Weeke (1973).

Figure 2.15. Double radial immunodiffusion of extract SGCG against anti-SGCG antiserum

The central well contained extract SGCG (1 mg/ml) suspended in PBS. Dilutions of anti-SGCG antiserum were arranged in the peripheral wells in an identical manner to that in Figure 2.14.





Reaction of SGCG extract against anti-SGCG antiserum showed at least three precipitin bands (Figure 2.15) of low titre (approximately 1/16). This result was consistent with the large number of soluble species demonstrated by SDS-PAGE of SGCG extract. No cross-reactivity between MAg and anti-SGCG antiserum could be shown. This finding implies that if the primary antigenic component of MAg extract is present in SGCG extract, it must be in only very small quantities. Such an interpretation is supported by the fact that anti-MAg antiserum also showed no reaction against SGCG extract.

It should be noted, however, that immunodiffusion is a relatively insensitive technique which depends largely on the solubility of the antigen which is being studied. Other techniques (e.g. immuno-electrophoresis in agarose gel) which also depend on antigen solubility have similar limitations. In the case of the extremely insoluble antigens investigated in this study, the usefulness of such methods is relatively limited, and a false impression of the specificity of anti-MAg antiserum could be gained from the results obtained. In order to overcome this problem, it was considered that haemagglutination and ELISA methods may be more useful in order to evaluate the antigenic determinants of anti-MAg antiserum.

Passive Haemagglutination. It was found that formalinized, tanned sheep red blood cells could be optimally coated with 0.1-0.2mg/ml of MAg, for use in haemagglutination assays. Titres of 1/1600-1/6400 were obtained for anti-MAg antiserum reaction against MAg-coated cells. This provided a quick method for titrating successive bleeds of antiserum, and it was found that a maximal titre of 1/6400 was obtained by the sixth injection. Titres were maintained at this level after subsequent booster injections.

Cross-reactivity of anti-MAg antiserum was checked by coating batches of cells with various antigens, and reacting them with the antiserum. SGCG-coated cells showed a reaction to a titre of 1/160 against anti-MAg antiserum, indicating that a small amount of cross-reacting protein was present in SGCG extract, despite negative results using immunodiffusion. No reactivity against tropoelastin or α-elastin coated cells could be demonstrated; anti-tropoelastin and anti-(α-elastin) antisera also did not agglutinate

Figure 2.16. Haemagglutination assay to demonstrate cross-reactivity of BSA with anti-MAg antisera

Haemagglutination assays were performed with formalinized, tanned sheep red cells coated with BSA, as described by Herbert (1973). Antisera were serially diluted from 1:20 to 1:2560 (wells 1 to 8). The antisera tested were as follows:

Row **A:** Mouse anti-BSA IgG (positive control), titre 1:320.

Row **B:** Pre-immune rabbit serum (negative control).

Row **C:** Anti-MAg antiserum (late bleed), titre 1:80.

Row **D:** Anti-MAg antiserum (early bleed), titre 1:640.

Note the significantly higher activity of early bleeds of anti-MAg antiserum in comparison to later bleeds. Well **10** contains uncoated cells in a 1:20 dilution of each antiserum. Wells **11** and **12** contain uncoated and coated cells respectively in saline.

Figure 2.17. Haemagglutination assay to demonstrate adsorption of contaminant anti-BSA activity from anti-MAg antiserum by preincubation with FCS

Haemagglutination assays were performed as in Figure 2.16. Antisera were serially diluted from 1:10 to 1:5120 (wells 1 to 10). The antisera tested were as follows:

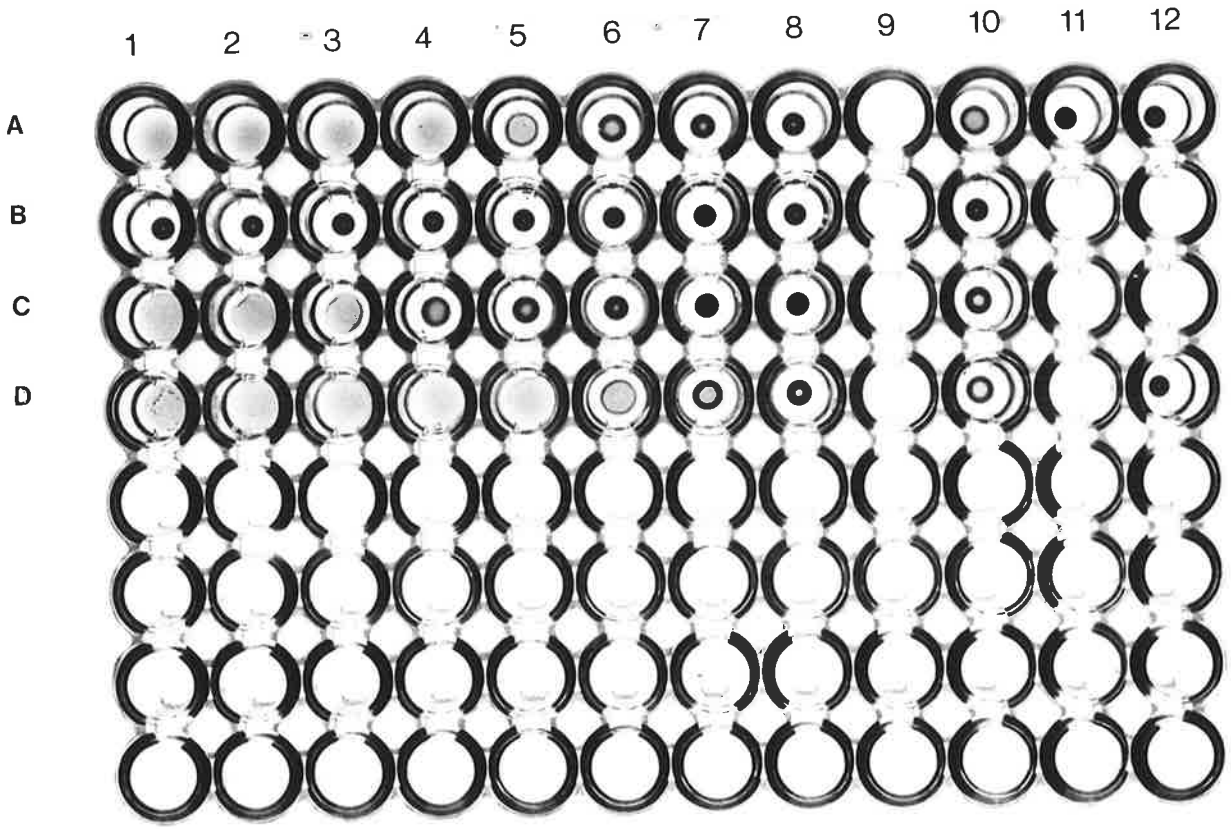
Row **A:** Mouse anti-BSA IgG (positive control), titre 1:640.

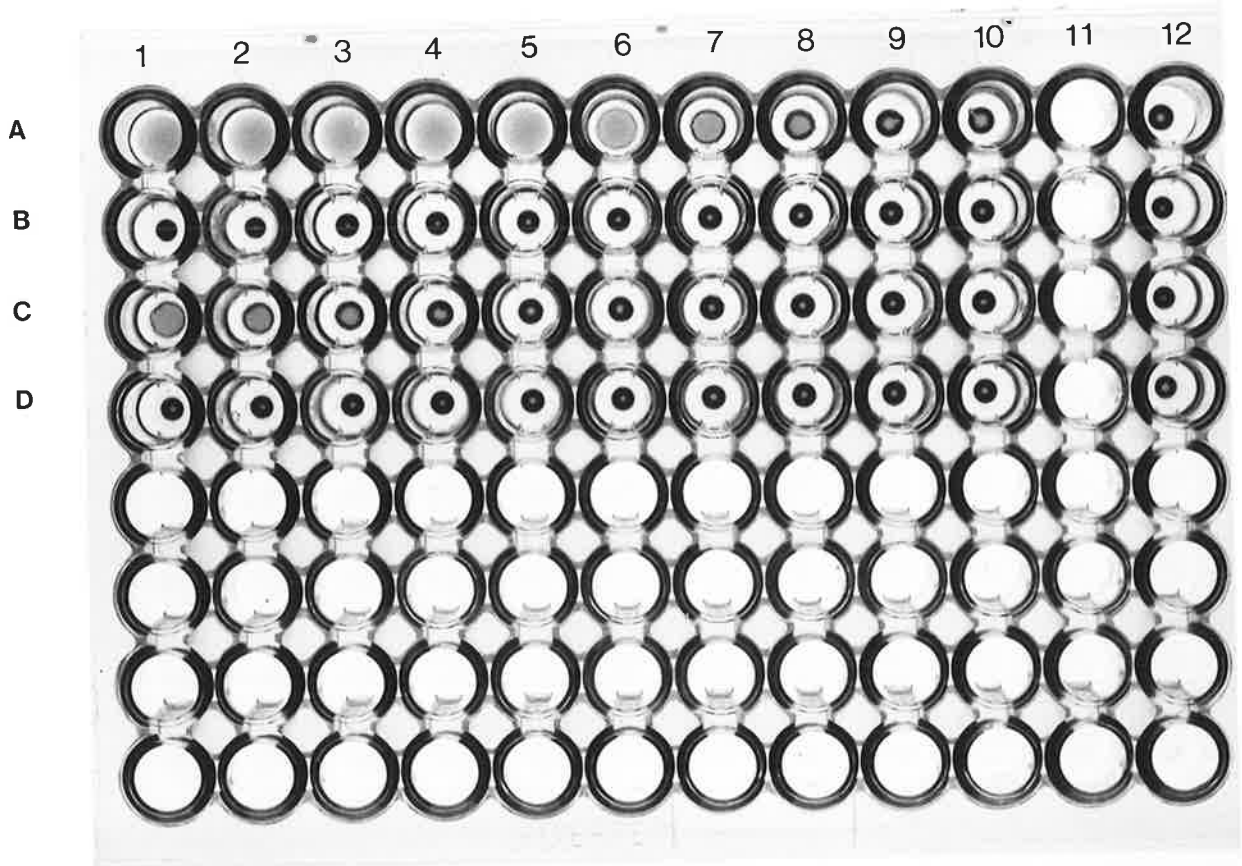
Row **B:** Pre-immune rabbit serum (negative control).

Row **C:** Anti-MAg antiserum (late bleed) before adsorption with FCS, titre 1:80.

Row **D:** Anti-MAg antiserum (late bleed) after adsorption with FCS, all wells negative.

Well **12** contains uncoated cells in a 1:10 dilution of each antiserum.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

MAg-coated cells. However, quite high titres were obtained against both BSA and FCS-coated cells. Titres against both antigens were similar, and it is possible that anti-BSA activity is responsible for most of the cross-reactivity shown against FCS. Early bleeds showed much higher activity against BSA than later bleeds (see Figure 2.16) from the same animal, and induction of relative tolerance to BSA may have occurred with repeated immunization. Pre-incubation of anti-MAg antiserum with FCS completely eliminated this cross-reactivity (see Figure 2.17), without lowering the titre against MAg more than one dilution. Although no band corresponding to BSA could be seen on SDS-PAGE of MAg, BSA is known to be an extremely potent immunogen, and even minute quantities of protein would be sufficient to induce activity of the magnitude shown in these studies. No precipitin lines were formed on immunodiffusion of anti-MAg antiserum against either BSA or FCS, and it was concluded that antibodies against these proteins did not contribute significantly to the precipitin lines formed by MAg extract against anti-MAg antiserum.

In view of these findings, it was considered necessary to investigate further cross-reactivity between anti-MAg antiserum and other matrix proteins. Haemagglutination has some drawbacks for such a survey; it is rather wasteful of precious antigen and some antigens investigated showed a tendency to cause auto-agglutination even at very low coating dilutions. These difficulties prompted investigation of ELISA as an alternative method of antigenic analysis.

Enzyme-Linked Immunosorbent Assay (ELISA). An indirect microplate ELISA method was used (as detailed in section 2.2.7) to study the specificity of anti-MAg antiserum. Optimal coating concentration of MAg was found to be approximately 15μg/ml as determined by a checkerboard titration technique (Voller et al., 1976). All other antigens were coated onto plates at similar concentrations. Only late bleeds of high titre (determined by haemagglutination) were used in these experiments. When anti-MAg IgG (prepared as described in section 2.2.8) was reacted against MAg extract, titres of 1/3200-1/6400 were routinely obtained; this level was very similar to that found by haemagglutination assay.

Cross-reactivity of anti-MAg IgG with BSA and FCS was confirmed, as was the abolition of such cross-reactivity by preincubation of anti-MAg IgG with FCS. Again, no significant lowering of titre against MAg was found after adsorption of anti-BSA and anti-FCS activity. The results of this experiment are shown in Figure 2.18.

No cross-reactivity was shown by reacting anti-MAg IgG against tropoelastin, α-elastin or collagen types I and III. However, cross-reactivity of some consequence was found against human plasma fibronectin. This was not altogether unexpected, as a protein of apparent molecular weight 210,000 had been shown to be one of the two major soluble species present in MAg extract (see section 2.3.3). Interestingly, cross-reactivity was highest against fibronectin prepared under denaturing conditions (i.e. with the use of 6M urea solutions). Fibronectin prepared under mild conditions, as described in section 2.2.9, showed only low cross-reactivity until it had been denatured with urea. A similar difference in antigenicity of native and denatured fibronectin has been noted by Vuento et al. (1980). Preincubation of anti-MAg IgG with denatured fibronectin resulted in the complete abolition of anti-fibronectin activity, with no lowering of titre against MAg extract. No precipitin lines were formed by reacting either fibronectin preparation with anti MAg IgG; this suggests that the sparingly soluble species of apparent molecular weight 160,000 shown to be present in MAg extract may be partly responsible for the precipitin line formed in immunodiffusion assays.

Anti-MAg antiserum was also shown to have low level activity against the extracts SG (titre 1/160) and SGCG (titre 1/80), confirming the results obtained with immunodiffusion and haemagglutination techniques.

Moderate activity (titre 1/160) was observed on reacting anti-MAg IgG with CL glycoprotein (see section 1.3.3) prepared by Dr M A Gibson of this department (Gibson and Cleary, 1982). This glycoprotein has an apparent molecular weight of 140,000, is collagenous in nature, and has been found in both foetal bovine aorta and ligamentum nuchae. It is the major PAS-positive species seen on SDS-PAGE analysis of 6M GuHCl extracts of these tissues. It is possible that the species of apparent molecular weight

Figure 2.18. Enzyme-linked immunosorbent assay to demonstrate adsorption of contaminant anti-FCS activity from anti-MAg antiserum

Enzyme-linked immunosorbent assay was performed by the method of Voller et al. (1976). Wells were coated with FCS (rows **A** to **C**) or MAg (rows **E** to **G**). Rows **D** and **H** were left uncoated for use as controls to monitor non-specific binding. Antisera were serially diluted from 1:100 to 1:12,800 in wells **1** to **8**. Wells **9** and **10** are blanks (serum omitted). Well **12** consists of negative controls with pre-immune rabbit serum at a dilution of 1:50. Well **11** was left unused. The antisera tested were as follows:

Row **A:** Pre-immune rabbit serum against FCS-coated wells.

Row **B:** Anti-MAg antiserum, before adsorption with FCS. Titre 1:800 against FCS-coated wells

Row **C:** Anti-MAg antiserum, after adsorption with FCS. Activity completely eliminated.

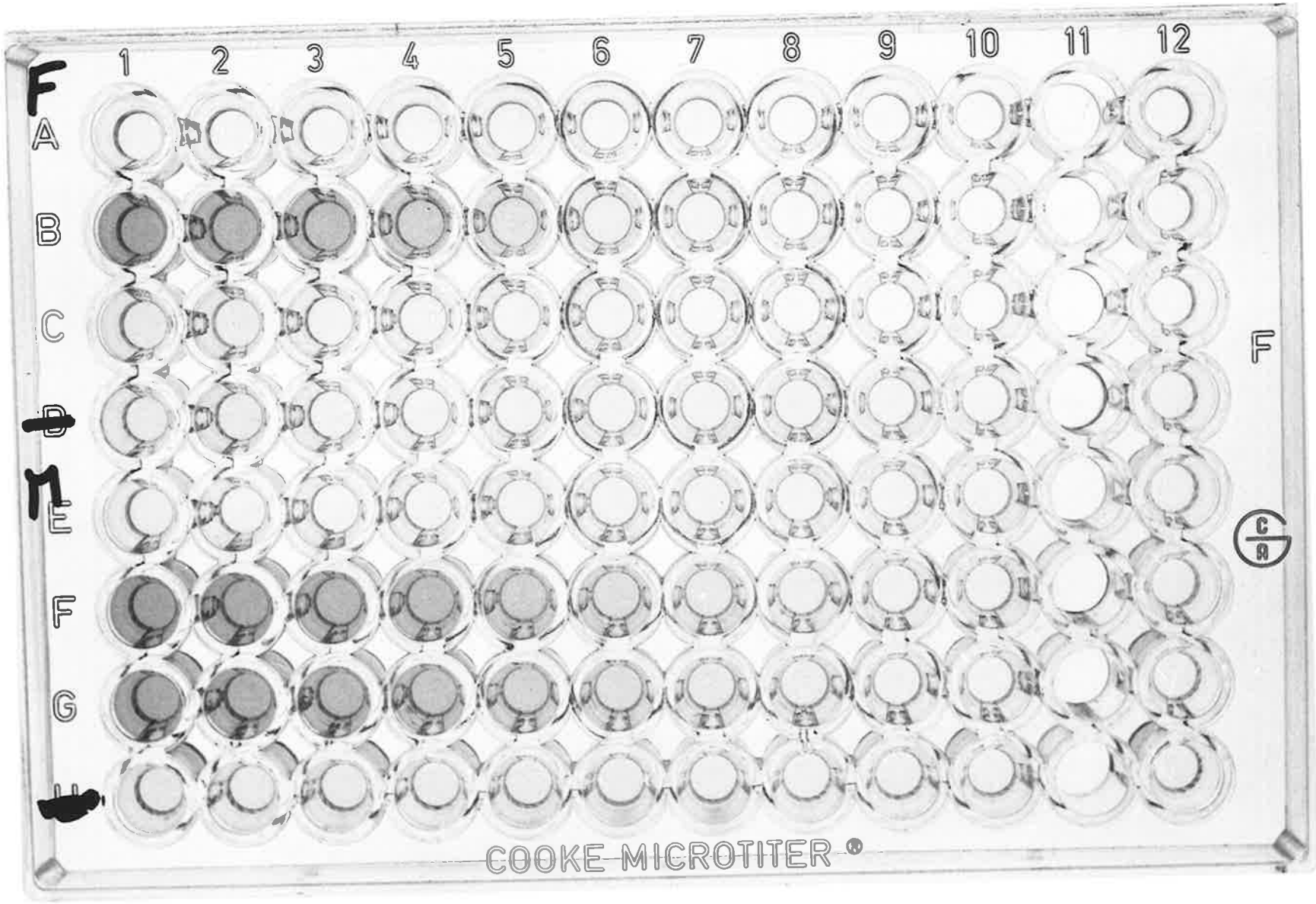
Row **D:** Anti-MAg antiserum against uncoated wells.

Row **E:** Pre-immune rabbit serum against MAg-coated wells.

Row **F:** Anti-MAg antiserum, before adsorption with FCS. Titre 1:3200 against MAg-coated wells.

Row **G:** Anti-MAg antiserum, after adsorption with FCS. Activity unchanged.

Row **H:** Anti-MAg antiserum against uncoated wells.


F
1 2 3 4 5 6 7 8 9 10 11 12
A
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G
H
F
COOKE MICROTITER

160,000 seen on SDS-PAGE of MAg extract (see section 2.3.3) may be identical with or related to CL glycoprotein. Such a relationship would be consistent with the observed antibody activity directed toward this protein.

In summary, it has been shown that, although anti-MAg antiserum appears 'mono-specific' as judged by immunodiffusion, more sensitive haemagglutination and ELISA assays have demonstrated considerable evidence of polyspecificity. In view of the heterogeneous nature of MAg (see section 2.3.3), this finding is not unexpected.

2.3.5 Adsorption of Non-specific Activity from Anti-MAg IgG

It has been shown, by a variety of techniques, that anti-MAg antiserum has significant activity against BSA, some FCS proteins and plasma fibronectin. It was possible to adsorb out this activity without significantly lowering the titre of antibody against the original extract. Therefore, it was considered necessary to eliminate non-specific activity before using anti-MAg IgG in subsequent immunohistochemical procedures (Chapter Three). This was achieved by affinity chromatography, as described in section 2.2.9. The IgG preparation so obtained, referred to as anti-MAg (ADS) IgG, showed no residual activity against any of the proteins tested above. It retained very high levels of activity against the original MAg extract (titre 1/3200), and, on the basis of all three immunological techniques used, was judged to be 'mono-specific'. This antibody preparation was used in subsequent immunohistochemical localization procedures, which are described in Chapter Three.

Subsequent to the completion of these experiments, it was found that anti-MAg contained low-level antibody activity (titre 1/160 on ELISA) against CL glycoprotein (Gibson and Cleary, 1982; 1983). However, insufficient quantities of CL glycoprotein were available to enable immunoadsorption of activity against this antigen from the purified anti-MAg (ADS) IgG preparation.

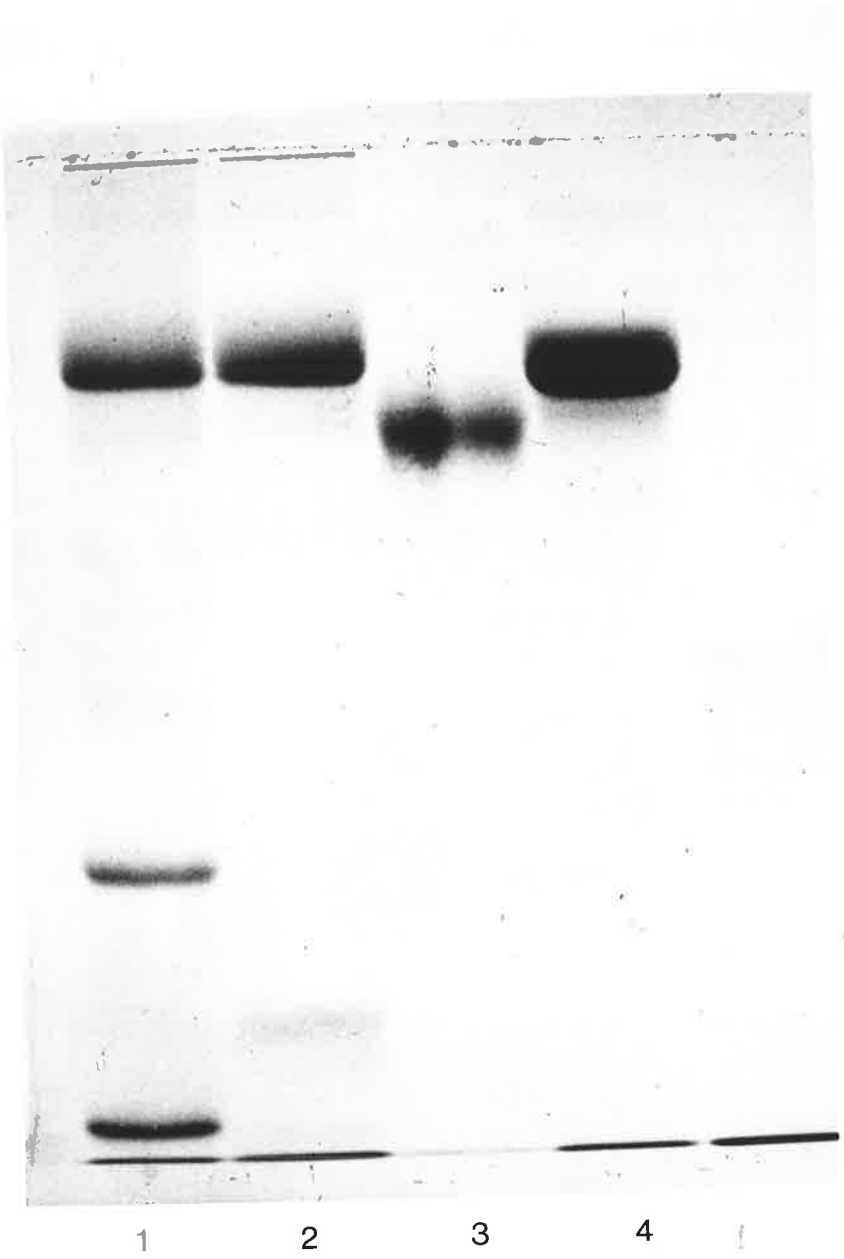
2.3.6 Assessment of the Purity of IgG Preparations

a. Goat anti-(rabbit IgG). Antibodies against rabbit IgG were raised in a goat as described in section 2.2.7. IgG prepared from this antiserum by DEAE-cellulose chromatography showed a single precipitin line on immunodiffusion against both whole rabbit serum and purified rabbit IgG. Titres of 1/6400 were obtained by haemagglutination assay against rabbit IgG-coated cells. SDS-PAGE of this goat IgG under non-reducing conditions showed a single protein band of apparent molecular weight 145,000 (see Figure 2.19). This agrees with the findings of Givol and Hurwitz (1969), and it was concluded that a pure IgG preparation had been obtained. This preparation was used for the production of enzyme conjugates for ELISA (see section 2.2.6), of fluorescein conjugates for immunofluorescence microscopy (see Chapter 3) and in immunoprecipitation experiments (see Chapter Four).

b. Rabbit anti-MAg IgG. SDS-PAGE of samples of rabbit anti-MAg IgG (prepared by the two methods detailed in section 2.2.8) showed a single species of apparent molecular weight 160,000 in each case (see Figure 2.19). No other polypeptides were demonstrated. Therefore both DEAE-cellulose chromatography and affinity chromatography on Protein A-Sepharose produced IgG preparations of equal purity. Both preparations gave very similar titres against MAg on ELISA (allowing for IgG concentrations). Protein A-Sepharose chromatography was preferred as it is technically simpler.

Figure 2.19. SDS-Polyacrylamide gel electrophoresis of purified IgG preparations
Purified IgG fractions from goat and rabbit antisera (prepared by the methods described in sections 2.2.7 and 2.2.8 respectively) were analyzed on a 6.5% slab gel run under non-reducing conditions. Samples (25μg in 25μl SPB) were applied to the gel as follows:

Lane 1: Bovine γ-globulin standard (Sigma: Cohn fraction II).
Lane 2: Rabbit IgG prepared by DEAE-cellulose chromatography.
Lane 3: Goat IgG prepared by DEAE-cellulose chromatography.
Lane 4: Rabbit IgG prepared by Protein A-Sepharose affinity chromatography.
Gels were stained with Coomassie Blue.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2.4 DISCUSSION

In comparing the findings of this study with those reported by other workers who have followed the Ross and Bornstein (1969; 1970) extraction procedure, cognizance must be taken of several important modifications that have been introduced into the methodology.

In the experiments reported here the tissues were very carefully homogenized and a preliminary saline extraction performed to remove salt-soluble and serum proteins. As elastin-associated microfibrils have been shown to be susceptible to proteolytic digestion (Ross and Bornstein, 1970), a cocktail of potent enzyme inhibitors was added to all buffers for extraction and dialysis, and the collagenase used in digestion steps was purified to eliminate non-specific protease activity.

Electron microscopic examination of extraction residues at each stage of the procedure has demonstrated conclusively that significant extraction of microfibrils by 6M GuHCl buffers occurs without the use of reducing agents. Similar findings have been noted by Sear et al. (1981b) in foetal bovine ligamentum nuchae, and by Gibson et al. (1982) in chick wing tendon. Experiments detailed in this chapter have shown that the preliminary GuHCl extracts, SG and SGCG, contained antigenically active material which cross-reacted with highly specific anti-MAg (ADS) IgG at low titre. This finding is consistent with the observed solubilization of microfibrils during these extraction steps. These observations are contrary to those reported by Ross and Bornstein (1969), who found that microfibrils were solubilized only in the presence of a reducing agent. Less rigorous homogenization of the ligament by them is likely to have contributed to this apparently contradictory finding by limiting the accessibility of microfibrils to extractants prior to the reductive step in their experiment.

Reductive GuHCl extraction, however, was shown to cause major disruption of elastic fibres, with the disappearance of recognizable microfibrils and alteration of the staining properties of residual amorphous elastin. Comparison with the published micrographs of Ross and Bornstein (1970) reveals similar, but less marked, changes in their residual material (their Figures 6 and 7). These appearances, together with the

relatively large amount of protein obtained in the MAg extract when compared to the previous (SGCGCG) GuHCl extract, implies that DTT is important in extracting a surface-associated component from elastic fibres. However, the identity of this component is not clear from the micrographs, and confident assertion that only elastin-associated microfibrils are being extracted is not possible. The observed disruption of residual elastic fibres may also indicate the presence of reducible cross-links remaining within immature amorphous elastin.

MAg extract proved to be highly insoluble in conventional biochemical buffers, a factor which greatly inhibited adequate analysis by the usual techniques. This has been a major problem with many of the 'microfibrillar protein' extracts reported in the literature (recently reviewed by Cleary and Gibson, 1983).

SDS-PAGE analysis of MAg extract was not satisfactory, as much of the applied sample failed to enter the analyzing gel, despite repeated attempts as described in section 2.3.3. Only two polypeptide bands could be discerned, with apparent molecular weights of 160,000 and 210,000. As low titre anti-fibronectin activity was detected in the antiserum raised to MAg, it is likely that the 210,000 dalton polypeptide band represents extracted matrix fibronectin. The 160,000 dalton molecular weight band may be related to CL glycoprotein (Gibson and Cleary, 1982), which has been shown to be the major glycoprotein component of 6M GuHCl extracts of foetal calf ligamentum nuchae. Significant activity directed toward purified preparations of CL glycoprotein was demonstrated to be present in anti-MAg antiserum (see section 2.3.4).

However, because of the difficulties outlined above, no further biochemical analysis of MAg extract was possible and it was concluded that the extract obtained was heterogeneous, despite the rigorous extraction procedure utilized. Subsequent work from this laboratory has been able to demonstrate convincingly the heterogeneity of similar extracts (Prosser et al., 1984).

It was considered that amino acid analysis of the MAg mixture was unlikely to provide useful information. Since the experiments of Ross and Bornstein (1969; 1970), it has been widely accepted that 'microfibrillar protein' preparations have a typical amino

acid analysis, characterized by large amounts of aspartic and glutamic acids as well as a significant cysteine content. A critical analysis of their data does not, however, support such a view. Ross and Bornstein, in fact, published two distinctly different amino acid analyses of 'microfibrillar protein' preparations of differing solubility. In addition, a high content of aspartic and glutamic acids has been found in many proteins (Reeck and Fisher, 1973), and the Ross and Bornstein amino acid pattern is that which may be expected of a mixture of proteins. Therefore, in the absence of a unique amino acid marker or other specific identifying property, amino acid analysis cannot by itself be regarded as evidence of the microfibrillar origin of protein extracts.

Kewley et al. (1977a,b) have performed similar experiments to those described in this chapter, using a less rigorous extraction schedule, to obtain a reductive GuHCl extract which they called 'microfibrillar protein' (see section 1.3.3). Although no SDS-PAGE pattern of this extract was published in the original work, the authors have acknowledged in another paper (Sear et al., 1977) that the Kewley 'microfibrillar protein' preparation was found to be quite heterogeneous when subjected to such analysis. It should also be noted that no proteinase inhibitors were incorporated into the buffers used during extraction of the original Kewley 'microfibrillar protein' preparation, a factor which may have led to considerable degradation of extracted proteins.

Antiserum developed to the original extract in Kewley's work was shown to produce at least three separate precipitin bands on immunodiffusion. The major precipitin band was excised, and used as an antigen in a secondary immunization experiment. The antibody thus obtained formed only a single precipitin band on immunodiffusion against the lyophilized original 'microfibrillar protein' extract, resuspended in saline. As no reaction was seen on immunodiffusion against serum proteins, α-elastin or proteoglycans, the authors concluded that a 'mono-specific' antibody had been produced. However (as noted in section 2.1) immunochemical assessment of the antibody preparation was undertaken only by immunodiffusion, which is a solubility-limited technique unlikely to be reliable for the assay of physiologically insoluble antigens such as those obtained in 'microfibrillar protein' extracts. It follows that the second 'mono-

specific' antibody produced by Kewley et al. (1977b) was directed only against those components of the original extract which were sufficiently soluble to diffuse into an agar gel. Furthermore, non-precipitating antibodies, or activity against insoluble components of the original 'microfibrillar protein' extract would not be detected by immunodiffusion. Therefore the claim for monospecificity of the Kewley anti-'microfibrillar protein' antiserum has not been firmly established.

In the study described in this chapter, antibodies to MAg extracts were raised in rabbits, and the resultant antiserum was shown to form a single precipitin band on immunodiffusion against the original antigen. This is in contrast to the findings of Kewley et al. (1977a), where a secondary immunization procedure was used to eliminate unwanted precipitin antibodies. MAg extract was found to be highly insoluble (see above and section 2.3.3.) in solutions of low ionic strength, and it was noted that substantial amounts of insoluble material remained within the antigen well during immunodiffusion experiments. In order to overcome this problem, other methods of immunochemical analysis were investigated. Haemagglutination assay, whilst sensitive and specific, proved extremely wasteful of antigen, and was therefore abandoned. ELISA, however, proved to be more suited to the study of MAg extract and its antiserum. ELISA techniques have a number of special advantages for such a study. Antigen insolubility is not a problem, and a wide range of different antigens can be directly compared on the same plate. Results can be standardized, and absolute end-points determined by measurement of the absorbance of the enzyme product. The technique itself is quick and easy, requires minimal manipulation, and is highly sensitive and specific. The characteristics of enzyme-immunoassays have been reviewed by Schurrs and Van Weemen (1977).

Use of the ELISA technique allowed more precise delineation of the specificities of anti-MAg antiserum. It was found that anti-MAg antiserum (despite forming only a single precipitin band on immunodiffusion) had significant reactivity towards components of foetal calf serum, fibronectin and CL glycoprotein. It was possible to remove contaminating activity against foetal calf serum and fibronectin by affinity chromatography, whilst retaining high titres against MAg extract. It was clearly demonstrated

that no cross-reactivity existed between this antibody preparation and types I or III collagen, porcine tropoelastin or bovine solubilized α-elastin. More recent work performed in this laboratory by Gibson and Cleary (1983) has also shown that anti-MAg antiserum does not possess activity toward collagen types I and III which have been denatured in GuHCl.

Some residual activity against CL glycoprotein (titre 1/160) remained in the final anti-MAg (ADS) IgG preparation, but immunoadsorption of this activity was not possible due to the very limited availability of this protein. This activity was, however, at quite a low level when compared to the overall activity directed against MAg (titre 1/3200). The presence of anti-(CL glycoprotein) activity in anti-MAg antiserum is of particular interest in considering the findings of Sear et al. (1981a,b) using the anti-'microfibrillar protein' antibody preparations of Kewley et al. (1977a,b). These authors showed that two distinct glycoproteins could be immunoprecipitated from the radioactively-labelled medium of cultured foetal calf ligamentum nuchae fibroblasts, which they called MFP I and MFP II (see section 1.3.4). The smaller of these glycoproteins, MFP I, had an apparent molecular weight of 150,000 and was found to be collagenous in nature. There is sufficient similarity between MFP I and CL glycoprotein to suggest that MFP I could be a soluble form of CL glycoprotein. Thus immunoprecipitation of MFP I with the Kewley antiserum probably indicates the presence within it of anti-(CL glycoprotein) activity, similar to that observed in anti-MAg antiserum.

Because of the different preparation method of anti-MAg antiserum, it was not possible to relate directly the specificities of this antibody to the anti-'microfibrillar protein' antiserum of Kewley et al. (1977a,b). Immunohistochemical studies were therefore undertaken to determine the tissue localization of anti-MAg antibodies, both to determine the origin of MAg, and to allow comparison with the work of Kewley and co-workers.

2.4.1 Conclusions

In conclusion, it has been shown that, despite rigorous extraction procedures, 'microfibrillar antigen' preparations are heterogeneous. The use of a more sensitive immunological assay (ELISA) has demonstrated that an antiserum produced to such an extract is polyspecific. It was found that the anti-MAg antiserum was not cross-reactive with interstitial collagens (both native and denatured), tropoelastin or solubilized amorphous elastin, and that contaminating antibody activity against components of foetal calf serum and fibronectin could be successfully removed by affinity chromatography. Although low-titre activity against CL glycoprotein remained, the resulting anti-MAg (ADS) IgG preparation was highly specific in its activity toward the original antigenic mixture. Unfortunately the difficulties involved in characterization of MAg extract, due to its physical properties, have not yet allowed adequate description of this heterogeneous antigen.

CHAPTER THREE

IMMUNOHISTOCHEMICAL LOCALIZATION OF ANTIBODIES TO MAg

3.1 INTRODUCTION

In Chapter 2, the preparation and immunochemical characterization of an antiserum raised against an extract of elastic fibres was described. The final antibody fraction obtained was shown to be highly specific in its activity against the original MAg extract and to lack cross-reactivity with serum proteins or other matrix proteins. Although ultrastructural evidence was adduced which implied that MAg was derived from the surface of elastic fibres, immunohistochemical localization in elastic tissues was considered necessary in order adequately to describe the origin of MAg extract. This was approached in two ways:

i) The histological organization of MAg was investigated using an indirect immunofluorescence technique. A number of tissues were investigated in order to gain an impression of the overall distribution, localization and tissue specificity of MAg. MAg was found to be closely related to the surface of elastic fibres in foetal calf ligamentum nuchae and aorta, as well as to the renal mesangium and interstitium and to 'pre-elastic' (Cotta-Pereira et al., 1977) fibres in the skin.

ii) Because immunofluorescence is a relatively low-resolution technique, localization to microfibrils necessitated the use of a direct ferritin-labelling technique to study the ultrastructural disposition of anti-MAg antibodies. Ferritin was chosen as an electron microscopic marker for a number of reasons: a) It gives discrete labelling and antibody localization can be directly studied without the need for detection by enzymatic techniques; b) Ferritin labelling does not obscure the underlying ultrastructure as does peroxidase labelling, and also obviates the problem of diffusion of reaction product (Novikoff et al., 1972); c) The use of a direct technique allows the closest approach possible of the marker label to the actual antigenic site. Results using this technique confirmed that MAg was of microfibrillar origin, and that no activity against either elastin or collagen was present.

A secondary objective of the study detailed in this chapter was to ascertain the distribution of a collagen-like (CL) glycoprotein antigen (isolated in work undertaken by Dr M A Gibson in this laboratory) which was shown in section 2.3.4 to cross-react at a

low level with anti-MAg antibodies. CL glycoprotein has a molecular weight on SDS-PAGE of about 140,000 daltons, and is the principal PAS- positive species found in GuHCl extracts of foetal calf aorta. The isolation and properties of this protein have been reported by Gibson and Cleary (1982). Gibson has succeeded in raising antibodies to CL glycoprotein in rabbits, and we have carried out a preliminary study of the localization of these antibodies, using immunofluorescence and immunoferritin techniques. These investigations have shown no relationship of anti-(CL glycoprotein) antibodies to elastic fibre microfibrils; however some common features with the localization of anti-MAg antibody, were apparent. This is an important finding, due to the biochemical similarities between this glycoprotein and the claimed microfibrillar protein, MFP I, described by Sear et al. (1981a,b) (see sections 1.3.3 and 1.3.4).

3.2 MATERIALS AND METHODS

3.2.1 Materials

Fluorescein isothiocyanate (isomer I) was obtained from Baltimore Biological Laboratories, Cockeysville, Maryland, USA. Biogel P-6 was supplied by Bio-Rad Laboratories, Richmond, California, USA. Sephadex G-75 and DEAE - Sephadex A-50 were from Pharmacia (South Seas) Pty Ltd, North Ryde, NSW. Millipore filtration membranes and cellulose acetate membrane electrophoresis requirements were supplied by Millipore Biomedica, Acton, Massachusetts, USA. Tissue-Tek OCT compound embedding medium was purchased from the Ames Co, a division of Miles Laboratories Inc, Elkhart, Indiana, USA. Ferritin, purified from horse-spleen (2x crystallized, cadmium-free) was obtained from Calbiochem-Behring Corp, La Jolla, California, USA. All other materials used were as specified in section 2.2.1.

3.2.2 Immunofluorescent Localization of Anti-MAg Antibodies in Tissue Sections

Anti-MAg antibodies were localized in a variety of tissues using an indirect immunofluorescent labelling technique. Only highly specific anti-MAg (ADS) IgG was employed in these studies. The characteristics of this antibody preparation are detailed in section 2.3.5.

a. Preparation of goat anti-(rabbit IgG) / fluorescein conjugate. Goat anti-(rabbit IgG) immunoglobulin was prepared as described in section 2.2.7. Conjugation to fluorescein isothiocyanate (FITC) was accomplished by the method of Goding (1976). FITC was dissolved in dimethyl sulphoxide (DMSO) to give a 1mg/ml solution, and 1.2ml of this solution was added dropwise to 60mg of goat IgG contained in 6ml of 0.1M sodium carbonate/bicarbonate buffer, pH 9.3. This, and all subsequent steps, were carried out in the dark at room temperature. Coupling was allowed to proceed for 2 hours, then unbound fluorochrome was removed (and the buffer changed) by gel filtration on a column (3cm x 30cm) of Biogel P-6 which was equilibrated with 0.01M potassium phosphate buffer, pH 8.0.

The first coloured band to emerge was collected, and loaded directly on to a column (1.6cm x 30cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The conjugated proteins were eluted with a linear 0.01M-1.0M salt gradient. The outflow reservoir contained 140ml of 0.01M phosphate buffer, pH 8.0, while the limit reservoir contained 140ml of the same buffer containing 1.0M NaCl. Fractions of approximately 5ml were collected, and optical densities of all samples were read at wavelengths of 280nm and 495nm.

The molecular fluorescein/protein (F/P) ratio was calculated as described by Thé and Feltkamp (1970a,b) and fractions having an F/P ratio of 1.7-3.4 were pooled, dialyzed extensively against PBS, pH 7.4, and concentrated by ultrafiltration. This conjugate preparation had a mean F/P ratio of 2.61, and was used in all subsequent labelling experiments.

Cellulose acetate membrane (CAM) electrophoresis (performed with a Millipore Zone Electrophoresis system using Phoroslide electrophoresis strips according to the manufacturer's instructions), showed that the conjugate contained no unbound fluorochrome or other serum proteins. Conjugate was sterilized by filtration through a 0.22µm Millipore membrane, and stored at 4°C. Stock conjugate contained 2.71mg/ml of protein (determined as described by Thé and Feltkamp, 1970a), and was diluted 1:5 with PBS, pH 7.4, before use, giving a working strength of approximately 0.5mg/ml of protein.

b. Preparation of tissues for sectioning. Tissues were dissected from foetal calves of 210-220 days gestation, within 30 minutes of the death of the mother. Blocks of ligamentum nuchae, thoracic aorta, kidney and skin were obtained. These were placed in Tissue-Tek OCT Compound embedding medium on slices of cork, and snap-frozen by immersion in an isopentane bath which had been cooled in liquid nitrogen. Specimens so obtained were transported to the laboratory on dry ice, and were stored at -20°C before use. The experiments reported in this study were performed on material collected no longer than one week before use.

Frozen sections, approximately 4μm in thickness, were cut on a cryostat and mounted on clean glass slides. Sections were allowed to dry, then washed for 10 minutes in PBS, pH 7.4, before use.

c. Staining procedures. Sections were incubated for 40 minutes with 10% non-immune goat serum in PBS and washed by immersion in a bath of PBS, with gentle stirring, for two 15-minute periods. The sections were then covered with 50μl of 1mg/ml solution of anti-MAg (ADS) IgG, prepared as described in section 2.2.9, and reaction was allowed to proceed overnight at 4°C. Controls were provided by treatment of some sections with normal (pre-immune) rabbit IgG or by omitting treatment entirely. In some experiments, the uptake of goat anti-(rabbit IgG)/fluorescein conjugate was blocked by incubating sections with unlabelled goat anti-(rabbit IgG) after treatment with anti-MAg (ADS) IgG to ensure that no non-specific uptake of conjugate occurred.

After washing for four 20-minute periods with PBS, sections were treated with fluorescein conjugate (which had been freshly diluted to working strength) for one hour, protected from light. Sections were washed as before, and mounted in 90% (v/v) glycerol buffered to pH 8.0 with carbonate-bicarbonate buffer. In later experiments, p-phenylenediamine (1mg/ml) was added to the buffered glycerol in order to prevent fading of fluorescence during microscopy (Johnson and Nogueira Araujo, 1981).

Specimens were examined with a Leitz Orthoplan microscope equipped with a Ploemopak 2 epi-illumination unit and Leitz filter block H. Results were photographed on Kodak Tri-X film rated at 800ASA, which was developed in Kodak HC-110 developer. Micrographs of control sections were taken from areas corresponding to those photographed on positive sections, and were exposed under identical conditions.

3.2.3 Ultrastructural Localization of Anti-MAg Antibodies by Immunoferritin Labelling

Ultrastructural studies were carried out using ferritin conjugated to anti-MAg antibodies as an electron-opaque marker. In order to gain maximum resolution, a direct technique was employed.

a. Preparation of ferritin-antibody conjugate. Ferritin-antibody conjugate was prepared by a modification of the method of Kirby and Parr (1979). Equine ferritin (Calbiochem) was further purified by centrifuging at 45,000g for 4 hours at a temperature of 4°C. The supernatant was discarded, and the procedure was repeated twice in order to collect very dense iron-containing ferritin, and remove apoferritin. The concentration of ferritin was determined by measuring its optical density at a wavelength of 440nm, and assuming $A_{1cm}^{1\%}$ to be 15.0 (Singer and Schick, 1961).

Ferritin (100mg in 20ml of 0.02M sodium phosphate buffer pH 7.0) was activated with 6mg of glutaraldehyde (Taab, EM Grade) for one hour at room temperature. Unreacted glutaraldehyde was then removed by gel filtration on a column (3cm x 3cm) of Sephadex G-25 equilibrated with the same buffer. The activated-ferritin eluate was collected, combined with 50mg of rabbit anti-MAg IgG (prepared as described in section 2.2.8) and allowed to conjugate at 4°C for 24 hours. Glycine was then added to a final concentration of 0.5M in order to block any unreacted aldehyde groups, and the mixture was again stood at 4°C for 24 hours. The conjugate was then centrifuged three times at 45,000g for 4 hours to remove unbound antibody, and redissolved in PBS containing 0.1% sodium azide. A small amount of insoluble ferritin aggregate was removed by centrifugation at 10,000g for 20 minutes at 4°C. Conjugates were stored at 4°C, and were used diluted to 25mg ferritin/ml. It should be noted that this method of ferritin conjugation produces a heterogeneous conjugate which contains a variable amount of polymerized ferritin.

The guidance and assistance of Dr Earl Parr (formerly of the Department of Anatomy in this University) during the preparation of these conjugates is gratefully acknowledged.

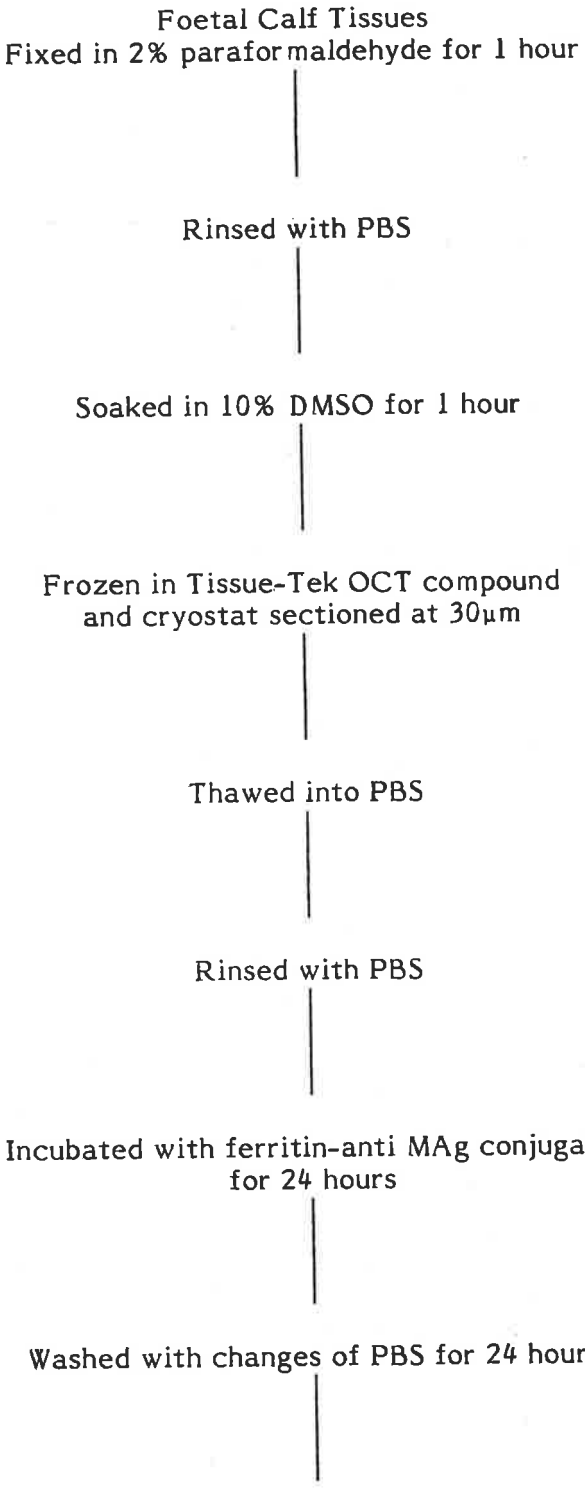
b. Immunoferritin labelling of tissue sections. Foetal calf ligamentum nuchae and thoracic aorta specimens were collected as described in section 3.2.2. The labelling procedure is summarized in Figure 3.1. Blocks of tissue approximately 5mm square were fixed in 2% paraformaldehyde (w/v) in PBS, pH 7.4, for $1\frac{1}{2}$ hours in order to immobilize tissue antigens in their native positions. The blocks were then soaked in 10% (v/v)

DMSO in the same buffer for one hour, before being frozen in Tissue-Tek OCT compound and sectioned on a cryostat at -20°C. Sections approximately 30µm thick were thawed into PBS, rinsed thoroughly, and then incubated with 10% (v/v) heat-inactivated NRS in PBS for one hour at 4°C. After rinsing with PBS, sections were incubated with ferritin-antibody conjugate (diluted to working strength) for 24 hours at 4°C, then washed with several changes of PBS over a further 24 hour period. Controls were prepared by incubating with ferritin-NRS IgG conjugate or free ferritin. Some sections were also preincubated with unlabelled anti-MAg IgG, in order to block the uptake of specific ferritin-anti MAg conjugate. Labelled sections were fixed in 2.5% glutaraldehyde (Taab) in 0.1M sodium cacodylate buffer, pH 7.4, for 2 hours at 4°C, then washed overnight in the same cacodylate buffer, before being post-fixed with osmium tetroxide, dehydrated and embedded in Spurr's epoxy resin, as described in section 2.2.3. Sections were cut and stained as before, and examined in a Philips EM 300 electron microscope operated at an accelerating voltage of 80kV.

The effect of fixation on antigenic distribution was studied by comparing the relative amount of labelling of tissues fixed with 1% or 2% paraformaldehyde, periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) containing 2% paraformaldehyde, or with 2.5% glutaraldehyde. Unfixed tissues were included for comparative purposes. Sections from these blocks were subjected to experimental procedures and conditions identical to those as described above.

3.2.4 Immunohistochemical Localization of Antibodies to a Collagen-like Glycoprotein Antigen from Aorta

As outlined in section 3.1, the tissue localization of antibodies directed against CL glycoprotein antigen, isolated from foetal calf aorta, was investigated using immunohistochemical techniques. Both the CL glycoprotein and antiserum to it [anti-(CL glycoprotein) antiserum] were prepared by Dr M A Gibson (Gibson and Cleary, 1982). ELISA of this anti-(CL glycoprotein) antiserum showed a titre of approximately 1/6,400. It did not cross-react with serum proteins or other matrix proteins. IgG was prepared



All steps apart from the last were carried out at $4^{o}C$.

Figure 3.1

Flow Diagram of Immunoferritin Labelling Procedure

from this antiserum by Protein A-Sepharose chromatography (see section 2.2.8 for details of methodology), and its activity assessed by ELISA as before (Gibson and Cleary, 1983). Its titre was found to be 1/6400 at a concentration of 10mg/ml.

Immunofluorescence microscopy. The histological localization of anti-(CL glycoprotein) antibodies in frozen sections of foetal calf aorta, was studied using an indirect immunofluorescence technique identical to that described in section 3.2.2. Anti-(CL glycoprotein) IgG was used at a working concentration of 3mg/ml in staining procedures. Control experiments were provided as before, and results were recorded photographically.

Immunoferritin labelling. Anti-(CL glycoprotein) IgG was coupled to ferritin in the manner outlined in section 3.2.3. Foetal calf aorta was lightly fixed, and the ultrastructural localization of ferritin-conjugated anti-(CL glycoprotein) antibodies determined as before. Controls were prepared by pre-incubating some sections with unlabelled anti-(CL glycoprotein) IgG in order to block uptake of specific conjugate, or by incubating sections with a ferritin-NRS IgG conjugate. All other procedures were the same as previously described.

3.3 RESULTS

3.3.1 Immunofluorescent Localization of Anti-MAg Antibodies

Ligamentum nuchae. Sections of ligamentum nuchae, treated with anti-MAg antibodies, showed strong positive fluorescence (see Figure 3.2) confined to the surface of the longitudinally aligned elastic fibres, which at this age are the dominant extracellular component of the ligament. No cellular fluorescence, or localization to collagen or other matrix components were observed. Controls prepared as described in section 3.2.2 showed barely perceptible non-specific fluorescence of the fibroblasts located between bundles of connective tissue. Due to the high specific fluorescence obtained with this technique, background autofluorescence of elastin was not a problem. No non-specific binding of fluorescein conjugate was noted in this tissue, or in any of those studied in the following experiments. Autofluorescence is a problem when adult ligament is studied.

Thoracic aorta. Fluorescence microscopy of control sections of aorta showed faint autofluorescence of aortic elastin, which appeared as rather indistinct fibres. Untreated sections of aorta showed a similar degree of autofluorescence. In contrast, sections treated with anti-MAg IgG appeared brightly fluorescent, with intense localization to the surface of the elastic fibres (positive control sections are shown in Figure 3.3). Both the circumferentially-oriented elastic lamellae of the media, and the longitudinally-oriented fibres of the intima showed a similar pattern, the fluorescence being apparently brighter at the edges of the elastic fibres, whilst the central portion of the fibre stained more uniformly. At high power, no cellular fluorescence could be detected, and no apparent localization of antibody to other components of the extracellular matrix was seen. The endothelial surface of some sections was noted to fluoresce brightly; however, a similar appearance was also occasionally noted in control sections, suggesting that this may be due to an 'edge' effect rather than specific antibody uptake.

On the basis of this highly specific localization in both aorta and ligamentum nuchae, it was concluded that anti-MAg IgG localized very precisely to a surface

constituent of elastic fibres, and had no affinity for fibroblasts, smooth muscle cells or other connective tissue proteins found in these tissues.

Skin. Control sections of foetal calf skin showed quite distinct yellowish, autofluorescence of the epidermis, especially in the stratum basale and stratum corneum. Autofluorescence was also observed in hair follicles, and in the condensed connective tissue of the perifollicular region. In other areas of the dermis and subcutaneous tissue, there was either no fluorescence, or a very weak, diffuse green autofluorescence. Tissues examined after staining with specific anti-MAg IgG showed similar autofluorescence in the areas described above. However, quite intense green, specific fluorescence was seen arranged along an extensive network of small fibres which continually branched and anastomosed in the dermis, and appeared to become finer and more intensely ramified in the area immediately subjacent to the dermo-epidermal junction. This fibrous network extended into the sub-dermal adipose tissue, apparently forming septae between groups of fat cells. These appearances are shown in Figure 3.4. No fluorescence was seen to be associated with the cells of the dermis or subcutaneous tissue.

Kidney. Kidney tissue which had received control treatments showed no fluorescence whatsoever. Bright immunofluorescence was seen after treatment with anti-MAg antiserum. In the renal cortex, fluorescence was associated chiefly with the glomeruli and peritubular connective tissue (Figure 3.5). Glomeruli showed intense fluorescence of the mesangial area, but this did not extend into the basement membranes of the adjacent glomerular capillaries. Moderately intense fluorescence was also apparent in the interstitial connective tissue surrounding the tubules and glomeruli. Cells of the tubules showed only a very weak, greenish fluorescence, as did those of the mesangium and glomerular endothelium. These appearances indicate that antigenic material is principally located in the mesangium and in the interstitium, but is not related to renal parenchymal cells or to glomerular basement membrane. Examination of the outer medullary zone of the kidney showed that fluorescence is similarly associated with peritubular connective tissue, especially at the edges of medullary rays (Figure 3.6). Again, only slight cellular fluorescence was evident.

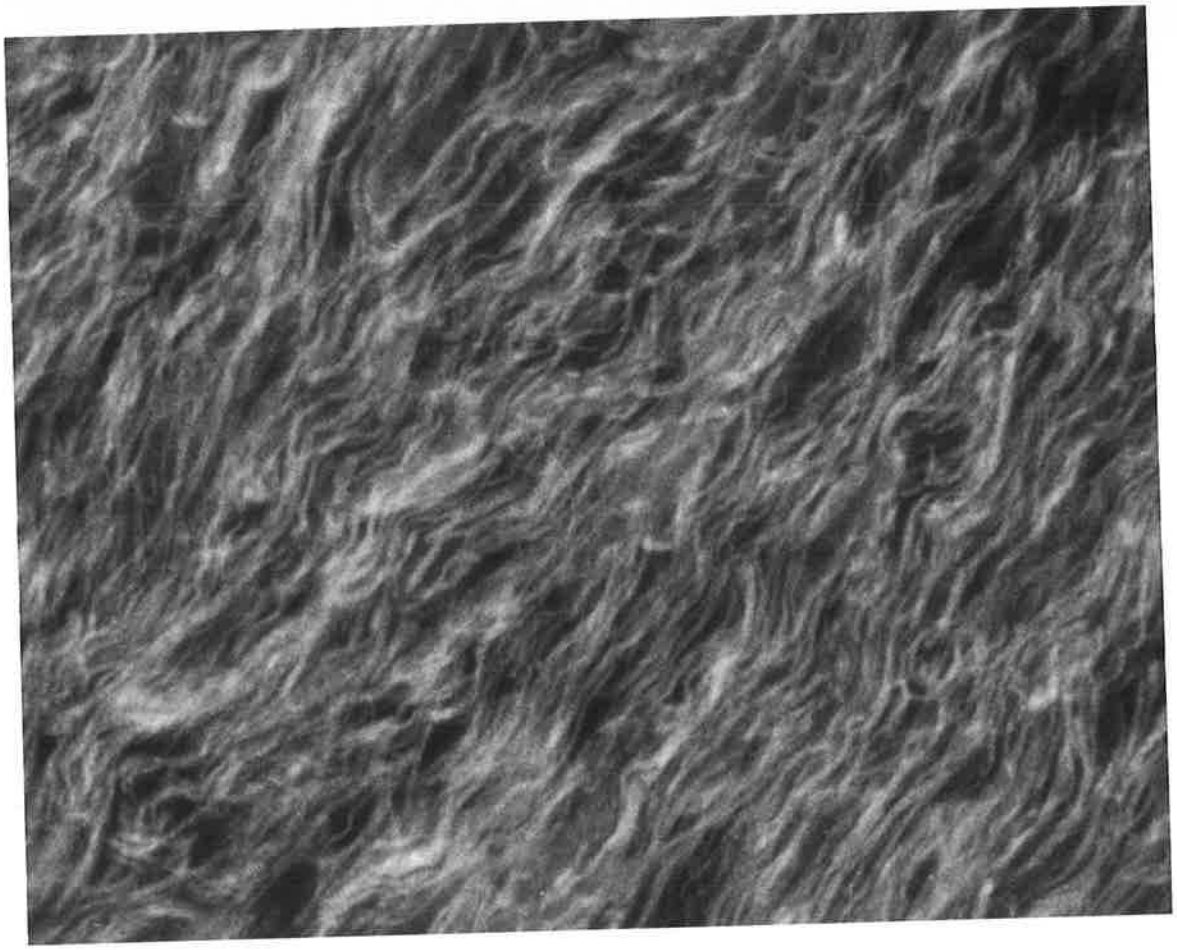
Figure 3.2. Immunofluorescent staining of foetal calf ligamentum nuchae with anti-MAg (ADS) IgG

Longitudinal sections of ligamentum nuchae.

Magnification X250.

a. Section treated with anti-MAg (ADS) IgG. Note the strongly positive fluorescence of longitudinally-oriented elastic fibres.

b. Control section treated with pre-immune rabbit IgG.



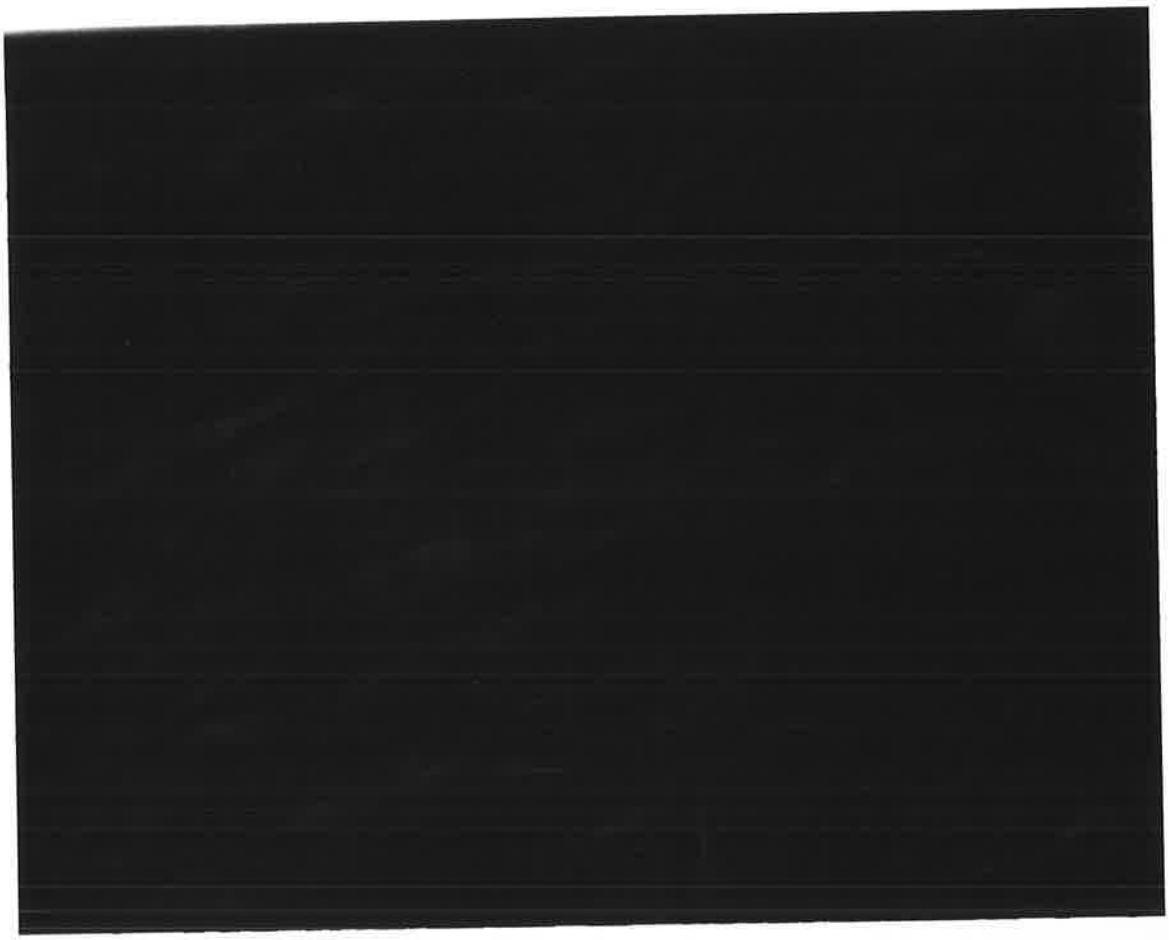


Figure 3.3. Immunofluorescent staining of foetal calf thoracic aorta with anti-MAg (ADS) IgG

Transverse section of the upper thoracic aorta showing intimal and medial regions. The lumen is toward the right of the micrograph.

Magnification X250.

a. Section treated with anti-MAg (ADS) IgG. Note the fluorescence of intimal and medial elastic fibres. Some fluorescence of the endothelial surface is apparent.

b. Control section treated with pre-immune rabbit IgG.

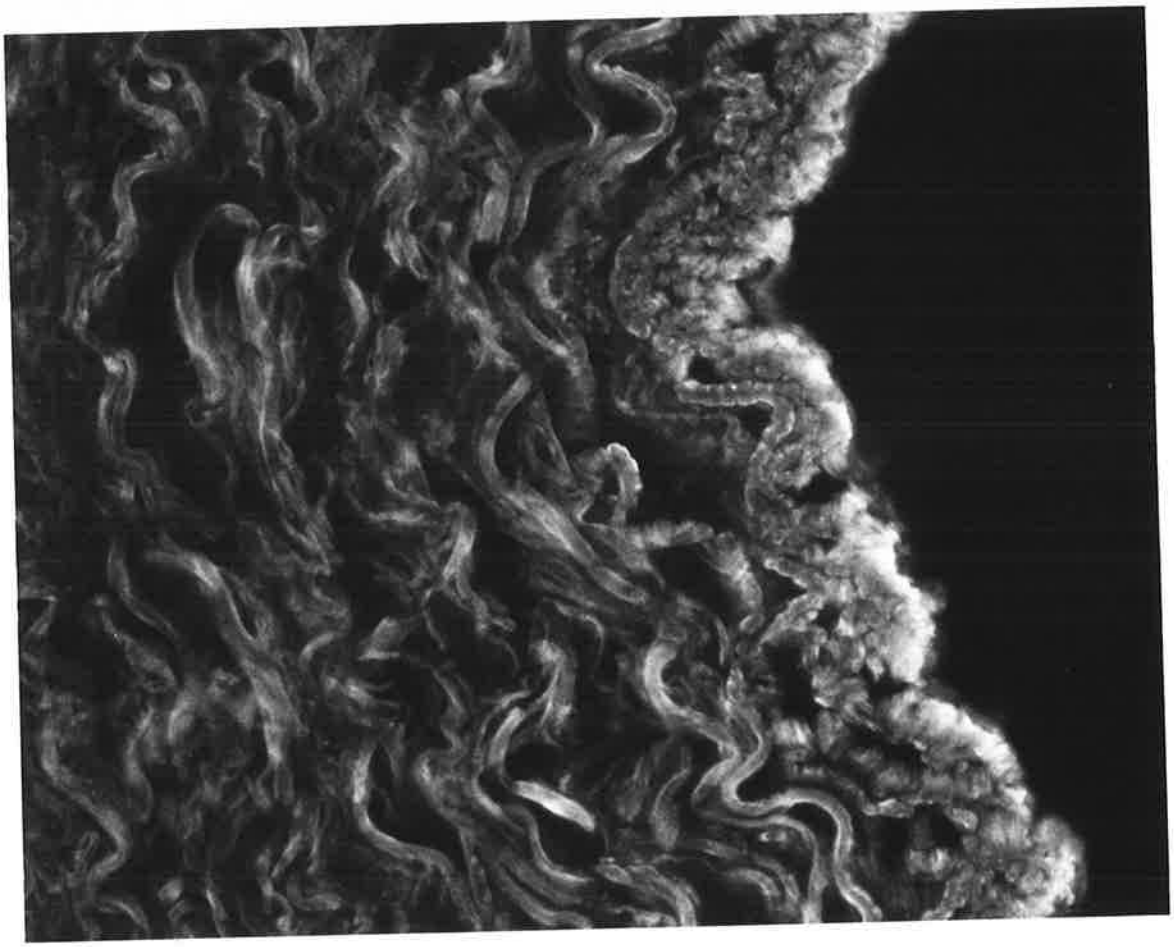
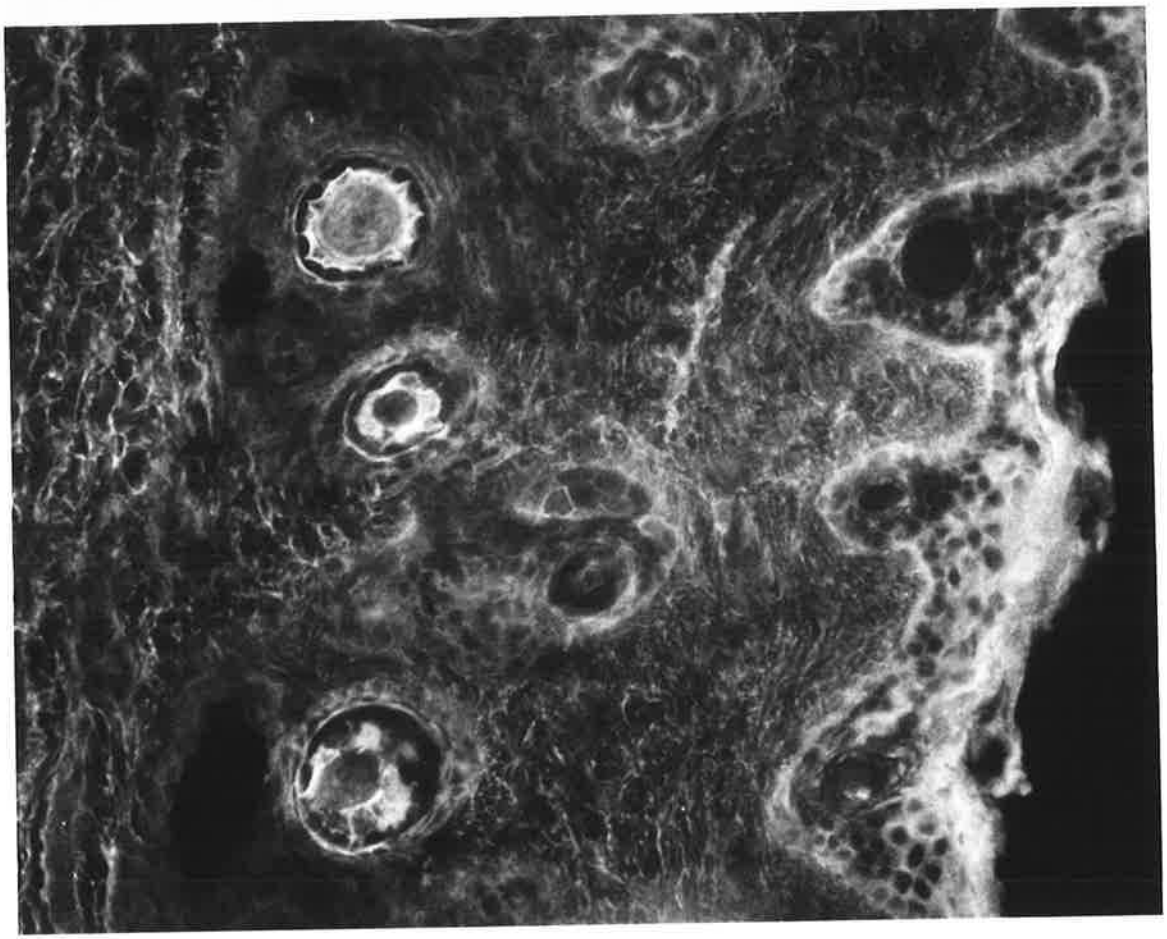


Figure 3.4. Immunofluorescent staining of foetal calf skin with anti-MAg (ADS) IgG
Sections of skin were taken from the scalp. The epidermal surface is toward the right of the micrograph.
Magnification X100.

a. Section treated with anti-MAg (ADS) IgG. Note the fluorescence of fine fibres in the papillary dermis which appear mostly to run parallel to the surface of the skin. These fibres appear to communicate via the reticular dermis with a plexus of larger fibres in the subcutaneous adipose tissue. Diffuse fluorescence of the epidermis and hair follicles is also seen. Similar staining was frequently observed in control sections and is probably non-specific.

b. Control section treated with pre-immune rabbit IgG.



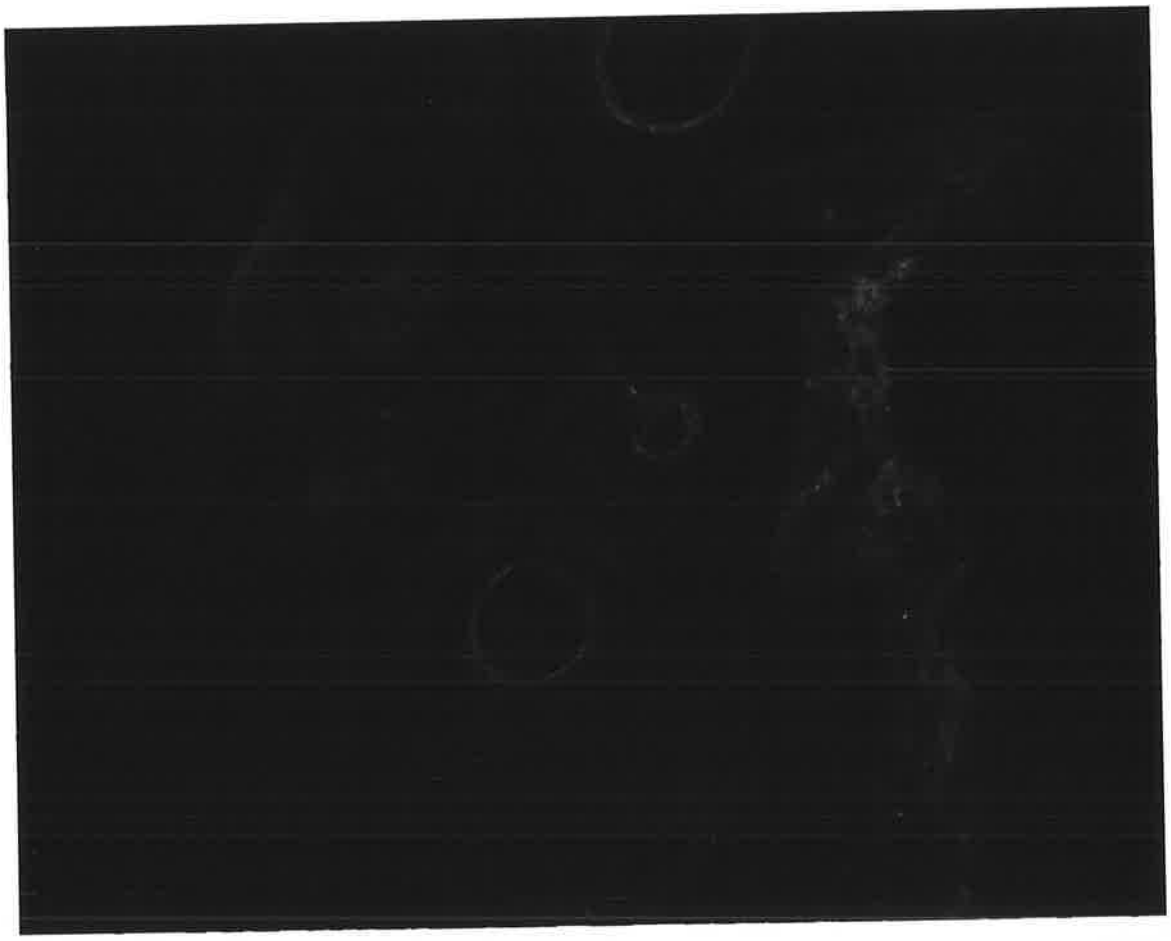


Figure 3.5. Immunofluorescent staining of foetal calf renal cortex with anti-MAg (ADS) IgG

Sections of renal cortex showing a glomerulus and adjacent convoluted tubules. Magnification X400.

a. Section treated with anti-MAg (ADS) IgG. Note the fluorescence of the mesangium, which does not extend to the peripheral capillary loops. Fluorescence of interstitial tissue related to Bowman's capsule and surrounding tubules can also be seen.

b. Control section treated with pre-immune rabbit IgG.

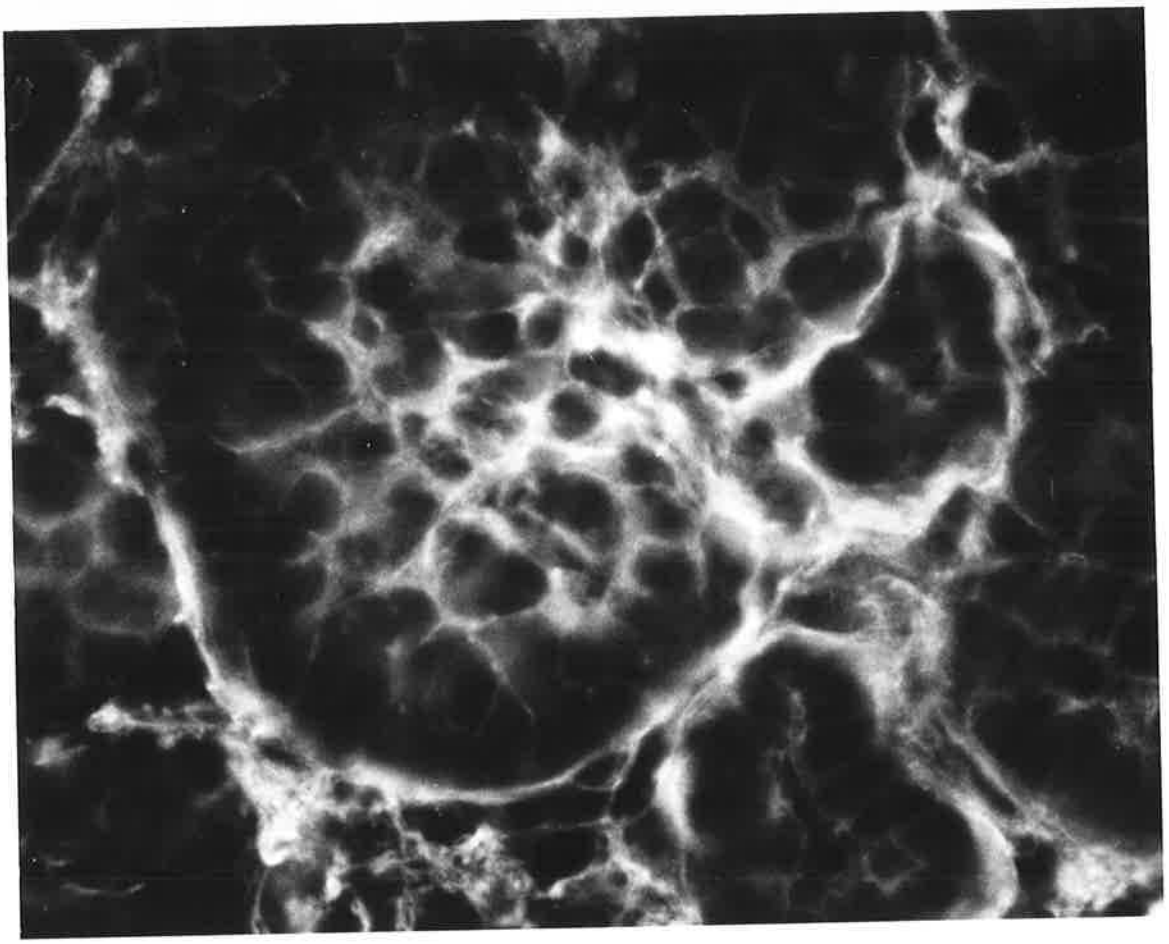


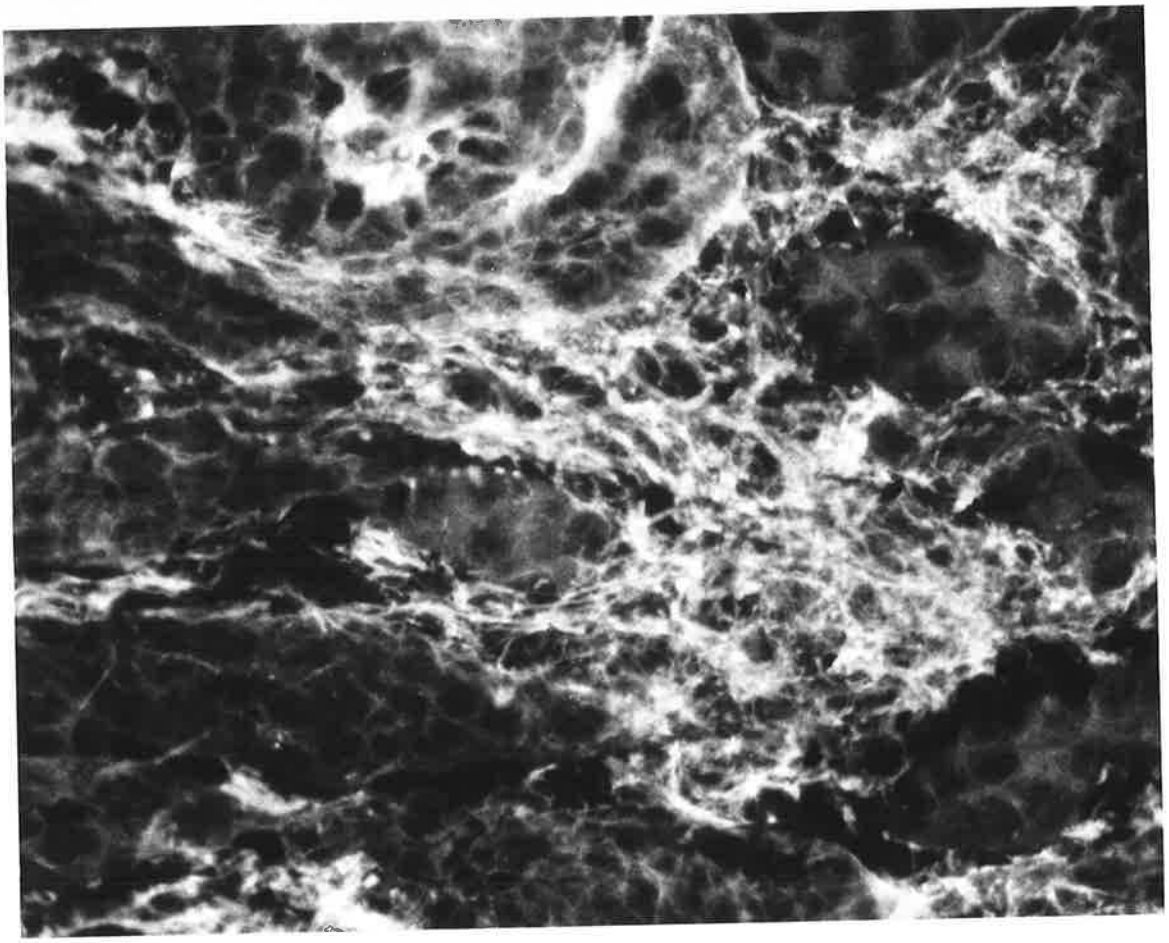
Figure 3.6. Immunofluorescent staining of foetal calf renal medulla with anti-MAg (ADS) IgG

Sections of the outer medullary zone showing part of a medullary ray and adjacent tubules.

Magnification X250.

a. Section treated with anti-MAg (ADS) IgG. Note the fluorescence of the more abundant interstitial connective tissue in this region.

b. Control section treated with pre-immune rabbit IgG.



Small elastic arteries in the medulla showed intense specific fluorescence, chiefly related to the intimal and medial regions of vessels. High power examination revealed that the fluorescence was chiefly associated with elastic fibres in the arterial walls. Less intense fluorescence was observed in the adventitial and perivascular connective tissues.

In conclusion, these studies suggest that material with similar antigenic properties to MAg can be found not only associated with the surface of elastic fibres, as seen in the aorta and ligament, but also in tissues not usually regarded as elastic.

No evidence was found in any tissue to suggest any relation to cellular components. On the basis of these studies, it appears that MAg (or an antigenically similar protein) is widely distributed in bovine tissues. High reactivity was obtained in all tissues studied, therefore anti-MAg antibodies are not tissue specific. Species specificity was not studied, but would be an important next step in the further investigation of this antigen.

3.3.2 Ultrastructural Localization of Anti-MAg Antibodies

Ligamentum nuchae. Most of the ferritin label observed in sections of ligamentum nuchae was found to be associated with elastic-fibre microfibrils. Ferritin was aligned precisely along the microfibrils, and at quite high density. In both longitudinal (Figures 3.7 and 3.8) and transverse (Figure 3.9) sections of elastin, in which areas of amorphous elastin could be seen to be devoid of microfibrils, no significant labelling of the exposed surface of the elastin was found.

Examination of the edges of the section revealed elastic fibres which had been cut during sectioning, exposing the amorphous elastin core. Again, no ferritin binding to polymeric elastin could be demonstrated at such sites. However, where linear densities were seen within the fibre, some ferritin binding did occur at the cut edge of the fibre corresponding to these areas of increased staining. In cross-section, it can be seen that elastic fibres in this tissue have quite extensive surface infoldings (see Figure 3.9), and the linear densities seen in longitudinally sectioned fibres probably represent microfibrils incorporated within such foldings; this would explain the observed reactivity with anti-MAg antibodies.

At high magnification labelled microfibrils did not reveal any ordered or periodic arrangements of ferritin-labelled antibodies corresponding to the regular organisation which has been inferred by Kewley et al. (1977a), using peroxidase-labelled anti-'microfibrillar protein' antibodies.

Ferritin-antibody conjugates showed no affinity for polymeric collagen fibrils, and no labelling of collagen bundles or individual fibres could be shown. However, significant binding did occur to thin filaments with a diameter of approximately 3-5nm which formed an extensive network between collagen fibres and around elastic fibres (Figures 3.7, 3.8 and 3.10). These filaments could be clearly distinguished from microfibrils, and ferritin appeared to associate specifically with them, rather than to collagen fibres. The amount of label was much less than that bound to adjacent microfibrils, and it would appear that reactivity with these filaments constituted only a minor portion of anti-MAg (ADS) activity.

Fixation with paraformaldehyde had no apparent effect on the distribution or relative intensity of antibody labelling observed. PLP fixation appeared to offer no significant advantage over fixation with paraformaldehyde alone, and was not used in later studies. Glutaraldehyde fixation was found to completely eliminate antigenic activity in fixed tissues, with no ferritin binding at all found after labelling with anti-MAg conjugate. The effect of glutaraldehyde in abolishing antigenicity has been reported in a number of systems (Williams, 1977); this action may be due to the very extensive cross-linking produced by this agent preventing access of antibody to binding sites.

Very poor preservation of cell ultrastructure was apparent in all experiments, despite many attempts to modify experimental conditions to prevent such damage (Figure 3.11). It is probable that use of the frozen section technique (see section 3.2.3) employed in this study was responsible for the cell damage observed. Unfortunately, intracellular contents bound ferritin non-specifically, and no observations could be made concerning the localization of anti-MAg antibodies to cell components. Large amounts of ferritin also bound to cellular debris spread throughout the matrix, as can be seen in Figure 3.12. This effect was seen in both positive and control experiments.

Control experiments using ferritin conjugated to pre-immune rabbit IgG (Figure 3.13) and unconjugated ferritin showed no non-specific binding to matrix components. Pre-incubation of sections with unlabelled anti-MAg (ADS) IgG almost completely prevented uptake of ferritin-labelled antibody (Figure 3.14), suggesting that the labelling patterns described above are produced specifically by antigen-antibody interaction.

Comparison of the labelling patterns produced by both the crude and highly specific antibody conjugates showed no qualitative or quantitative differences in ferritin distribution (Figures 3.7 and 3.8). It was therefore concluded that the non-specific activity detected in the studies described in Chapter 2 did not materially contribute to the observed antibody localization.

Thoracic aorta. anti-MAg (ADS) antibodies were found to localize in aortic sections in a similar way to that described above for ligamentum nuchae. Ferritin was observed to be principally associated with elastic fibre microfibrils, which in aorta at this age appear to be less abundant than in ligamentum nuchae. No binding to amorphous elastin occurred (Figures 3.15 and 3.16), labelling of elastic fibres being restricted to areas containing recognizable microfibrillar material. This observation was confirmed, as before, by examination of the cut edges of the elastic fibres.

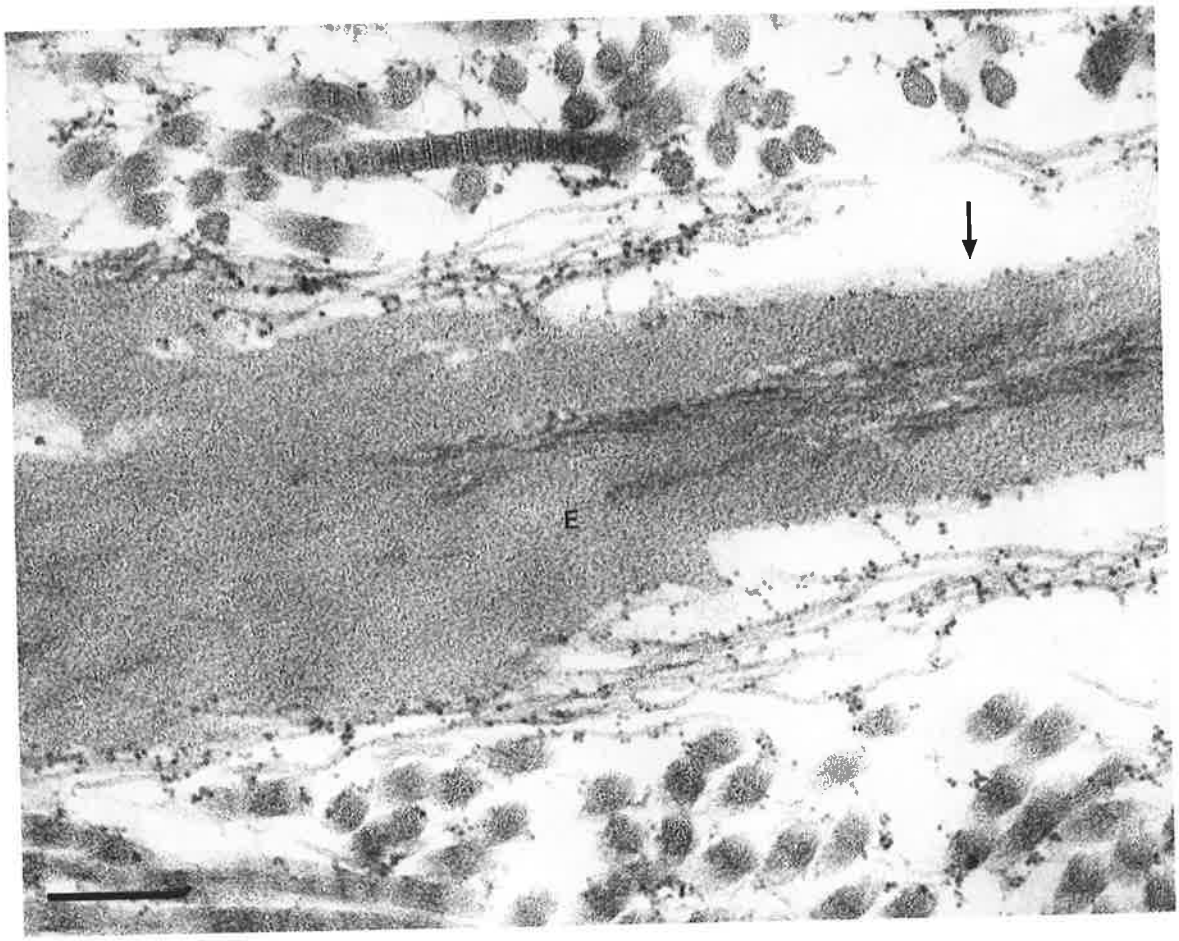
The small matrix filaments which have been described in ligamentum nuchae (see above) were apparently more abundant in aortic tissue, with a concomitant increase in the amount of ferritin-antibody conjugate which bound to them (Figure 3.15). This was a constant finding in a number of experiments. Collagen appeared not to take up labelled antibody, but ferritin was often observed between collagen fibrils and bundles, seemingly related to these small interconnecting filaments.

Cell preservation was also a problem in aortic tissue, probably due to frozen sectioning the tissue. Cellular debris non-specifically bound ferritin, again preventing any observations of localization to subcellular components (Figure 3.16).

Control experiments confirmed the specificity of the labelling obtained.

Figure 3.7. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin-labelled anti-MAg IgG (crude IgG fraction)
Longitudinal section of an elastic fibre (E) showing dense localization of ferritin label along microfibrils. Note the small amount of labelling of thin matrix filaments running between collagen fibrils, and the minimal labelling of exposed areas of elastin devoid of microfibrils (↑). Tissue fixed with 2% (w/v) paraformaldehyde. X85,000. Scale = 200nm.

Figure 3.8. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin-labelled anti-MAg (ADS) IgG (affinity-purified IgG fraction)
Longitudinal section of elastic fibre (E) and surrounding collagen fibrils. Note the association of ferritin label with microfibrils, which appears unchanged by elimination of contaminant antibody activity. Minor labelling of thin filaments between collagen fibrils persists. Tissue fixed with 2% (w/v) paraformaldehyde. X65,000. Scale = 200nm.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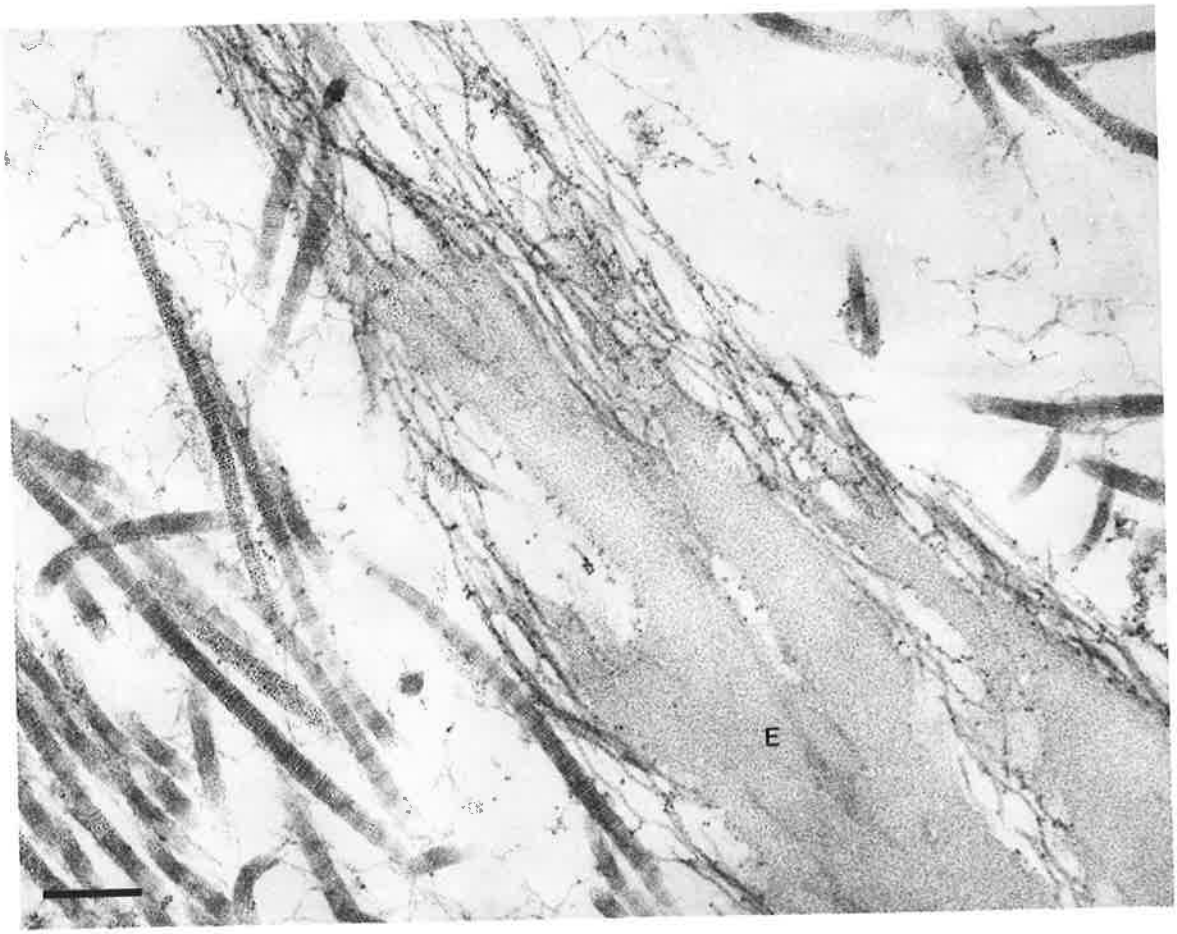
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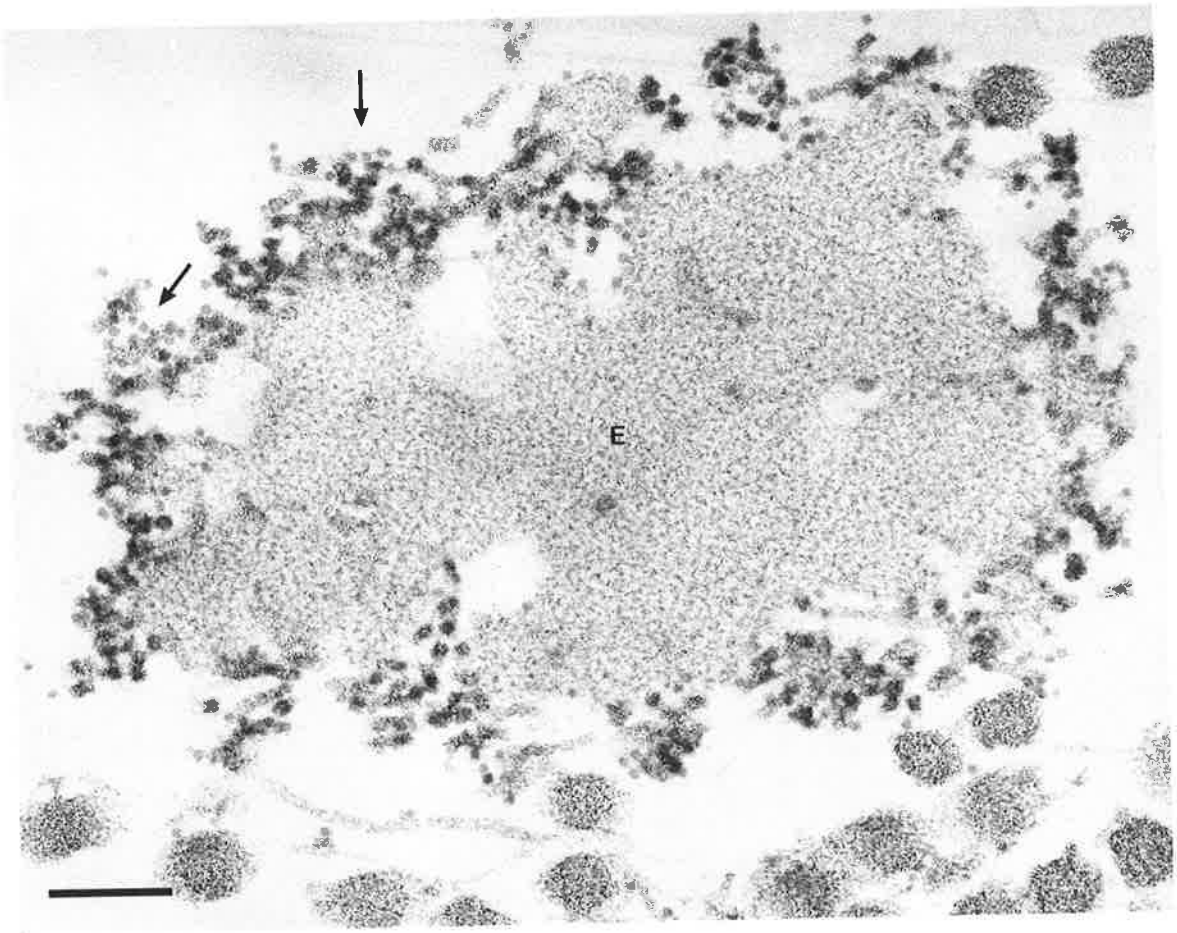
Figure 3.9. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin-labelled anti-MAg (ADS) IgG

Transverse section of an elastic fibre (**E**), showing attachment of ferritin label (↑) to microfibrils. Ferritin is seen as relatively electron-lucent round particles attaching to the more electron-dense microfibrils, here seen in cross section. Tissue fixed with 2% (w/v) paraformaldehyde. X160,000. Scale = 100nm.

Figure 3.10. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin-labelled anti-MAg (ADS) IgG

Longitudinal section of an elastic fibre (**E**) and surrounding collagen fibrils. Microfibrils have ferritin label attached to them. Note also the additional sparse labelling of thin matrix filaments (↑). X110,000. Scale = 100nm.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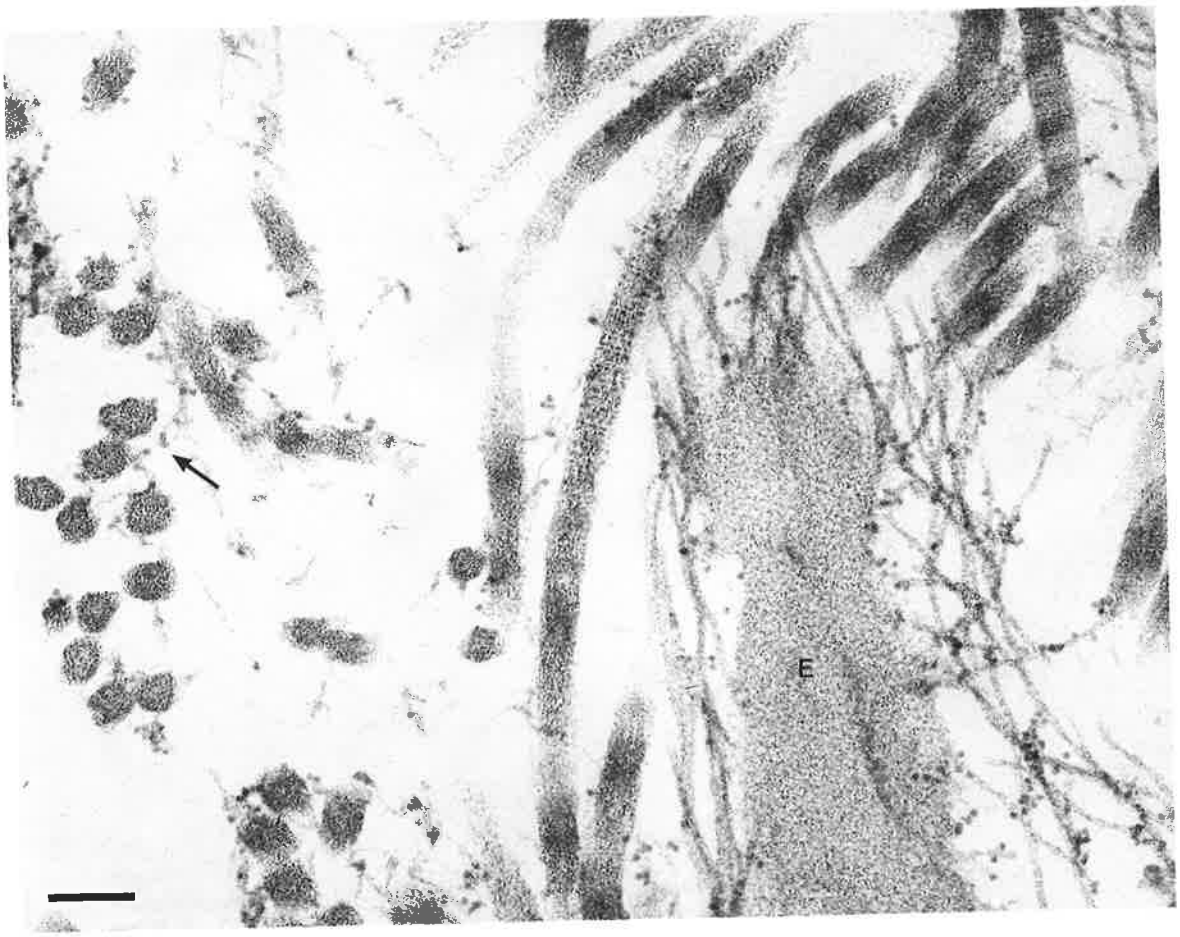
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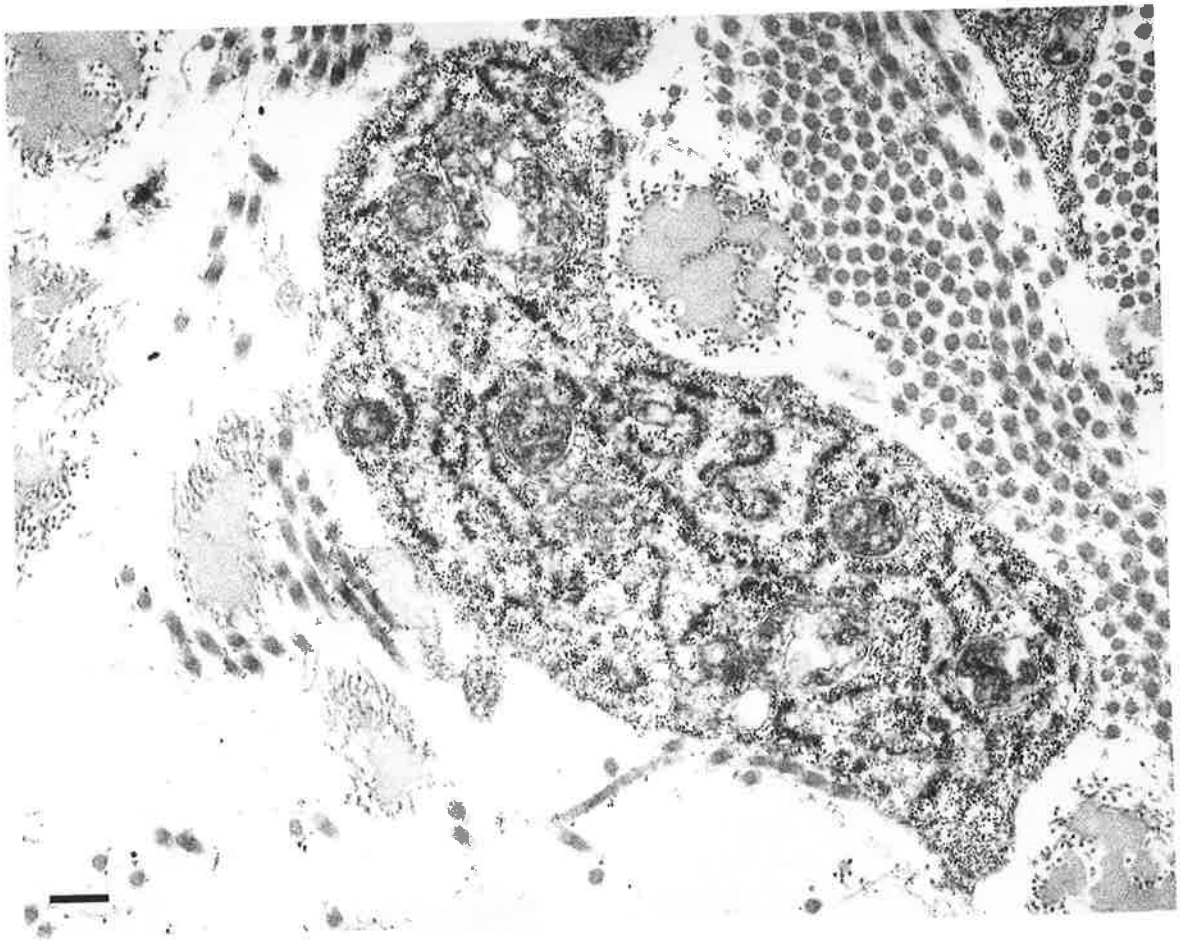
Figure 3.11. Transmission electron micrograph of frozen sectioned foetal ligamentum nuchae after overnight incubation with unconjugated ferritin

Control section. Note the poor ultrastructural preservation of cellular detail. Large amounts of ferritin bound non-specifically to the contents of degenerate fibroblasts (centre and top right). Some non-specific binding is seen in the matrix adjacent to these cells. Tissue fixed with 2% (w/v) paraformaldehyde. X40,000. Scale = 200nm.

Figure 3.12. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin-labelled anti-MAg (ADS) IgG

Tangential section of an elastic fibre (E) and collagen fibrils. Ferritin label can be seen attached to microfibrils and to thin matrix filaments. Note the clump of cellular debris at the right of the micrograph (↑) to which large quantities of ferritin have bound. Tissue fixed with 2% (w/v) paraformaldehyde. X85,000. Scale = 200nm.



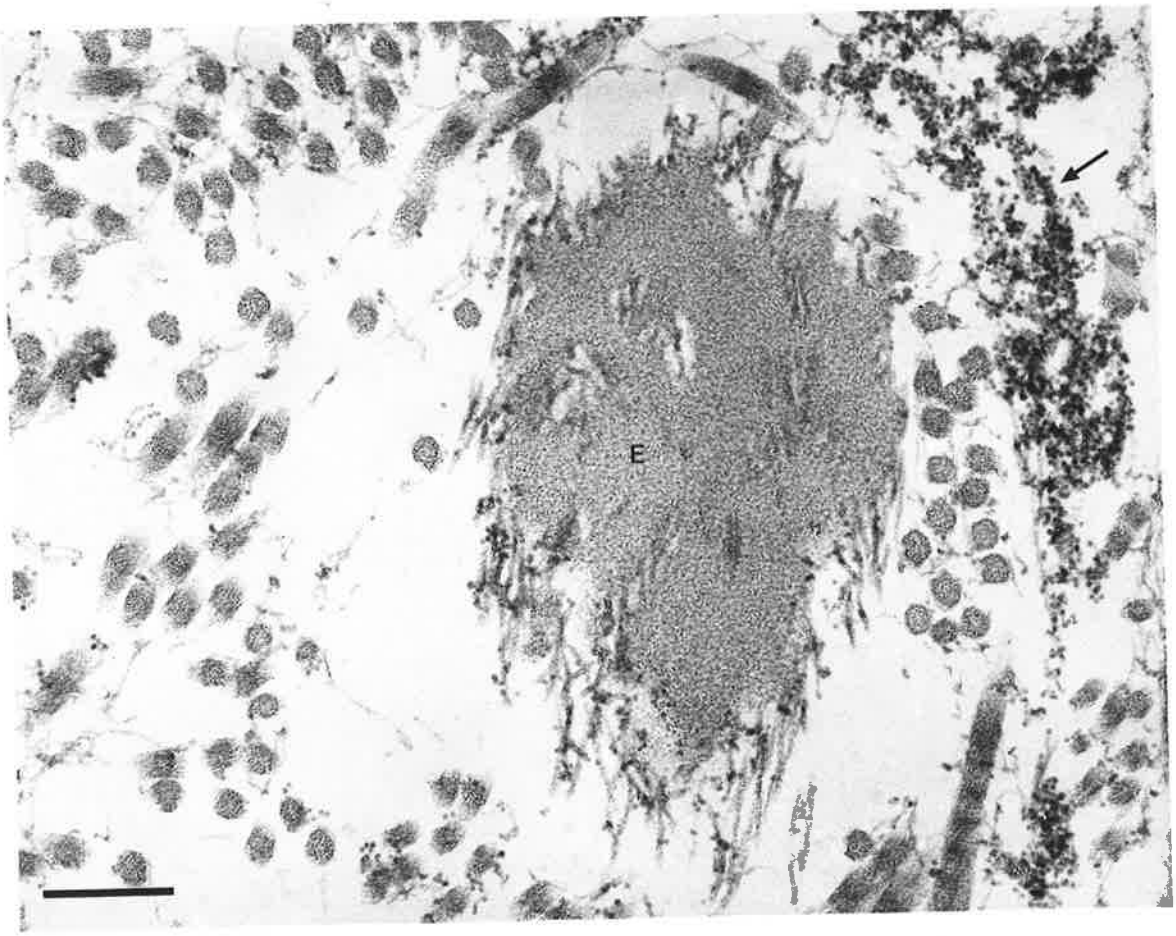

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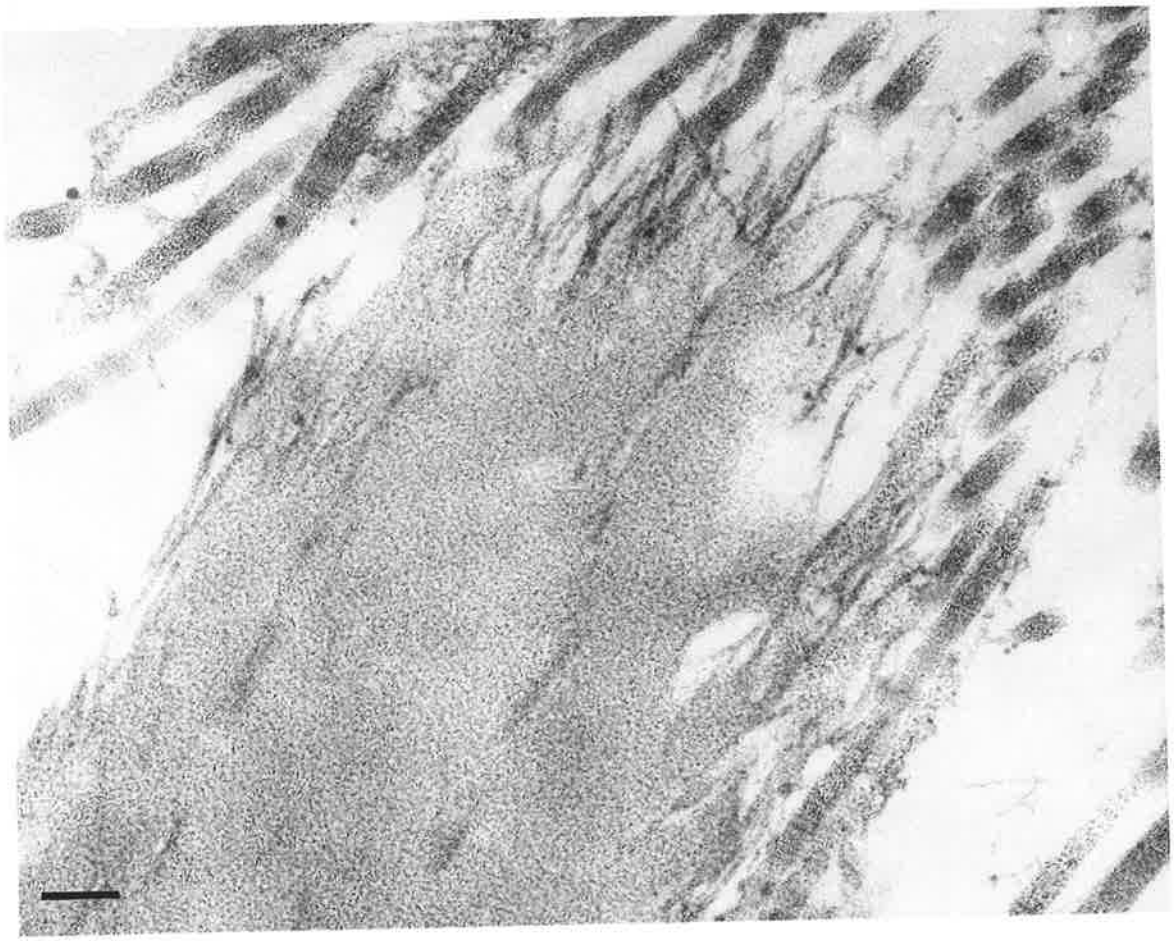
Figure 3.13. Transmission electron micrograph of foetal calf ligamentum nuchae after reaction with ferritin conjugated to pre-immune rabbit IgG

Control section. Note the almost complete absence of ferritin label compared with Figures 3.7 and 3.8. Tissue fixed with 2% (w/v) paraformaldehyde. X100,000. Scale = 100nm.

Figure 3.14. Transmission electron micrograph of foetal calf ligamentum nuchae treated with unlabelled anti-MAg (ADS) IgG before reaction with ferritin-conjugated antibody

Control section. The uptake of ferritin-labelled anti-MAg (ADS) IgG has been completely blocked. Tissue fixed with 2% (w/v) paraformaldehyde. X125,000. Scale = 100nm.



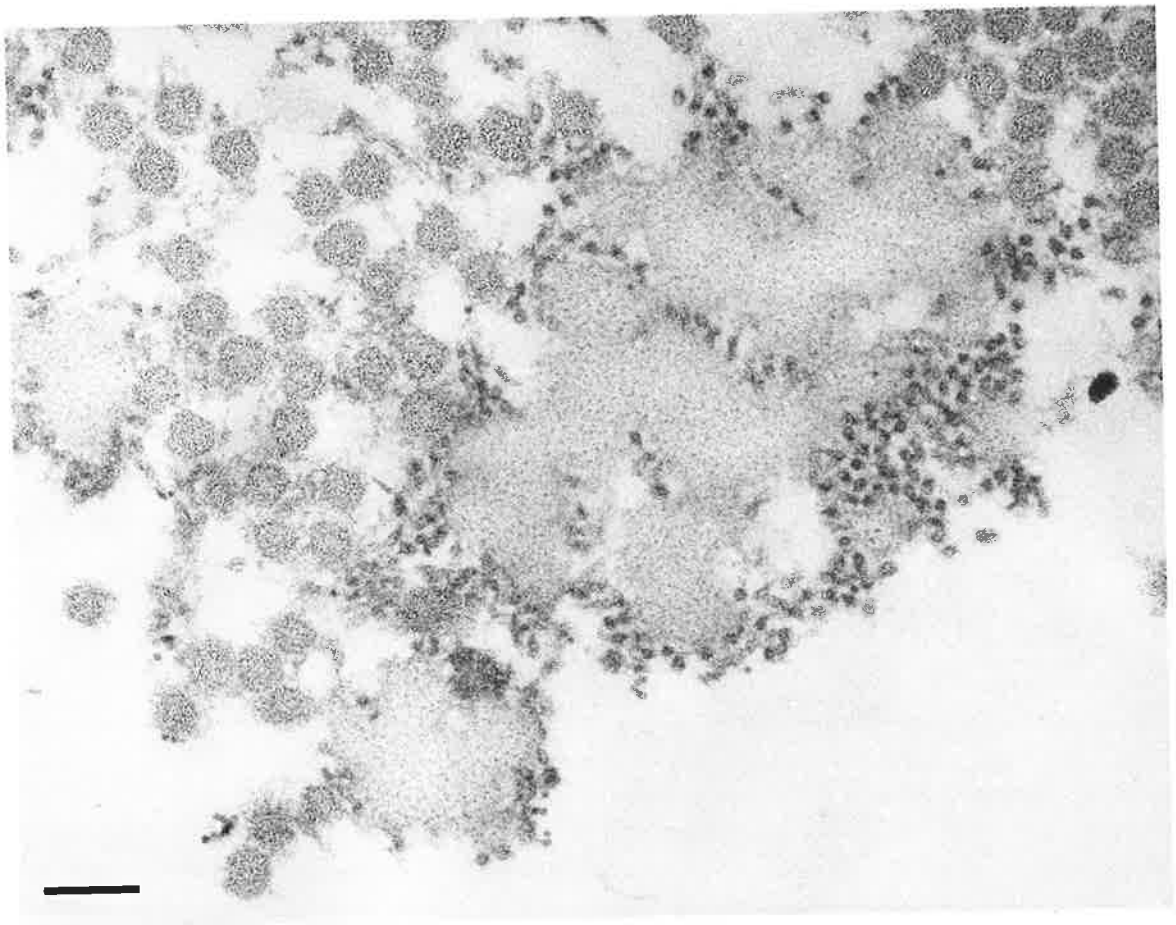
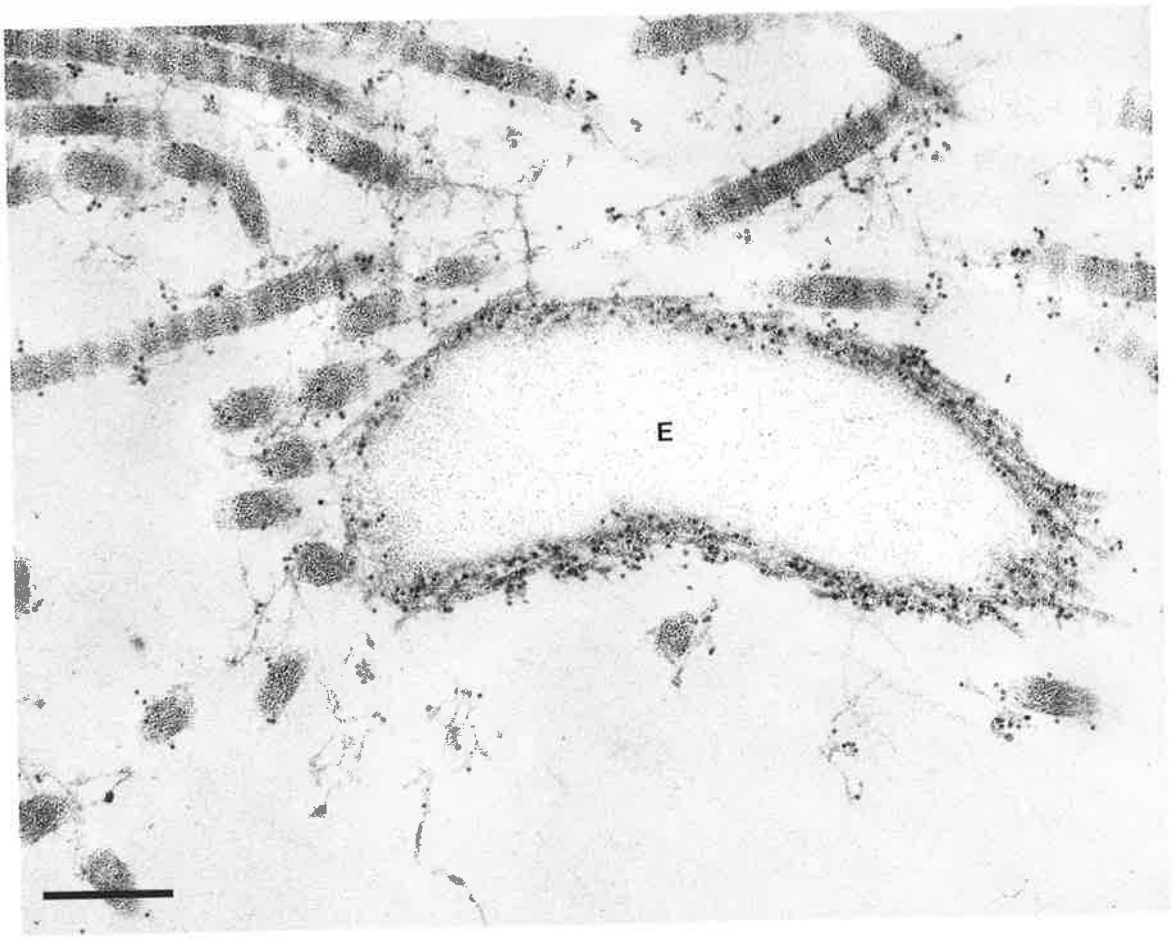


Figure 3.15. Transmission electron micrograph of foetal calf aorta after reaction with ferritin-labelled anti-MAg (ADS) IgG

Longitudinal section of an elastic fibre (E) showing dense ferritin labelling of microfibrils. Note the significant localization of ferritin to thin matrix filaments, which appear to be more abundant in aorta than in ligamentum nuchae (cf. Figures 3.7 and 3.8). Tissue fixed with 2% (w/v) paraformaldehyde. X85,000. Scale = 200nm.

Figure 3.16. Transmission electron micrograph of foetal calf aorta after reaction with ferritin-labelled anti-MAg (ADS) IgG

Tangential section of a large elastic fibre (E) showing ferritin labelling of microfibrils. Note the absence of label in areas where elastin appears devoid of microfibrils (↟). A large amount of ferritin is seen bound to a clump of cellular debris (↟↟). Similar non-specific binding of ferritin to cellular contents was noted in control specimens. Fixed with 2% (w/v) paraformaldehyde. X70,000. Scale = 200nm.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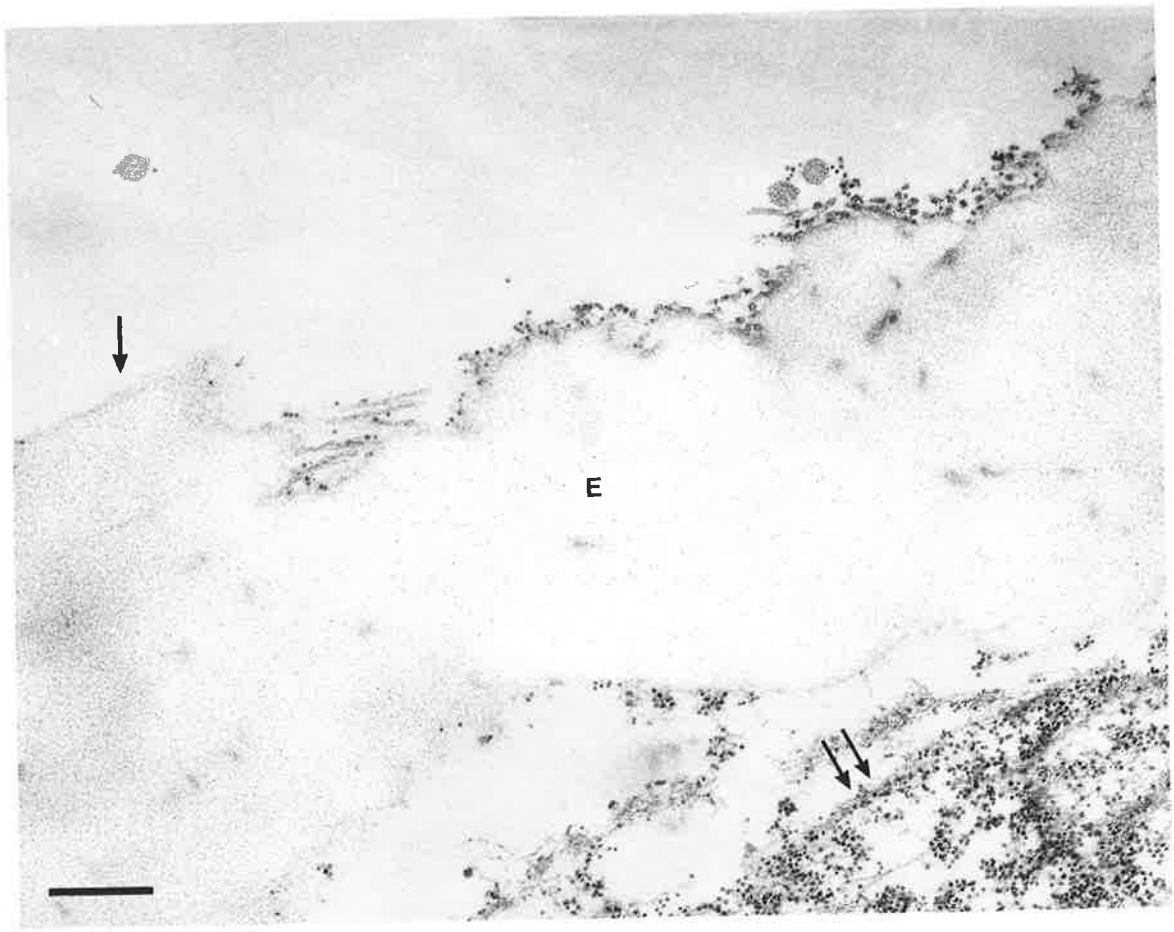
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3.3.3 Immunohistochemical Localization of Antibodies to CL Glycoprotein

The localization of antibodies to foetal calf aortic CL glycoprotein was studied by immunofluorescence and immunoferritin techniques.

Immunofluorescence microscopy. Positive results were obtained using immunofluorescence microscopy (Figure 3.17), but the amount of anti-(CL glycoprotein) antibody binding to sections appeared to be relatively small when compared to that obtained in experiments with anti-MAg antibody. The reason for this finding is not clear. However, the isolation of aortic GP involves preparative electrophoresis in the presence of SDS, and it is possible that antibodies prepared against CL glycoprotein which has been denatured in this way may possess low cross-reactivity against native protein. Experiments are proceeding to obtain material prepared under less severe conditions in order to clarify this problem.

Strikingly, no localization to elastic fibres was seen in aortic sections, and the overall picture is quite different from that seen with the anti-MAg antibodies (cf. Figure 3.3). Due to the relatively low fluorescence with this antibody, autofluorescence of elastic fibres is more prominent, but comparison with control sections allows easy distinction between specific binding and autofluorescence. Antibody was found to localize in two distinct areas: Firstly, the intercellular matrix between elastic lamellae in the media of the aorta showed a diffuse fluorescence, which did not appear in control sections, and did not appear to be due to non-specific conjugate binding. The very even intensity of binding suggested that antigen in this area was ubiquitously dispersed throughout the non-elastic matrix components, especially in areas where collagen is the dominant element. Secondly, a rather brighter, patchy linear fluorescence was observed in association with medial smooth muscle cells. This appeared to be extracellular, and appeared to be related to the external surface of the cells.

It was concluded that anti-(CL glycoprotein) antibodies do not localize to elastic fibres, are principally directed against an extracellular matrix component which could not be histologically identified, and may bind to a basement membrane moiety. However, the study reported here is preliminary and these findings should be regarded as provisional only.

Immunoferritin labelling. No observations regarding the binding of antibody to cellular components could be made due to the inadequate tissue preservation as described in section 3.3.2. No confident assessment of antibody localization to basement membrane was possible, due to the invariable association of such material with cellular debris which bound large quantities of ferritin non-specifically.

Some specific binding was observed to the thin filaments which formed a loose network in the extracellular matrix (Figure 3.18). Small amounts of ferritin were aligned along these filaments, especially where they were found in close proximity to bundles of collagen fibrils. Occasional ferritin molecules were seen to bind to collagen fibres but this may be due to the intimate association of such matrix filaments with collagen, which in places exceeds the 'resolution' offered by immunoferritin techniques. This binding could be blocked by preincubation of specimens with unlabelled anti-(CL glycoprotein) antibody (Figure 3.19). Few ferritin molecules were observed to bind to elastin-associated microfibrils. A similarly low level of binding of ferritin to elastin-associated microfibrils was also observed in control sections, suggesting that such uptake is non-specific. Control specimens treated with free ferritin or ferritin-NRS IgG conjugates confirmed the specificity of the binding described above.

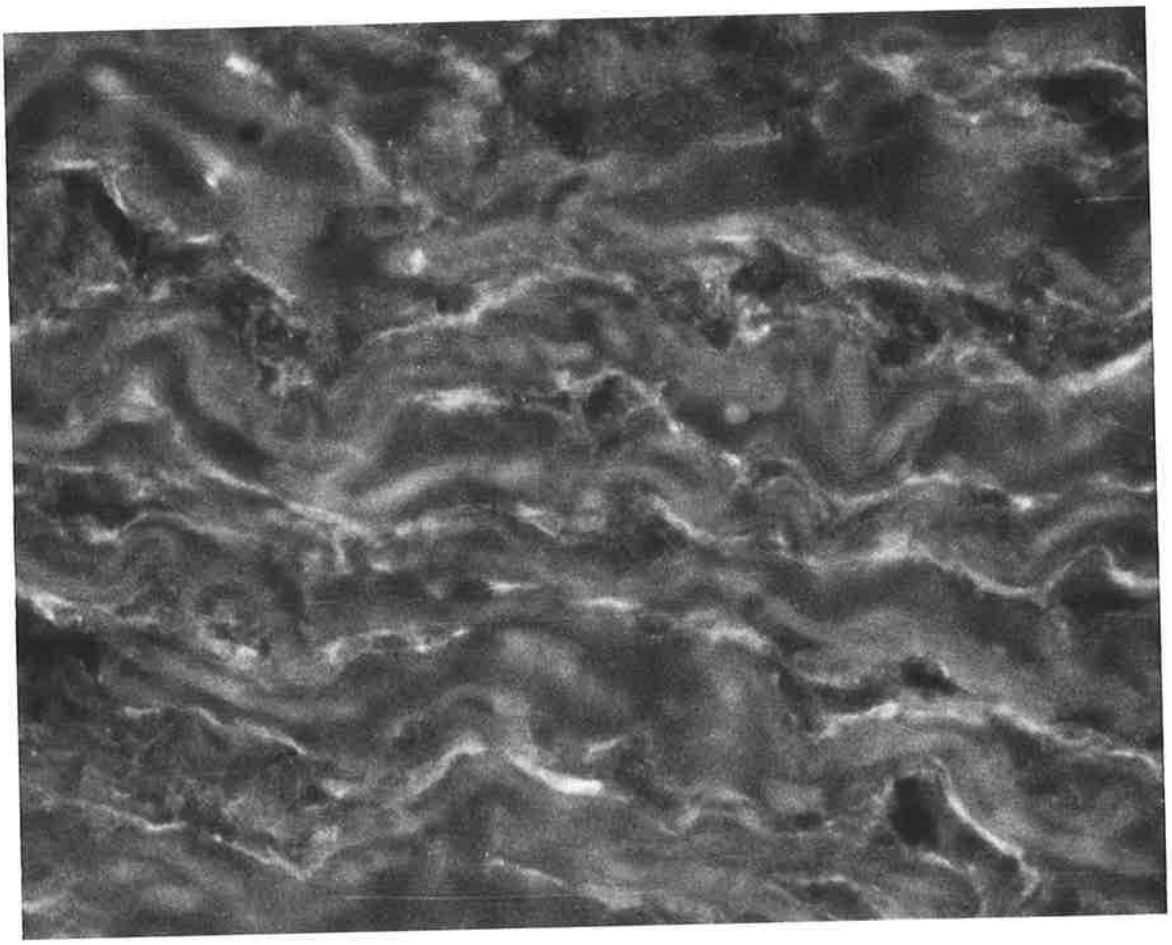
Figure 3.17. Immunofluorescent staining of foetal calf thoracic aorta with anti-(CL glycoprotein) IgG

Transverse section of the upper thoracic aorta showing the medial region only. The lumen is toward the top of the micrograph.

Magnification X400.

a. Section treated with anti-(Cl glycoprotein) IgG. Note the diffuse fluorescence of interstitial connective tissue with stronger patchy fluorescence apparently aligned along the surface of smooth muscle cells. No fluorescence of the elastic lamellae is evident.

b. Control section treated with pre-immune rabbit IgG.



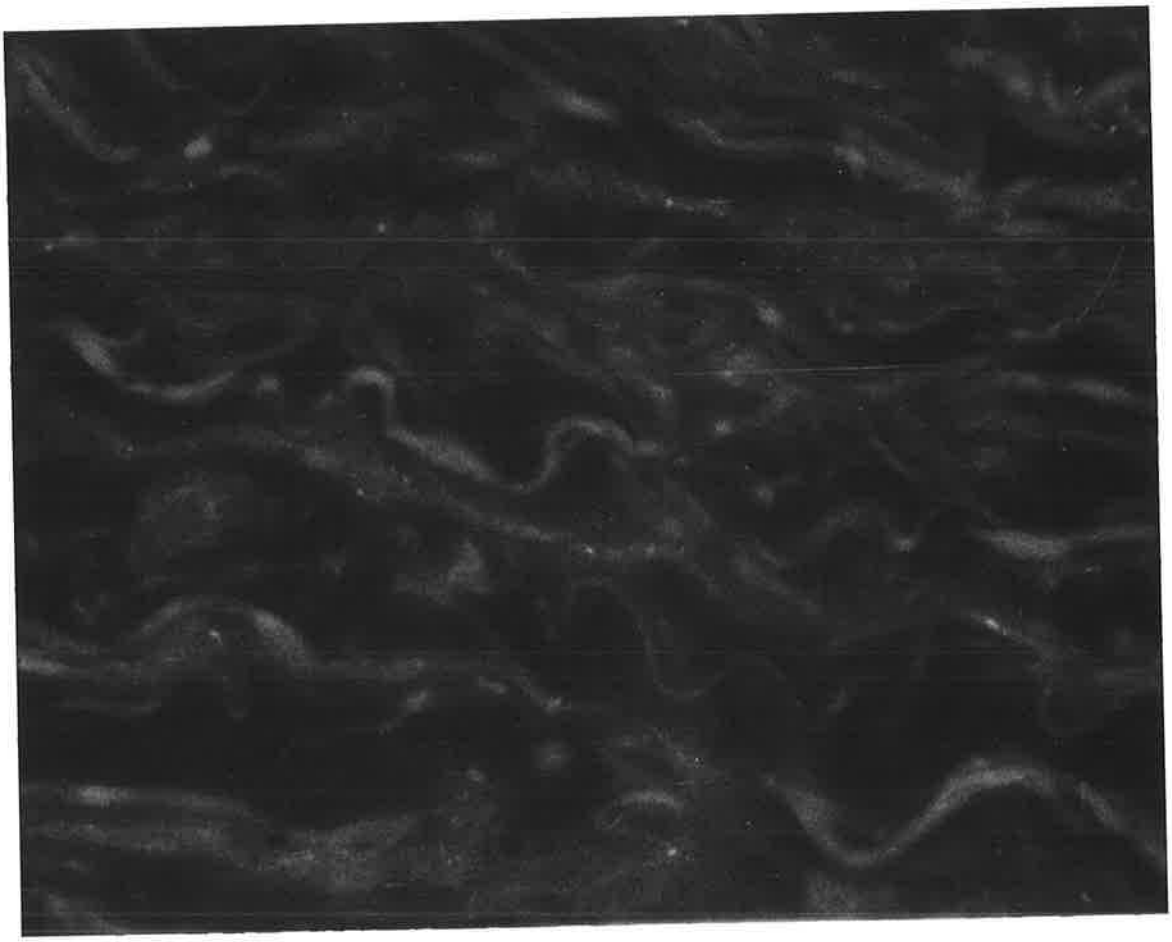
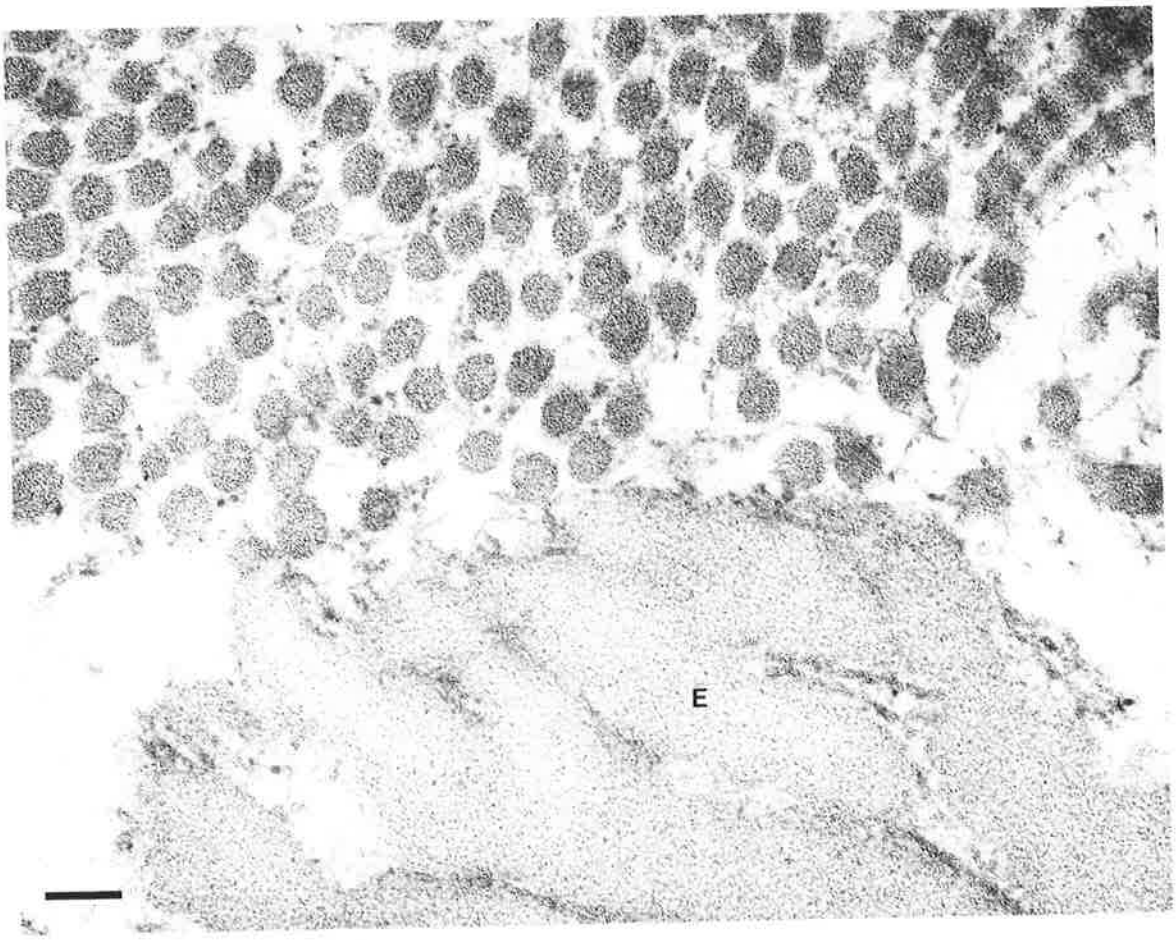


Figure 3.18. Transmission electron micrograph of foetal calf thoracic aorta after reaction with ferritin-labelled anti-(CL glycoprotein) IgG
The section shows part of an elastic fibre (**E**) and an adjacent bundle of collagen fibrils. Note the ferritin labelling of thin filaments running between collagen fibrils. No ferritin was observed to bind to elastin-associated microfibrils. Tissue fixed with 2% (w/v) paraformaldehyde. X100,000. Scale = 100nm.

Figure 3.19. Transmission electron micrograph of foetal calf aorta treated with unlabelled anti-(CL glycoprotein) IgG before reaction with ferritin-conjugated antibody
Control section. The uptake of ferritin-labelled anti-(CL glycoprotein) has been completely blocked. Tissue fixed with 2% (w/v) paraformaldehyde. X130,000.
Scale = 100nm.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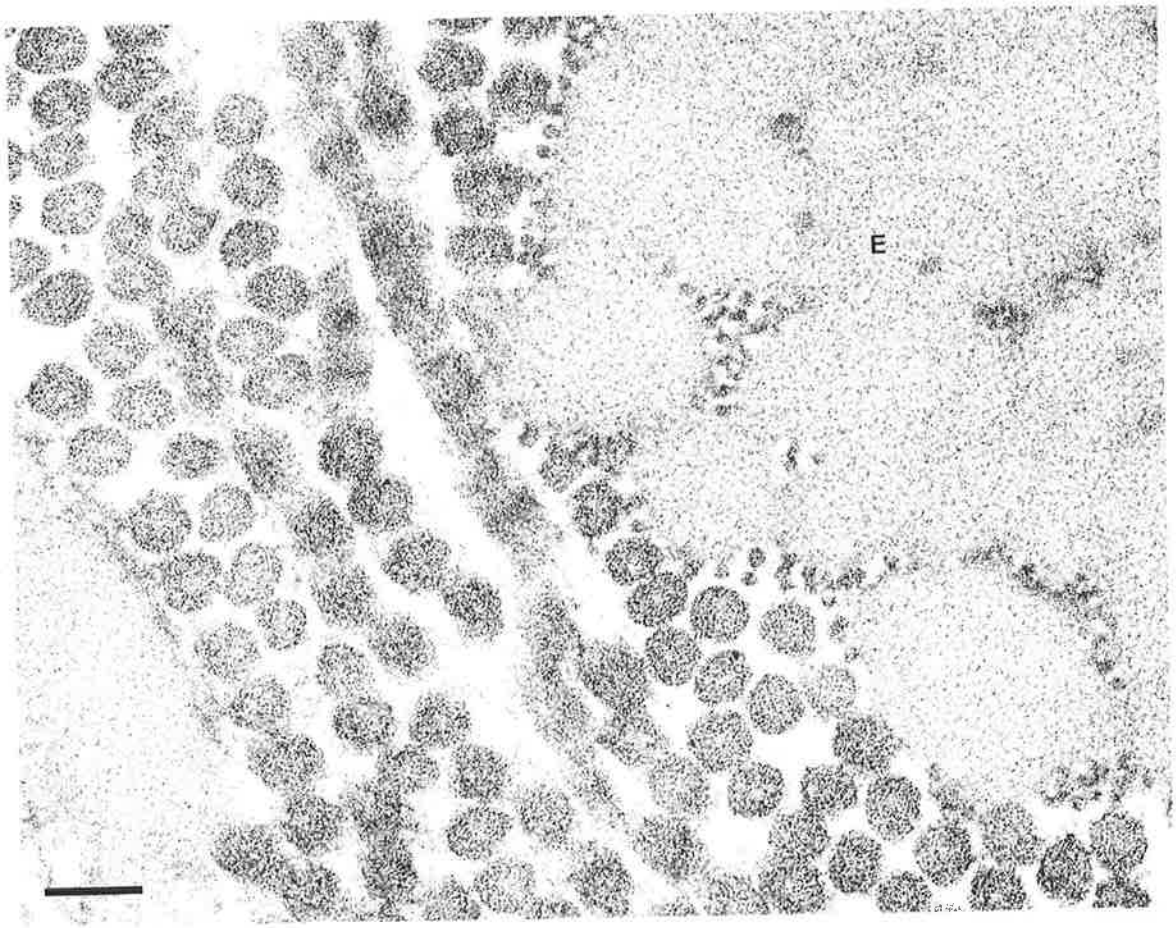
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3.4 DISCUSSION

3.4.1 Immunofluorescent Localization of Anti-MAg Antibodies

Immunofluorescence labelling of ligamentum nuchae and thoracic aorta from foetal calves clearly showed that anti-MAg antibodies specifically localized to the surface of elastic fibres in both tissues. No cellular, basement membrane or other matrix components were observed to bind antibody, and it was concluded that anti-MAg antibodies were primarily directed toward a surface component of the elastic fibres in these tissues.

Whilst this finding is similar to that reported by Kewley et al. (1977a,b) using their anti-'microfibrillar protein' antiserum (anti-'MFP'), it should be noted that these authors used only purified elastic fibres from ligamentum nuchae in their experiments, rather than tissue sections of the ligament. Therefore, no conclusions could be drawn from their experiments concerning the specificity of binding of anti-'MFP' antibodies to other cellular and matrix components present in intact ligamentum nuchae. Nor were any experiments performed on bovine aortic tissues with their antiserum. These are critical omissions in their studies and severely limit interpretation of their results.

However, Kewley and his co-workers observed binding of their anti-'MFP' antibody to basement membrane structures and to reticulin fibres in a variety of other tissues. These included bovine spleen, human kidney and mouse kidney, liver, spleen and heart muscle (Kewley et al., 1977b).

The anti-MAg antibody labelling obtained in foetal bovine kidney was confined primarily to the mesangial region of the glomerulus, with less intense specific fluorescence of the interstitial connective tissue surrounding the tubules and glomeruli. Microfibrils which are ultrastructurally identical to elastin-associated microfibrils have been demonstrated in the mesangium of human kidney (Farquhar, 1978; Hsu and Churg, 1979). It is possible that similar microfibrils may be responsible for the observed localization of anti-MAg antibodies to the mesangial region in the present study, thereby implying an immunochemical relationship to elastin-associated microfibrils. Further studies at an electron microscopic level would be necessary to confirm such a relationship.

However, the pattern of fluorescent labelling produced by anti-MAg antibodies in the kidney was significantly different from that reported by Kewley et al. (1977b) in that they found additional binding of anti-'microfibrillar protein' antibody to basement membrane structures of the tubules and glomerular capillaries. There are several possible reasons for these observed differences in antibody binding. Firstly, as discussed in section 2.4, the antibody specificities of the Kewley 'anti-microfibrillar' antiserum and the anti-MAg antiserum are not directly comparable because of differences in technique, both in extraction of the original antigens and the subsequent immunization and assay procedures used in the preparation and characterization of the resultant antisera. Secondly, Kewley and his co-workers utilized human and mouse kidney as substrates for antibody binding, rather than foetal bovine tissues as were used in the present study. Therefore it is possible that species and/or age differences in the distribution of tissue antigens might have resulted in the different labelling patterns observed. Finally, the Kewley anti-MAg antiserum has been shown to precipitate two glycoproteins from the medium of cultured foetal calf ligamentum nuchae fibroblasts (Sear et al., 1981a,b). The smaller of these glycoproteins, designated 'MFP I', closely resembled CL glycoprotein (Gibson and Cleary, 1982) in those of its biochemical properties which are known. Preliminary studies of the tissue localization of anti-(CL glycoprotein) antibodies, described in section 3.3.3, have shown that CL glycoprotein is immunologically unrelated to elastin-associated microfibrils. Further work by Gibson and Cleary (1983) has demonstrated localization of antibodies to the intercellular matrix of a number of tissues, as well as to vascular smooth muscle cell basement membrane and splenic reticulin fibres. These latter localizations closely parallel the non-elastic tissue distribution of immunofluorescent labelling with the Kewley antiserum. Therefore, some of the observed differences between the antibody binding obtained with anti-MAg antibodies, and that reported by Kewley et al. (1977a,b), may be due to significantly greater contamination of the Kewley antiserum with anti-(CL glycoprotein) activity.

The distribution of anti-MAg antibody binding in sections of foetal bovine skin is of interest in that it corresponds closely to the pattern formed by oxytalan, elaunin and

elastic fibres in skin as described by Cotta-Periera et al. (1976; 1977). It has been suggested that these fibres may represent sequential stages in elastogenesis, and they have been shown to be ultrastructurally similar to elastin-associated microfibrils (Cotta-Periera et al., 1977). It is thus an attractive hypothesis that the localization demonstrated in this study is indicative of an immunological identity between the microfibrillar components of oxytalan and elaunin fibres, and elastin-associated microfibrils. Ultrastructural localization of anti-MAg antibodies is necessary, however, for such an interpretation to be confirmed.

In conclusion it can be seen that the observed localization of anti-MAg antibodies is consistent with the reported tissue distribution of elastin-associated microfibrils. It also binds to regions containing microfibrils (found in non-elastic tissues) which share similar ultrastructural characteristics. Comparison of the distribution of anti-MAg antibodies with the distribution of the Kewley anti-'microfibrillar protein' antibody (Kewley et al., 1977a,b) reveals that their antibody is much more widespread in its tissue localization, binding not only to elastic fibres, but also to basement membrane structures and reticulin fibres. These findings suggest significant contaminant antibody activity within the Kewley antiserum, rendering it less specific in its tissue labelling properties than anti-MAg antiserum.

3.4.2 Ultrastructural Localization of Anti-MAg Antibodies

Direct labelling of foetal calf ligamentum nuchae sections with ferritin-labelled anti-MAg antibodies revealed strong localization of antibody to elastin-associated microfibrils, but no binding was observed to the amorphous component of elastin itself. Collagen fibrils also remained unlabelled, but a small amount of ferritin was observed to bind to thin filaments which appeared to connect collagen fibrils to each other, and to adjacent elastic fibres. Sections of foetal calf thoracic aorta bound ferritin-labelled anti-MAg antibodies in a qualitatively similar pattern to that seen in ligamentum nuchae. Such binding could be blocked completely by pretreatment of sections with unlabelled anti-MAg antiserum, thus confirming the specificity of the antibody labelling obtained.

Experiments using both crude and immunoadsorbed anti-MAg IgG-ferritin conjugates showed no significant differences in the antibody labelling of both foetal calf ligamentum nuchae and thoracic aorta. These findings indicate that the contaminant antibody activity detected in the crude anti-MAg antiserum, directed against components of FCS and fibronectin, did not significantly affect the pattern of antibody labelling. Only purified immunoadsorbed anti-MAg (ADS) IgG was used in the studies described in this chapter. Unfortunately considerable non-specific uptake of ferritin by cellular debris was evident in both antibody-labelled and control sections. Acceptable cellular preservation could not be achieved despite repeated attempts, probably due to the use of a frozen section technique. Therefore no observations concerning specific interaction of anti-MAg antibodies with cellular components or basement membrane structures could be made. These problems proved insurmountable in more complex tissues such as kidney and skin, and experiments on these tissues were not pursued further. Such problems may, however, be resolved by the application of the recently described low temperature embedding techniques using Lowicryl media (Roth, 1983).

Kewley et al. (1977a) have also studied the ultrastructural localization of their anti-'microfibrillar protein' antiserum, using an indirect immunoperoxidase technique on 17-day-old chick aorta. The authors found that peroxidase reaction product localized to elastin-associated microfibrils in a regular arrangement, which suggested to them an underlying periodic organization of microfibrillar subunits. Collagen fibrils also showed significant surface staining. Similar findings have been reported by Streeten and her co-workers for the localization of antibodies both to sonicated bovine ocular zonules (Streeten et al., 1981) and to an anti-'microfibrillar protein' antiserum [produced by the authors (Streeten and Licari, 1983), from a 'microfibrillar protein' antigen preparation supplied by Kewley's group in Manchester]. An indirect immunoperoxidase technique was also used in these experiments, and the authors were able to confirm that the peroxidase reaction product demonstrated periodic localization along elastin-associated microfibrils at measured intervals of 35-45nm. Again, significant labelling of collagen fibrils was seen to occur. Both antibodies produced identical patterns in a number of

tissues, including human and bovine ciliary body, calf ligamentum nuchae and chick aorta.

There are some major points of difference between the findings described in this chapter, and those of Kewley et al. (1977a), Streeten et al. (1981) and Streeten and Licari (1983). Although anti-MAg antibodies showed strong localization to elastin-associated microfibrils, they did not bind to collagen fibrils in either of the tissues studied. Streeten and Licari (1983) have suggested that staining of collagen by their anti-'microfibrillar protein' antibody might be due to diffusion of reaction product from adjacent elastic fibres. However, the consistent nature of the staining obtained with three separate antibody preparations militates against such an explanation, and it seems more likely that contaminant antibody activity is responsible for the observed collagen labelling. It should be noted that anti-(CL glycoprotein) antibodies showed no direct localization to collagen fibrils themselves, attaching instead to the thin matrix filaments which were seen to run between them. It therefore seems unlikely that anti-(CL glycoprotein) antibody activity would be responsible for the labelling of collagen fibrils observed by Kewley et al. (1977a). The presence of other contaminant antibody activity is consistent with the widespread immunofluorescent localization of the Kewley anti-'microfibrillar protein' antiserum, which was discussed in detail in the preceding section.

Ferritin-labelled anti-MAg antibodies failed to show any distinct periodicity in their pattern of localization, in contrast to the 35-45nm 'macroperiodicity' demonstrated by Streeten et al. (1981) and Streeten and Licari (1983) using an indirect immunoperoxidase technique. This discrepancy may in part be due to the differences in the methods employed. The steric displacement of ferritin-IgG conjugates has been estimated to be of the order of 9-25nm (Voak and Williams, 1971; Dimmock et al., 1972; Williams, 1971). As the diameter of the elastin-association microfibrils is approximately 12nm, it is obvious that the resolution obtained with ferritin-labelling techniques would not allow observations about antigenic frequency on such small structures. The 'resolution' of the horseradish peroxidase-labelled antibody technique used by Streeten and her co-workers is, as yet, unestimated.

Immunoperoxidase techniques are also subject to 'blurring' of localization due to diffusion of the enzyme product. This is a particular problem at high magnifications (Novikoff et al., 1972). The amount of enzyme product deposited is also dependent on the time and temperature of incubation of sections with the substrate (Williams, 1977). It is conceivable that these factors may have given rise to the very heavy, clumped distribution of reaction product (which obscures the underlying ultrastructure) observed by Streeten and her co-workers. Further data concerning the effects of varying the enzyme incubation periods may help to confirm or refute this hypothesis.

Less prominent, but significant, ferritin-labelling of the thin filamentous fibrils (3-5nm in diameter) running between collagen fibrils was consistently observed in sections labelled with anti-MAg antibodies. Such labelling appeared to be specific, and was not observed in control sections. The localization of anti-MAg antibodies to these thin matrix filaments is very similar to that obtained with ferritin-labelled anti-(CL glycoprotein) antibodies as described in section 3.3.3. It is therefore possible that the observed labelling of these filaments may be due to the small degree of activity against CL glycoprotein now known to be present in the anti-MAg (ADS) antiserum (see section 2.3.4). This interpretation could be tested by adsorption of anti-MAg antiserum with CL glycoprotein and subsequent comparison of the labelling patterns obtained using this absorbed antibody with that described above. However, insufficient amounts of CL glycoprotein were available at the time to allow this experiment to be carried out.

Myers et al. (1973) have shown that fine filaments of similar ultrastructural morphology display an affinity for ruthenium red stain. The authors suggested that this finding was indicative of the presence of glycoprotein components within such filaments, which were found to interconnect collagen fibrils in a variety of tissues, including rat aorta, rat and human synovium and rabbit articular cartilage. Similar observations have been reported by Kischer and Shetlar (1974) and Shipp and Bowness (1975). Ruthenium red is not, however, a specific stain for glycoproteins, and will in fact bind to any polyanionic material (Luft, 1971). More convincing evidence of the presence of glycoprotein moieties has recently been presented by Fanning and Cleary

(1984). These authors showed that fine matrix filaments in foetal calf aorta and ligamentum nuchae bind alkaline bismuth subnitrate only after periodic acid oxidation. These histochemical observations are therefore consistent with the presence of CL glycoprotein as a component of these matrix filaments.

3.4.4 Conclusions

The experiments described in Chapters Two and Three have established that MAg extract, despite its demonstrated heterogeneity, contains antigenically active components which are related to elastin-asssociated microfibrils. Although the preparation of MAg involved the use of powerful protein denaturants, immunohistochemical studies have demonstrated clearly that anti-MAg antiserum contains significant antibody activity toward native elastin-associated microfibrils, with only minor activity toward other matrix antigens. The use of a sensitive ELISA technique has shown that such activity is not directed toward tropoelastin or solubilized amorphous elastin. The antiserum was shown to be active against antigens in a number of foetal bovine tissues, and in more recent work from this laboratory, to cross-react with adult bovine tissues as well as with those of other species, including humans (Prosser et al., 1984).

Both light and ultrastructural studies suggest that anti-MAg antiserum is more specific in its tissue binding than any previously described anti-'microfibrillar protein' antibody preparation (Kewley et al., 1977a,b; Streeten et al., 1981; Streeten and Licari, 1983). A very low level of contaminant antibody activity against CL glycoprotein was present in the final immunoadsorbed anti-MAg (ADS) IgG preparation. However, the studies described in section 3.3.3 have shown that antibodies directed toward CL glycoprotein localized in a quite different tissue distribution to that observed with anti-MAg antiserum. Comparison with the findings of Kewley et al. (1977a,b) implies significant contamination of their anti-'microfibrillar protein' antibody preparation with antibodies against other matrix antigens, including CL glycoprotein.

Anti-MAg antibodies, therefore, represent a means of immunochemically identifying components of elastin-associated microfibrils, and of studying the relationship between

such microfibrils and morphologically similar microfibrils in non-elastic tissues. This antiserum should prove useful in identifying specifically-microfibrillar components in tissue extracts, thereby allowing purification and biochemical characterization of such components.

CHAPTER FOUR

STUDIES OF FOETAL CALF LIGAMENTUM NUCHAE FIBROBLAST CULTURES

4.1 INTRODUCTION

The extraction of a 'microfibrillar' antigen (MAg) and subsequent preparation of an antiserum against this extract (anti-MAg) has been described in Chapter Two. However the isolation and characterization of 'microfibrillar' moieties from intact tissues is hampered by a number of factors; such extracts are invariably denatured due to the strong solvents employed, are often degraded by non-specific proteases released during extraction procedures, and are usually extremely insoluble.

In an attempt to obviate these difficulties a number of investigators have turned to tissue culture as an alternative to extraction of intact tissue (see section 1.6). It is known that soluble forms of connective tissue components (e.g. procollagen and tropoelastin) are secreted into the medium by connective tissue cells in culture and these macromolecules can be relatively easily retrieved in undenatured form. Thus it is possible that cultured foetal calf ligamentum nuchae fibroblasts (which have been shown to elaborate both elastin and collagen) might secrete precursors of elastin-associated microfibrils into the medium, or that such precursors might be more easily extracted from the cell layer matrix.

This approach has been utilized by Sear and his co-workers (Sear et al., 1981a,b). They described the precipitation of two macromolecules from the medium of cultured foetal calf ligamentum nuchae fibroblasts, using an anti-'microfibrillar protein' antiserum raised against 'microfibrillar protein' extracted from intact ligament (Kewley et al., 1977a,b). One of these species, which they called MFP I, was a collagenous glycoprotein (apparent molecular weight 150,000 daltons) which was claimed to be a subunit of microfibrils on the basis of the 'mono-specificity' of the antiserum employed. As outlined in Chapter Three, there are significant differences between anti-MAg antiserum and the 'anti-microfibrillar protein' antiserum described by Kewley et al. (1977a,b).

In order to examine the potential of this in vitro system for exploring the process of elastinogenesis, studies were made of foetal calf ligamentum nuchae fibroblast cultures. Some experiments were planned such that the results obtained could be

compared directly with those reported by Kewley et al. (1977a,b) and Sear et al. (1981a,b). Several approaches were employed.

i) Ultrastructural studies of the cell layer of such cultures were undertaken to determine the extracellular matrix characteristics. Although large numbers of morphologically-typical microfibrils were found, no formation of amorphous elastin was detected despite lengthy periods in culture under scorbutic conditions.

ii) Radioactively-labelled precursor molecules were used to label newly-synthesized macromolecules released into the medium. [^{3}H]-proline was used as a protein label for several reasons: It labels collagenous molecules, including MFP I, which are rich in proline; significant quantities are incorporated into tropoelastin; and it is present to some extent in all proteins so far studied, making it a useful general protein label. In addition [^{3}H]-fucose was used to detect the biosynthesis of glycoproteins, after the work of Sear et al. (1981a,b). Labelled species were precipitated from spent culture medium with $(NH_4)_2SO_4$ and analyzed by SDS-PAGE.

iii) Immunoprecipitation experiments were performed utilizing anti-MAg antiserum in an attempt to precipitate radioactively-labelled macromolecules, immunochemically related to MAg, from the spent medium of fibroblast cultures. No cross-reacting material could be detected.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM), antibiotics (supplied as a stock solution of penicillin 5000IU/ml and streptomycin 5mg/ml), foetal calf serum and Falcon plastic tissue culture flasks ($25cm^2$ and $75cm^2$ growth areas) were supplied by Flow Laboratories (Australia Pty Ltd), Stanmore, Sydney, NSW.

Difco 1:250 trypsin was manufactured by Difco Laboratories of Detroit, Michigan, USA, and supplied by Flow Laboratories in Australia.

Soluble bovine insulin (as an 80IU/ml sterile solution) was obtained from Commonwealth Serum Laboratories, Parkville, Victoria.

Aprotinin (Trasylol) was supplied in sterile ampoules as a solution of 10,000 kallikrein inhibitor units (KIU)/ml by Bayer Pharmaceutical Company, Botany, NSW.

Epon 812 epoxy resin kits were purchased from TAAB Laboratories, Emmer Green, England.

The radiochemicals, L-[6-^{3}H]-fucose (25Ci/mmol) and L-[5-^{3}H]-proline (29Ci/mmol) were supplied by Amersham (Australia) Pty Ltd, Surry Hills, Sydney, NSW.

Ascorbic acid and dimethylsulphoxide (analytical grades) were manufactured by BDH Chemicals, Poole, UK.

Type I collagen was prepared in this laboratory from bovine skin by the method of Epstein (1974).

All other materials and reagents were obtained from sources previously detailed in sections 2.2.1 and 3.2.1.

4.2.2 Culture of Foetal Calf Ligamentum Nuchae Fibroblasts

Foetal calves of 200-220 days gestation were obtained at the abattoir and transported to the laboratory on ice. Nuchal ligaments were excised within two hours of maternal death using aseptic technique and rinsed in warm phosphate buffered saline (PBS-A; see Table 4.1) containing penicillin (50IU/ml) and streptomycin (50µg/ml).

Table 4.1

Composition of Phosphate Buffered Saline A

Component	mg/L
NaCl	8,000.0
KCl	200.0
Na_2HPO_4	1,150.0
KH_2PO_4	200.00

This formulation is based on that described by Dulbecco and Vogt (1954).

Small blocks of ligament were cut and finely minced with scissors under sterile conditions. Pieces of minced ligament were then placed in Falcon plastic culture flasks ($75cm^2$ growth surface area; approximately 20 pieces of ligament per bottle) with a few drops of FCS and incubated for 3 hours at 37°C, in an atmosphere of 5% CO_2:95% air, to allow for adhesion of the tissue explants. DMEM (see Table 4.2), supplemented with 10% (v/v) FCS and antibiotics as above, was then added to each flask (10ml). The medium was subsequently changed every 2-3 days.

After 48 hours outgrowths of fibroblastic cells began to appear from the explants, spreading laterally to reach confluence in 7-10 days. Confluent cultures were sub-cultured by trypsinization as follows: The cell layers were rinsed twice in warm PBS-A (5ml) and then incubated with 3ml of PBS-A containing 0.025% (w/v) trypsin (Difco 1:250) at 37°C for 5 minutes. Cells were detached from the flask by gentle tapping and 4ml of DMEM containing 10% (v/v) FCS was added to inhibit further activity of the trypsin. Trypsinized cells were then suspended by gentle aspiration with a sterile pipette, transferred to centrifuge tubes and collected by centrifugation at 500g for 5 minutes. The supernatant was carefully aspirated and the cells resuspended in fresh DMEM/10% (v/v) FCS. Cell density was determined with a haemocytometer (Fuchs-Rosenthal) and cells were inoculated into fresh flasks at a density of 3×10^6 cells per $75cm^2$ flask.

Table 4.2

Minimum Essential Medium (Dulbecco's Modification)

Component	mg/L	Component	mg/L
$CaCl_2.2H_2O$	264.0	L-Aspartic acid*	53.20
$Fe(NO_3)_3.9H_2O$	0.10	L-Cystine	48.0
KCl	400.0	L-Glutamic acid*	58.80
$MgSO_4.7H_2O$	200.0	L-Glutamine	580.0
NaCl	6,400.0	Glycine*	30.0
$NaH_2PO_4.H_2O$	140.0	L-Histidine HCl	38.4
$NaHCO_3$	3,700.0	L-Isoleucine	105.0
Glucose	4,500.0	L-Leucine	105.0
Ca pantothenate	4.0	L-Lysine	146.0
Choline chloride	4.0	L-Methionine	30.0
Folic acid	4.0	L-Phenylalanine	66.0
i-Inositol	7.20	L-Proline*	46.0
Nicotinamide	4.00	L-Serine*	42.0
Pyridoxal HCl	4.00	L-Threonine	95.0
Riboflavin	.40	L-Tryptophan	16.0
Thiamine HCl	4.00	L-Tyrosine	72.0
L-Alanine*	35.60	L-Valine	94.0
L-Arginine	84.0	Phenol red	17.0
L-Asparagine.H_2O*	60.0		

DMEM was supplied by Flow Laboratories Ltd. The formulation is based on that of Dulbecco and Freeman (1959) and Smith et al. (1960), modified by the addition of the 'non-essential' amino acids marked '*'.

Subcultures were incubated and the medium changed as before, confluence being reached in 5-7 days after seeding. In order to minimize any possible effect of repeated subculture on phenotypic expression (see section 1.6), experiments were performed only on cells that were three subcultures removed from the primary culture.

4.2.3 Storage of Fibroblast Stocks

Cells selected for storage were obtained from confluent secondary cell cultures by trypsinization. After washing in fresh DMEM/10% (v/v) FCS, the cells were resuspended at a density of 1 x 10^6 cells/ml in DMEM/20% (v/v) FCS containing 10% (v/v) DMSO as a cryoprotectant. Aliquots (2ml) of cell suspension were placed in plastic ampoules, packed in a plastic box containing polystyrene crumbs to ensure a slow initial cooling rate, and stored in a -70°C freezer.

Rehabilitation of frozen cells was achieved by rapid thawing at 37°C for 10 minutes. The contents of one ampoule were then transferred to a 75cm^2 plastic culture flask, fresh DMEM/10% (v/v) FCS (5ml) added, and the flask incubated as described above. After 6 hours, the medium was changed to remove any remaining DMSO. Cultures were refed at 2-3 day intervals and subcultured by trypsinization.

4.2.4 Morphology and Electron Microscopy of the Cell Layer

Tertiary subcultures of ligamentum nuchae fibroblasts were seeded in 25cm^2 plastic culture flasks at an initial density of 1 x 10^6 cells/flask. Phase-contrast micrographs of cultures were taken two days after seeding and at confluence using an Olympus phase-contrast inverted microscope. Cultures were maintained as previously described, and divided into two groups on reaching confluency: the first group received DMEM/10% (v/v) FCS containing antibiotics only, whilst the second group was also supplemented with freshly-prepared ascorbic acid (50µg/ml). One flask from the unsupplemented group was processed for electron microscopy at 18, 30 and 65 days after confluency.

However, cultures supplemented with ascorbic acid could not be maintained in culture for lengthy periods, as after a short time (usually of 5-6 days) the cell layer began to retract from the sides of the flask and roll inwards to form clumps.

Cell layers were fixed and processed in situ by a modification of the method of Brinkley et al. (1967). Cell layers were rinsed three times with warm PBS-A, then fixed for one hour at room temperature with 2.5% (w/v) glutaraldehyde in 0.067M phosphate buffer (pH 7.3) containing 2mM $CaCl_2$. The fixed cell layer was washed, post-fixed with 1% (w/v) osmium tetroxide, dehydrated and embedded as summarized in Figure 4.1.

Embedding was performed with a 1:1 (w/w) mixture of Spurr and Epon 812 epoxy resins. Spurr's resin was prepared as described in section 2.2.3. The formulation of Epon 812 utilized was as follows:

DDSA	20.8g
MNA	4.6g
Epon 812	24.6g
DMP 30	0.5g

Meticulous attention was paid to careful resin mixing before use.

After polymerization, small pieces were cut from the flask with a jeweller's saw and stuck, edge-on, to wooden stubs with cyano-acrylate cement. This enabled examination of the full thickness of the cell layer. Blocks were trimmed and sectioned, mounted on rhodium-plated copper 300 mesh grids, stained and examined as described in section 2.2.3.

4.2.5 Detection of Glycoproteins Released into the Medium by Ligamentum Nuchae Fibroblasts in Culture

Tertiary subcultures of ligamentum nuchae fibroblasts were grown to confluency in $75cm^2$ plastic flasks as described in section 4.2.2. Confluent cultures were then incubated with medium containing radioactively-labelled precursor molecules in order to study newly-synthesized macromolecules released by the cells into the culture medium.

The basic incubation medium used in all experiments (hereinafter called 'maintenance medium') consisted of DMEM containing 0.2% (v/v) FCS and supplemented with

Glutaraldehyde-fixed cell layer rinsed X4 with 0.1M Na cacodylate buffer (pH 7.3) in situ

↓

Post-fixed with 1% (w/v) OsO_4 in Na cacodylate buffer, one hour

↓

70% ethanol, two changes, each 5 minutes

↓

90% ethanol, two changes, each 5 minutes

↓

100% ethanol, two changes of 5 minutes each, followed by two changes of 15 minutes each

↓

Infiltrated for 1 hour with 1:1 mixture of Spurr and Epon 812 epoxy resins

↓

Resin changed, enough fresh resin being added to cover culture layer to a depth of 2-3mm

↓

Holes burnt in tops of culture flasks, then incubated overnight at 70°C to polymerize resin

All manipulations were carried out at room temperature.

Figure 4.1

Embedding of Cell Culture Layers in situ for Electron Microscopic Examination

soluble insulin (0.4 units/ml), aprotinin (Trasylol; 100 KIU/ml), penicillin (50IU/ml) and streptomycin (50µg/ml). At confluency, the cell layer was washed free of growth medium with warm serum-free DMEM (10ml/flask). The culture flasks were then split into two groups and pre-incubated with maintenance medium (10ml) for 4 hours in order to remove excess FCS from the cell layer. Proline was omitted from the maintenance medium used in experiments in which cultures were incubated with radio-labelled proline.

Each group of flasks was subsequently incubated with radio-labelled precursors in maintenance medium for 24 hours at 37°C. One group was incubated with L-[6-^{3}H]-fucose (200µCi/flask), in order to label newly-synthesized glycoproteins and the other group with L-[5-^{3}H]-proline (100µCi/flask) to label predominantly collagenous molecules.

The labelled medium was then removed from the cell layer and proteinase inhibitors NEM (5mM), PMSF (5mM), benzamidine (5mM) and EDTA (10mM) added to the final concentrations indicated. The medium was cooled to 4°C, centrifuged for 10 minutes at 1200g to sediment any contaminating cells, and macromolecules were precipitated by the slow addition of solid $(NH_4)_2SO_4$ to give a solution of 80% saturation. After standing at 4°C for 18 hours, precipitated material was collected by centrifugation for 90 minutes at 30,000g in a Sorvall RC-2B refrigerated centrifuge and analyzed by PAGE. Precipitate was first dissolved in sample preparation buffer (SPB) containing 2M urea (see Table 2.2). Duplicate samples were applied to 6.5% cylindrical gels and electrophoresis was carried out under reducing conditions using the technique described in section 2.2.4. Localization of labelled macromolecules was determined by freezing the unstained gel (fixed with 7% (v/v) glacial acetic acid in distilled water) and slicing it with a parallel array of razor blades to provide slices 1.15mm thick (Choules and Zimm, 1965). Slices were air-dried in scintillation vials and digested in a 30% (v/v) solution of H_2O_2 containing 1% (v/v) NH_3 for 18 hours at 65°C (Goodman and Matzura, 1971). Scintillation mixture (10ml) comprising 2,5-diphenyloxazole (3.33g/l) dissolved in a 2:1 mixture of toluene and methyl cellosolve was added and radioactivity measured in a Beckman liquid scintillation counter, model LS 100.

The mobilities of labelled macromolecules were plotted and compared to those of concurrently electrophorezed protein standards as described in section 2.2.4. Type I collagen, prepared from bovine skin, was also electrophorezed for comparative purposes.

4.2.6 Immunoprecipitation Experiments with Anti-MAg IgG

Attempts were made to immunoprecipitate [^{3}H]-labelled macromolecules from spent ligamentum nuchae fibroblast culture medium using anti-MAg (ADS) IgG (prepared as described in section 2.2.9). The anti-MAg (ADS) IgG used in these experiments was shown by ELISA assay to have titres of 1/3200 against MAg, with undetectable non-specific activity.

Culture medium labelled with either [^{3}H]-fucose or [^{3}H]-proline was obtained from experiments as described in section 4.2.5. Medium (10ml) was concentrated 5-fold in an Amicon stirred ultrafiltration cell with a YM-10 membrane and a small amount of precipitate removed by centrifugation for 10 minutes at 18,000g in a Beckman Microfuge. The concentrated medium (2ml) was then divided into two equal portions. To one portion was added 90µl of anti-MAg IgG (concentration 11.4mg IgG/ml), and to the other 100µl of pre-immune rabbit IgG (concentration 9.8mg IgG/ml) as a control. Each sample was incubated for 2 hours at 37^{o}C and then for a further 15 hours at 4^{o}C to allow immunoprecipitation to occur.

Goat anti-(rabbit IgG) immunoglobulin, prepared as described in section 2.2.7, was then added to precipitate any unreacted rabbit IgG and soluble immune complexes. Preliminary experiments showed that a 4-fold excess of goat anti-(rabbit IgG) immunoglobulin was required to precipitate optimally rabbit IgG from solution. Therefore 400µl of the 10mg/ml solution of goat anti-(rabbit IgG) immunoglobulin was added to each sample, and incubation was continued for a further 3 hours at 37^{o}C, during which time a heavy flocculent precipitate was formed. Immunoprecipitate was collected by centrifugation for 10 minutes at 18,000g, carefully washed twice with 62mM Tris-HCl buffer, pH 6.8 (2ml aliquots) and dissolved in 750µl of SPB containing 2M urea. Aliquots (100µl)

of each sample were measured for radioactivity before analysis by PAGE as described in section 4.2.5.

4.3 RESULTS

4.3.1 Macroscopic Appearance of Bovine Foetal Ligamentum Nuchae Fibroblast Cultures

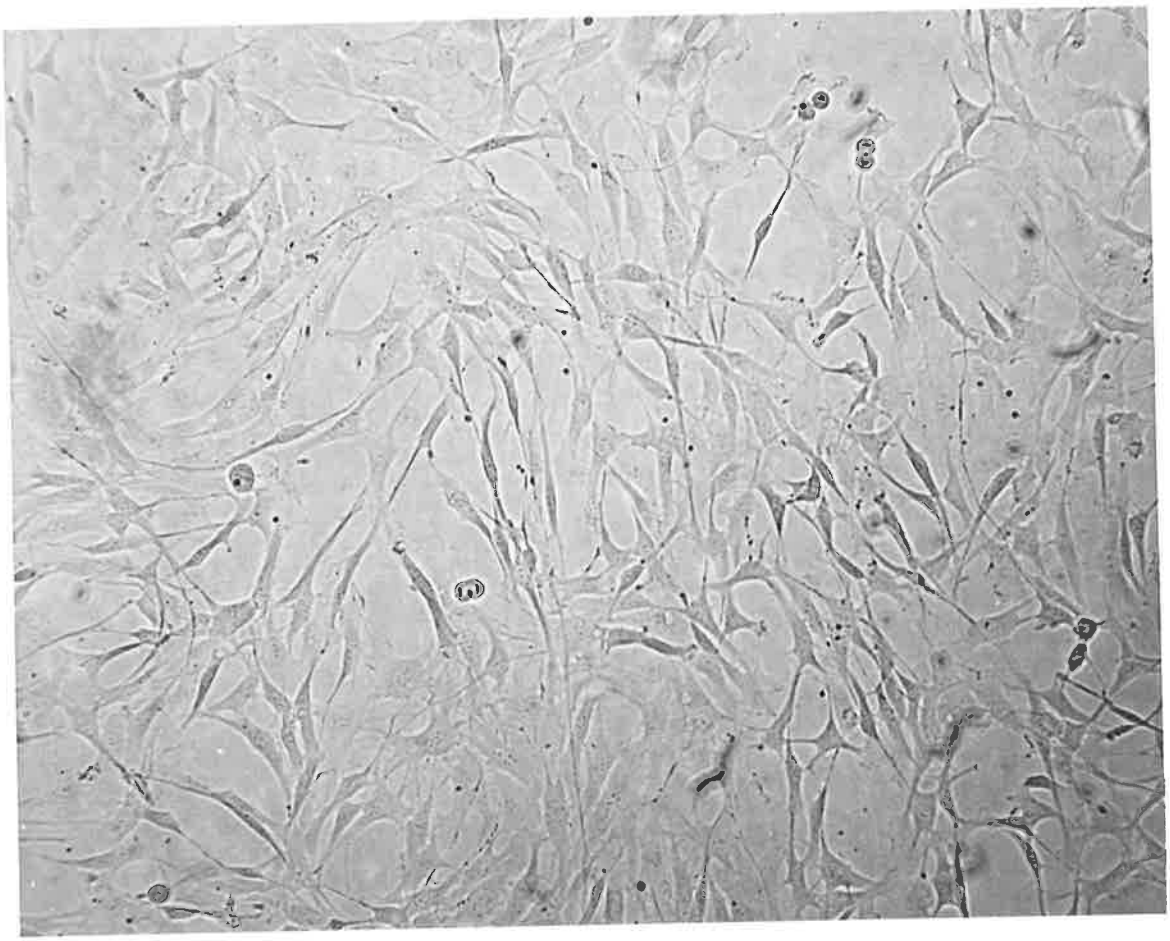
Before attaining confluence, all cultures were maintained with DMEM supplemented with 10% (v/v) FCS. Soon after seeding, cells exhibited a varied morphology, ranging from typical bipolar, elongated fibroblasts to polygonal cells with short processes (Figure 4.2). Very few detached or degenerate cells were seen; mitotic activity was frequent, and confluence was reached within 5-7 days after seeding a $75cm^2$ culture flask with 2×10^6 cells (Figure 4.3). As described in section 4.2.4, the cultures were then divided into two groups, one receiving DMEM/10% (v/v) FCS whilst the other was also supplemented with ascorbic acid (50µg/ml).

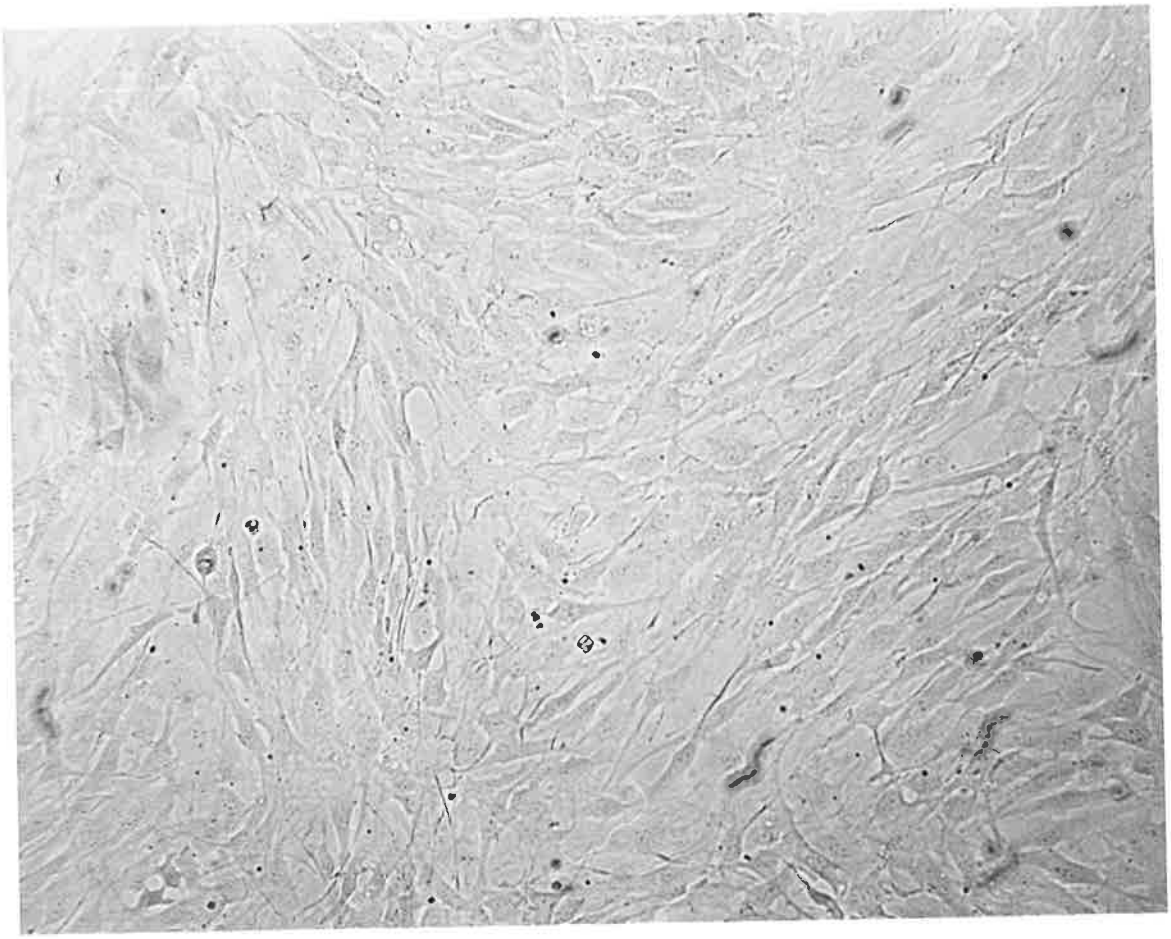
Unsupplemented cultures continued to grow as multi-layered cell sheets, maintaining a normal morphology and active metabolism (as indicated by a decrease in medium pH between changes) for very long periods of time.

It was only possible to maintain cultures supplemented with ascorbic acid for short periods of time, due to cell layer detachment and the subsequent formation of dense cell aggregates. Although centrifugal fibroblast outgrowth occurred from these cell clumps, the cells at the centre of such clumps rapidly became non-viable and necrotic. It was felt that little meaningful information could be obtained from study of these cell knots because of the unpredictable nature of such effects as relative nutrient deprivation to cells within the knots, the possible proteolysis of newly formed intercellular matrix by lysosomal enzymes from necrotic cells, and contamination of the cell layer and matrix with necrotic debris. Therefore study of cultures supplemented with ascorbic acid was abandoned.

Figure 4.2. Phase contrast micrograph of a foetal calf ligamentum nuchae fibroblast culture one day after seeding (second subculture)
Magnification X100.

Figure 4.3. Phase contrast micrograph of a foetal calf ligamentum nuchae fibroblast culture at confluence (second subculture)
Magnification X100.





4.3.2 Ultrastructural Studies of Unsupplemented Cultures

a. Cellular morphology

At 18 days post-confluence (Figure 4.4) the cell layer consisted of multiple layers of longitudinally-attenuated fibroblastic cells with elliptical nuclei containing dispersed chromatin. Rough endoplasmic reticulum (RER) distended with granular, amorphous material was especially prominent in cells situated deeply in the cell layer. Lamellar accumulations of Golgi saccules were readily identified and both coated and smooth vesicles were seen which seemed to arise from the Golgi complex. In some places these vesicles appeared to approach and merge with the plasma membrane, apparently discharging their contents (Figures 4.4 and 4.5) into the extracellular matrix.

Microtubules were seen to run through the cytoplasm in apposition to the plasma membrane, but were not a prominent feature. Dense bundles of microfilaments were frequently observed in the cytoplasm, being especially well developed close to the plasma membrane and most strikingly at the tips of cell processes (Figure 4.6). No pericellular basement membrane was found.

The appearance of cells 30 days after confluence corresponded closely to the above description, but the cytoplasm contained multiple aggregates of electron-dense glycogen granules and occasional lipid droplets (Figure 4.7). A few lysosomes were also seen, and the RER was less prominent. Some vacuolization of the cytoplasm was also evident, usually in superficial cells; the significance of this finding is uncertain, but is probably artefactual. Discharging vesicles were again noted.

By 65 days post-confluence the cell layer was much thicker than before (Figure 4.8). Although viable cells were still present on the upper surface of the culture, considerable cell breakdown had occurred in the deeper parts of the cell layer and cellular debris was observed scattered throughout the intercellular matrix. Intact cells showed extensive vacuolization with increased numbers of lysosomes. However RER was still present, and secretory activity appeared to be preserved.

b. Matrix morphology

Cultures examined 18 days after confluence showed a relatively sparse intercellular matrix (Figure 4.4), consisting primarily of randomly oriented bundles of microfibrils. These microfibrils were approximately 12nm in diameter and showed an indistinct periodicity when sectioned longitudinally (Figure 4.5). At higher power (Figure 4.9) cross-sectioned microfibrils appeared tubular with a central lucent core and electron-dense coat, corresponding closely to the typical appearance of elastin-associated microfibrils. In some areas where microfibrils were cut tangentially to the plane of the section, stain particles appeared to spiral across the surface of the cut fibre (Figure 4.9).

No other formed elements could be clearly identified, and collagen fibrils were not seen. However in some areas patches of amorphous granular, electron-dense material were seen in close apposition to the cell membrane, especially in areas of high intracellular microfilament density (Figure 4.6). This appearance is consistent with that described by Hedman et al. (1978) for fibronectin-containing structures in human skin fibroblast cultures, and may represent fibronectin deposition. Fibronectin-like material was also found by Jones et al. (1980) in their study of foetal calf ligamentum nuchae fibroblast cultures.

By 30 days post-confluence, large tracts of microfibrils were evident (Figure 4.7), often associated with patches of fibronectin-like material close to the cell membrane. However, despite the formation of quite dense arrays of microfibrils (Figure 4.10), careful searching did not reveal any deposition of elastin within these aggregates. In some sections, what appeared to be collagen fibrils were interspersed with microfibril aggregates.

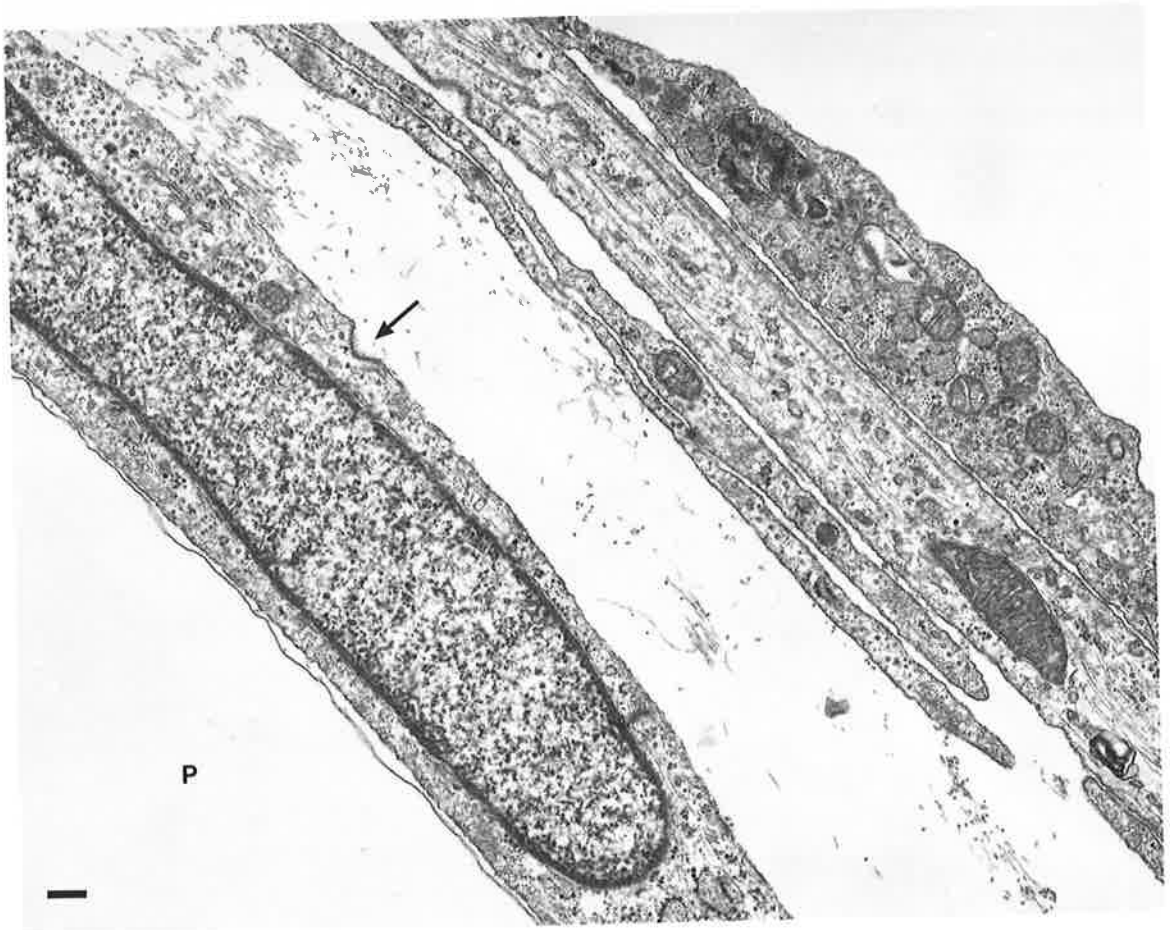
Large amounts of microfibrillar material were found in cultures observed 65 days after confluence, but at no stage was there any evidence of amorphous elastin deposition, or the formation of recognizable elastic fibres. In some fields (Figure 4.11), longitudinal bundles of microfibrils appeared to coalesce, but this is probably due to the thickness of the section. Collagen fibrils were found very infrequently, despite careful observation.

Figure 4.4. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 18 days after confluence

Longitudinal section through the cell layer showing general features. Note the very sparse extracellular matrix. A coated vesicle (↑) which has merged with the cell membrane can be seen. The plastic culture suface (P) is at the left of the micrograph. X25,000. Scale = 200nm.

Figure 4.5. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 18 days after confluence

Detail of a cell and adjacent matrix. Note the predominance of microfibrils in the matrix, some of which show indistinct periodicity in longitudinal section (↑). Several pino/exocytic vesicles can be seen (bottom left). The plastic culture (P) surface is at the top left of the micrograph. X65,000. Scale = 200nm.


P

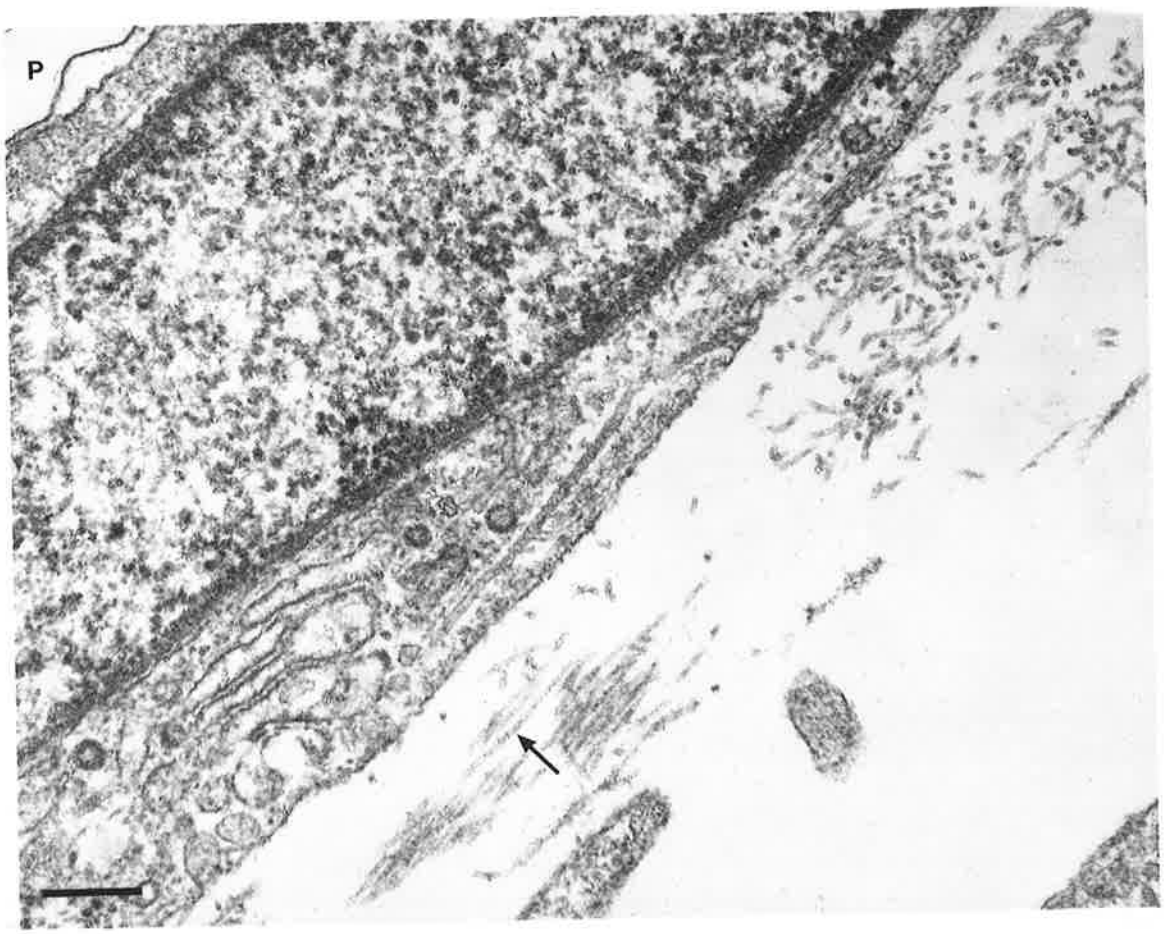
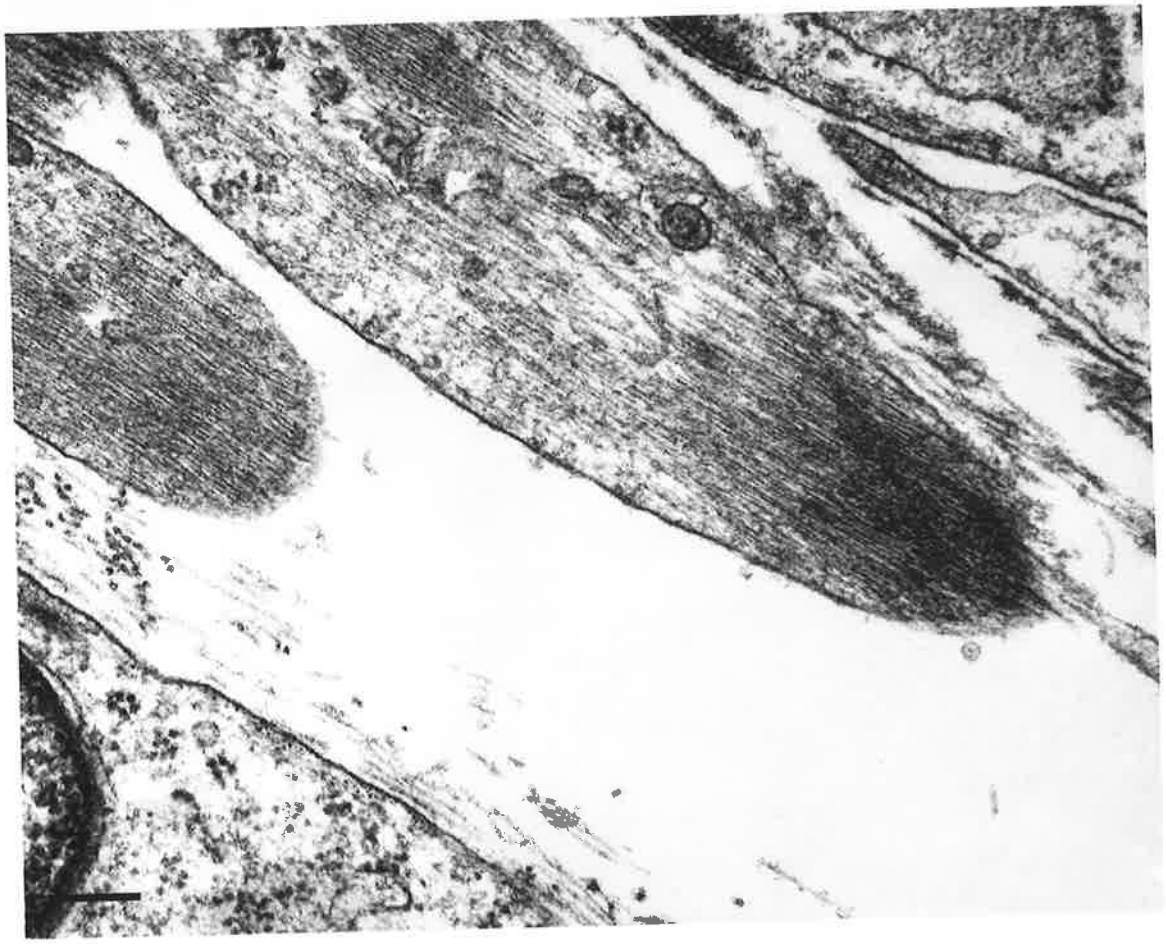

P

Figure 4.6. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 18 days after confluence

Note the dense accumulations of microfilaments at the tips of cell processes, associated with strands of granular, amorphous extracellular material which may represent fibronectin. X60,000. Scale = 200nm.

Figure 4.7. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 30 days after confluence

Note the lipid droplet (L) in a cell which appears to be vacuolated. Aggregates of microfibrils are present in the extracellular matrix. The plastic culture surface is toward the top right of the micrograph. X40,000. Scale = 200nm.



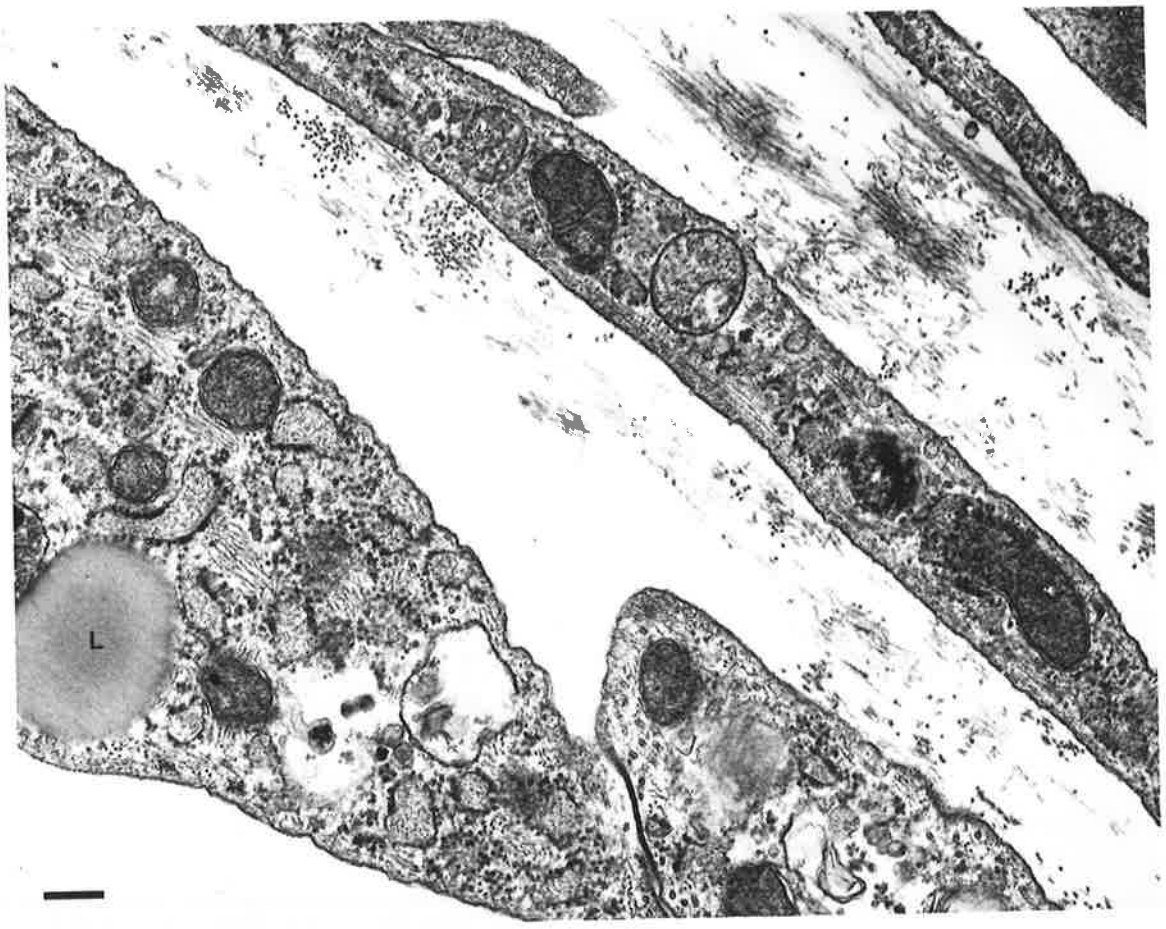
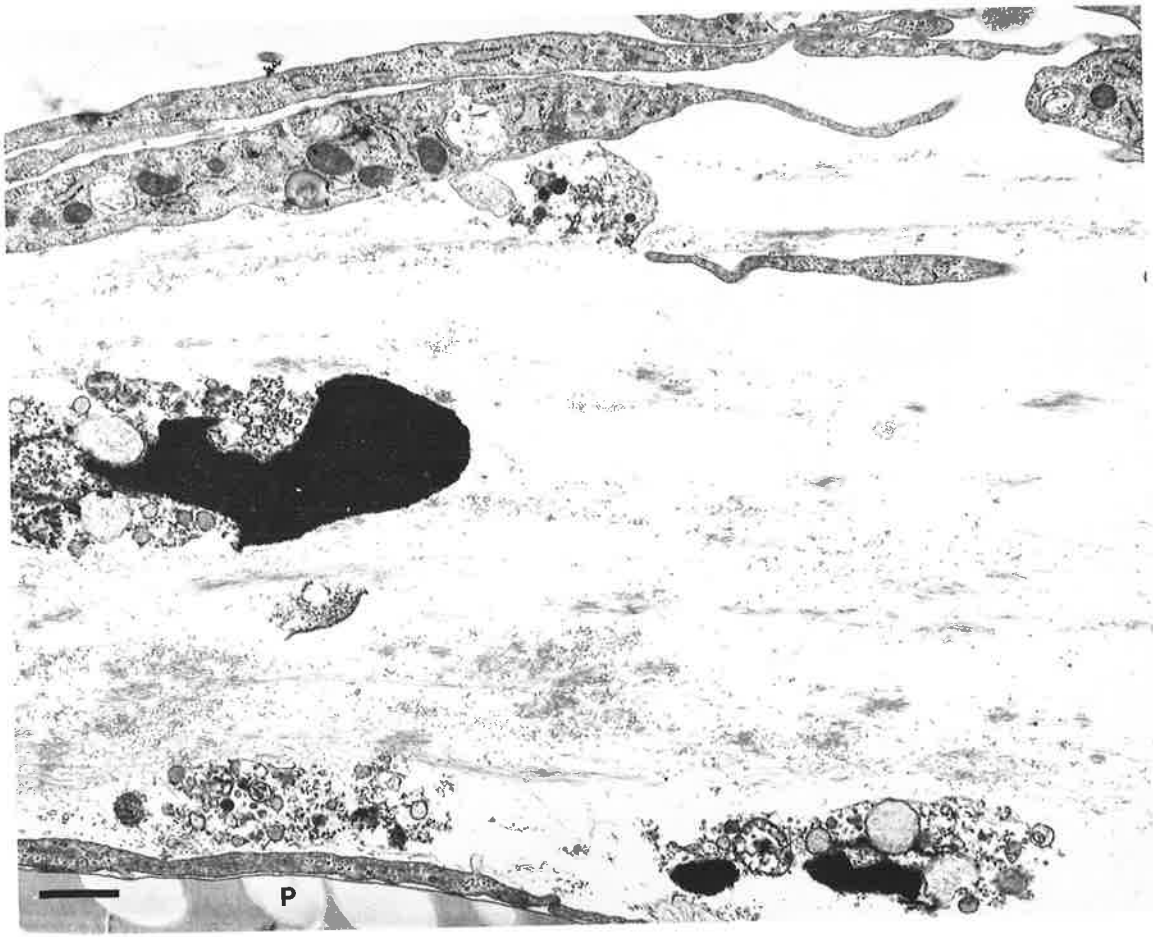

L

Figure 4.8. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 65 days after confluence

The cell layer appears quite thick and contains several degenerating cells. Superficial cells appear to be viable. The extracellular matrix is composed almost entirely of tracts of microfibrils, but no amorphous elastin is present. The plastic culture surface (**P**) is at the bottom of the micrograph. X10,000. Scale = 1μm.

Figure 4.9. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 18 days after confluence

High-power view to demonstrate tubular appearance of microfibrils in cross-section (↟). Stain particles appear to 'spiral' across the surface of a tangentially-sectioned microfibril (↟↟). X95,000. Scale = 100nm.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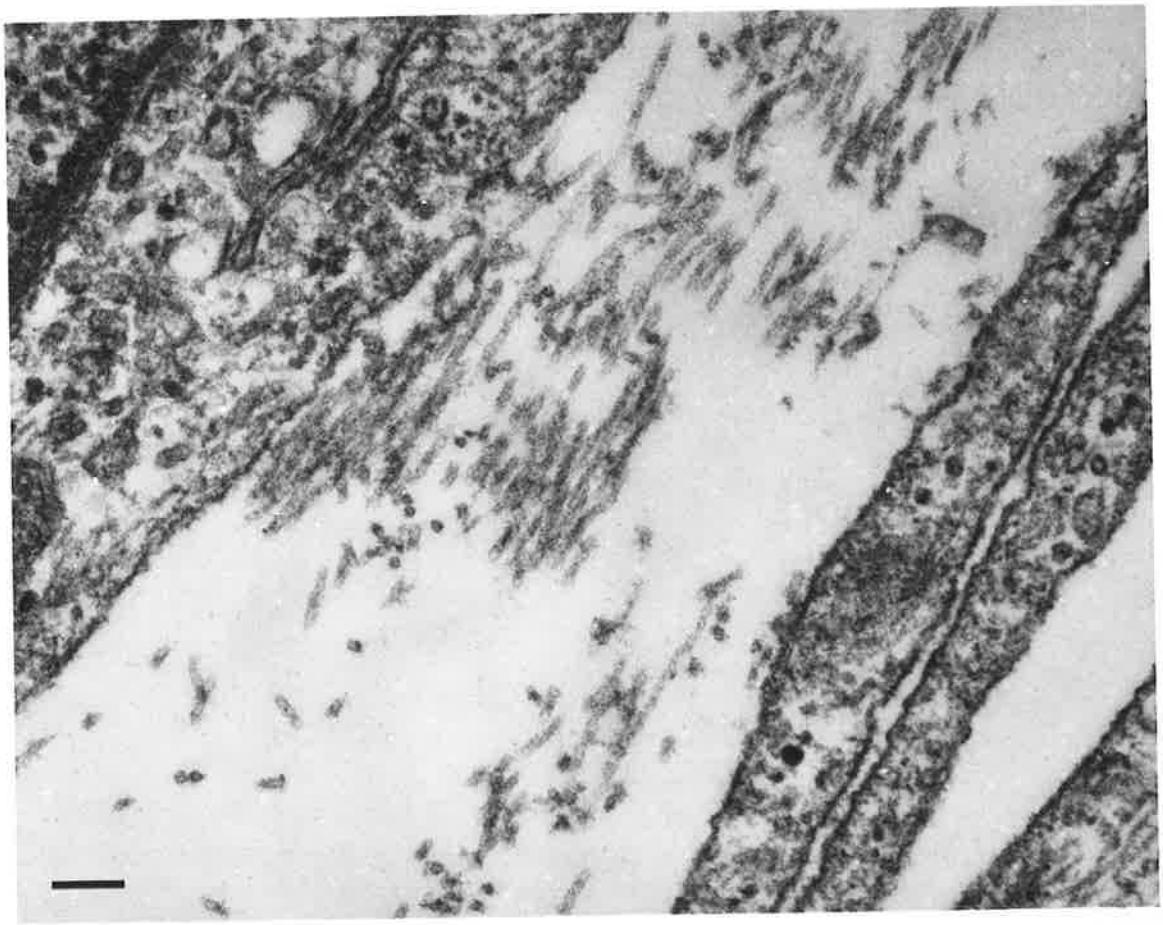
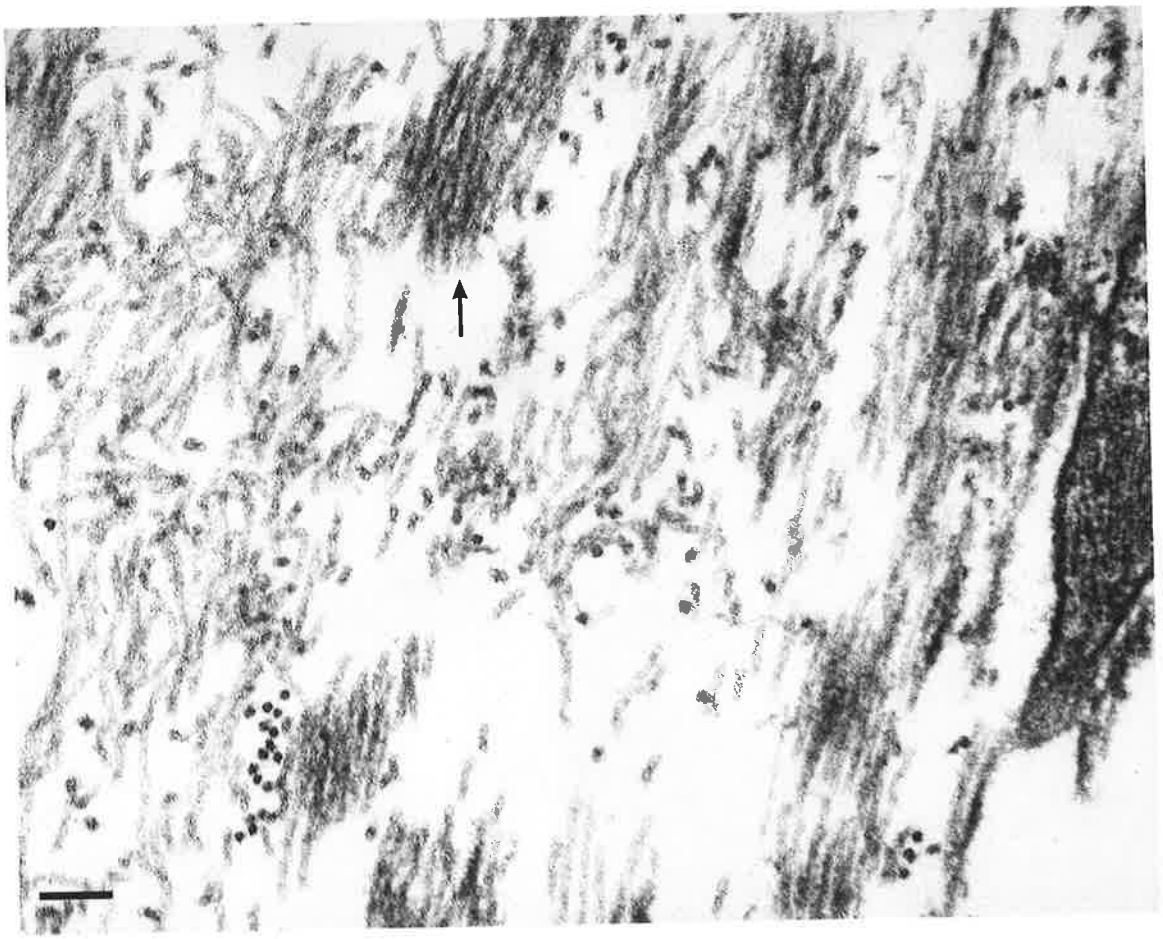


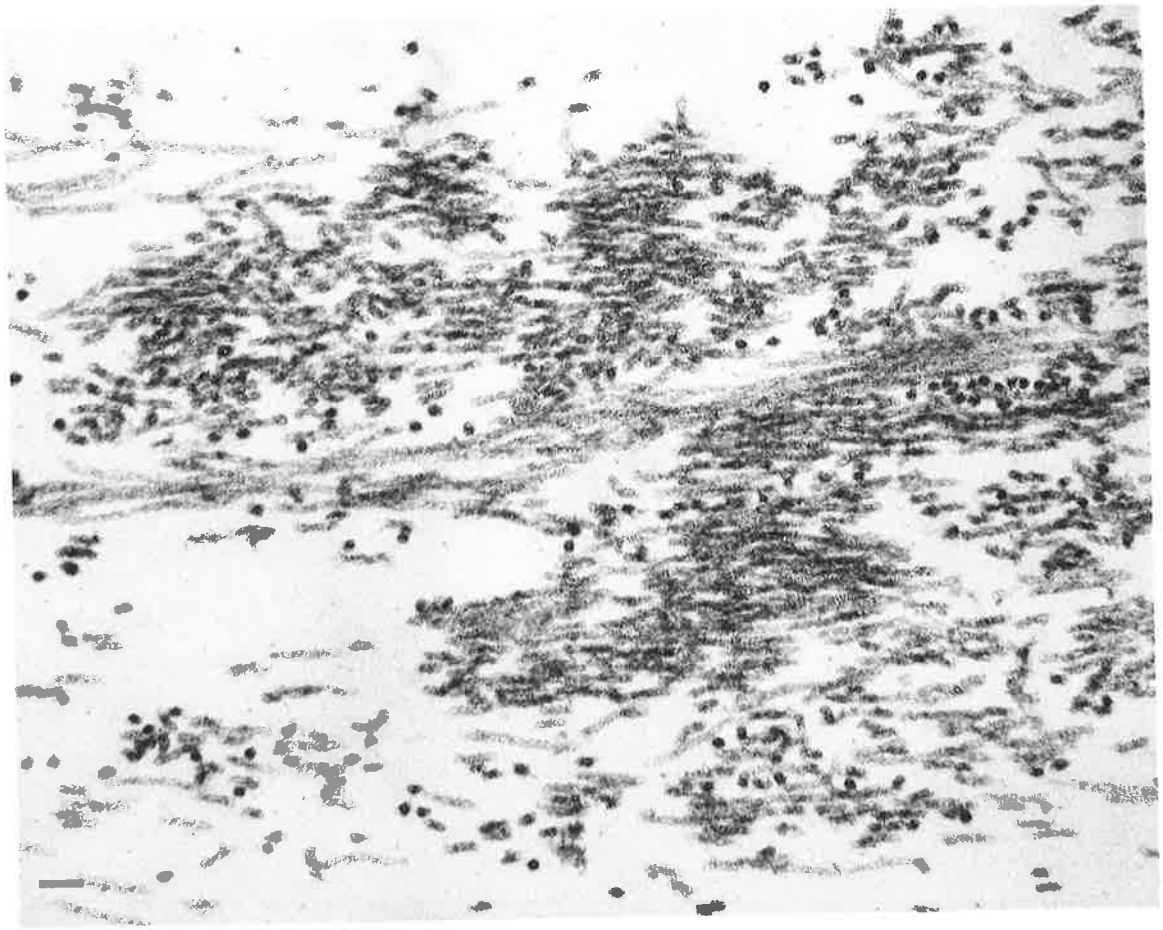
Figure 4.10. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 30 days after confluence

High-power view to demonstrate dense aggregates of microfibrils in the extracellular matrix. Apparent coalescence of microfibrillar bundles (↑) is probably artefactual due to section thickness. X95,000. Scale = 100nm.

Figure 4.11. Transmission electron micrograph of a foetal calf ligamentum nuchae fibroblast culture at 65 days after confluence

Similar view to that in Figure 4.10. Note the absence of amorphous elastin within microfibrillar bundles. X55,000. Scale = 100nm.





4.3.3 Glycoprotein Biosynthesis by Foetal Calf Ligamentum Nuchae Fibroblasts in Culture

Newly-synthesized macromolecules released into the medium by foetal calf ligamentum nuchae fibroblasts were labelled with either [^{3}H]-proline or [^{3}H]-fucose, and precipitated with 80% saturated $(NH_4)_2SO_4$ as described in section 4.2.5. The resulting material was analyzed by SDS-PAGE and labelled macromolecules localized by gel slicing, followed by assay for radioactivity.

Figures 4.12 and 4.13 show the electrophoretic patterns derived from [^{3}H]-proline and [^{3}H]-fucose labelled medium respectively. Seven major [^{3}H]-proline labelled species (designated LP1-LP7) and five [^{3}H]-fucose labelled species (designated LF1-LF4) can be distinguished; their apparent molecular weights are given in Table 4.3.

Band LP1 (apparent molecular weight 225,000) co-migrated with the [^{3}H]-fucose labelled species LF1 and with authentic human plasma fibronectin chains. Sear et al. (1981a) have shown that ligamentum nuchae fibroblasts secrete appreciable quantities of fibronectin in culture, which can be labelled with both fucose and proline. These findings suggest that species LP1/LF1 are fibronectin monomers.

Species LP2 and LP3 (apparent molecular weights 180,000 and 160,000 respectively) were by far the predominant [^{3}H]-proline labelled macromolecules found in the medium. Fibroblast cultures are known to secrete procollagen molecules into the medium (Prockop et al., 1979; Sear et al., 1981a), and the size and high [^{3}H]-proline activity of LP2 and LP3 would therefore suggest that they represent type I collagen pro-α1 and pro-α2 chains. In addition, species LP4 and LP5 co-migrated with authentic α1 and α2 chains from bovine skin type I collagen, having apparent molecular weights of 135,000 and 120,000 respectively (Freytag et al., 1979). No [^{3}H]-fucose labelled macromolecules corresponding in size to LP2-LP5 were detected, an observation consistent with species LP2-LP5 being type I collagen chains (Bornstein and Sage, 1980).

Bovine ligamentum nuchae fibroblasts readily elaborate soluble elastin in culture (Mecham et al., 1981) and it is possible that the [^{3}H]-proline labelled species LP6 (apparent molecular weight approximately 75,000 represents newly synthesized

tropoelastin. However, there are two closely spaced [^{3}H]-fucose labelled species, LF3a and LF3b (apparent molecular weights 75,000 and 70,000 respectively), which correspond approximately in size to LP6; as tropoelastin has been shown to be non-glycosylated (Grant et al., 1971), this finding casts some doubt on the true nature of species LP6. Further experiments (perhaps using anti-tropoelastin antibodies) are necessary in order to clarify this point.

LF2 was the only [^{3}H]-fucose labelled macromolecule for which no corresponding [^{3}H]-proline labelled polypeptide was identified. This species had an apparent molecular weight of about 155,000, electrophorezed as a sharp peak, and had the highest [^{3}H]-fucose activity of any found in the medium. Although it is similar in size to the 'MFPI' glycoprotein described by Sear et al. (1981a), the absence of an analogous [^{3}H]-proline labelled species suggests that LF2 is not related to this protein. In order to clarify this question, immunoprecipitation experiments on samples of labelled medium were undertaken using anti-MAg (ADS) IgG. These experiments are described below in section 4.3.4.

Species LP7 and LF4 are very similar in size (about 45,000 daltons), migrating slightly slower than ovalbumin. The nature of these species is uncertain; whilst they may represent a single medium glycoprotein, it is also possible that limited proteolysis of large macromolecules in the medium is responsible for their appearance. However, as is the case with the other tentatively-identified species discussed above, further fractionation and analysis is necessary before any certainty is possible.

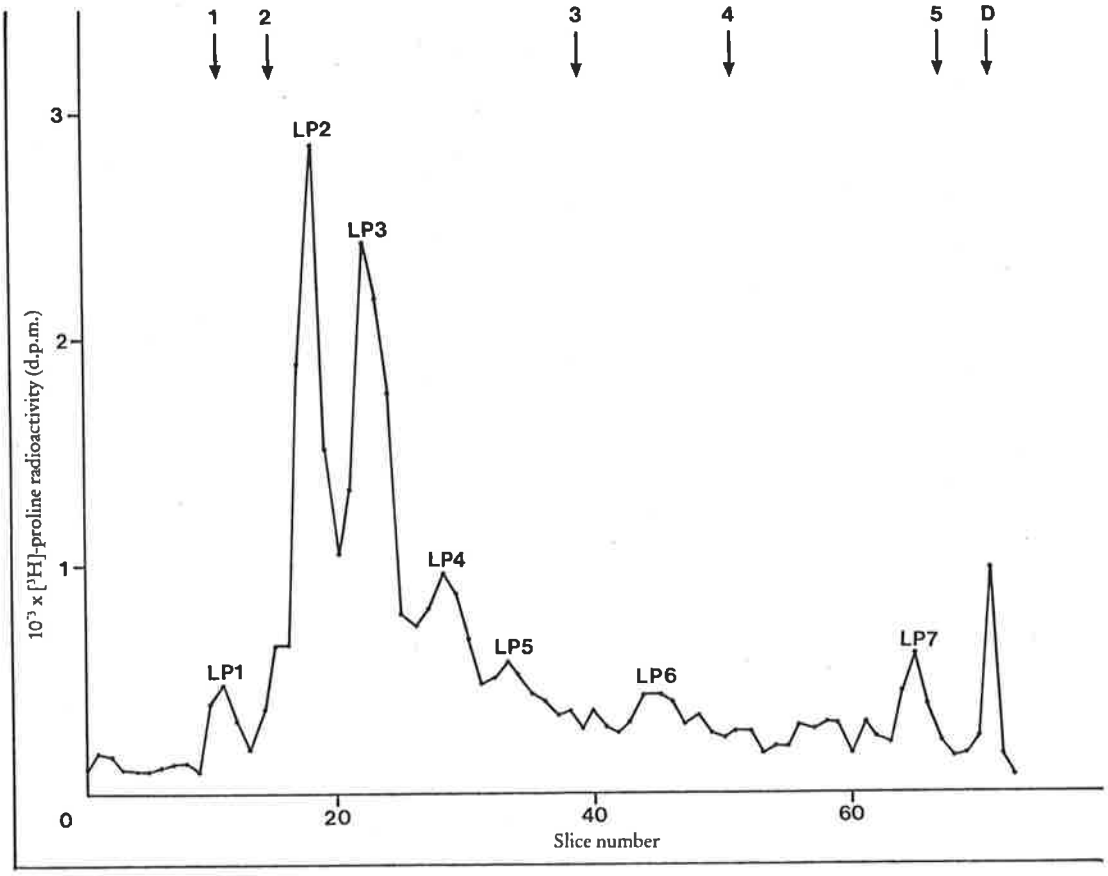
In conclusion, it should be noted that only small amounts of radioactive precursor were incorporated into newly-synthesized macromolecules as judged by the measured radioactivity. This is particularly so in the case of [^{3}H]-fucose (Figure 4.13). The [^{3}H]-labelled precursors were checked for activity by measuring 5µl of each stock solution in a scintillation counter. In both cases adequate activity was present, and both had been stored for less than 18 months. It is possible that the conditions under which labelling was carried out inhibited synthetic activity in some way, but time and available materials did not allow a full investigation of the effects of different media on the

Figure 4.12. SDS-polyacrylamide gel electrophoresis of [^{3}H]-proline labelled macromolecules secreted into the medium by cultures of foetal calf ligamentum nuchae fibroblasts

Proteinaceous macromolecules were precipitated with 80% saturated $(NH_4)_2SO_4$ before analysis on 6.5% cylinder gels. The gels were sliced and assayed for radioactivity as described in section 4.2.5. The positions of co-electrophorezed standards are indicated: **1**, rabbit skeletal muscle myosin; **2**, human plasma fibronectin; **3**, phosphorylase a; **4**, bovine serum albumin; **5**, ovalbumin. **D** indicates the position of the bromophenol blue tracking dye. [^{3}H]-proline labelled macromolecules of interest are shown as **LP1-LP7.** The apparent molecular weights of these species are recorded in Table 4.3.

Figure 4.13. SDS-polyacrylamide gel electrophoresis of [^{3}H]-fucose labelled macromolecules secreted into the medium by cultures of foetal calf ligamentum nuchae fibroblasts.

The technique used is identical to that in Figure 4.12. Protein standards and tracking dye are indicated as above. [^{3}H]-fucose labelled macromolecules of interest are shown as **LF1-LF4.** The apparent molecular weights of these species are recorded in Table 4.3.


1
2
3
4
5
D
LP1
LP2
LP3
LP4
LP5
LP6
LP7
10^{-3} x [^{3}H]-proline radioactivity (d.p.m.)
Slice number

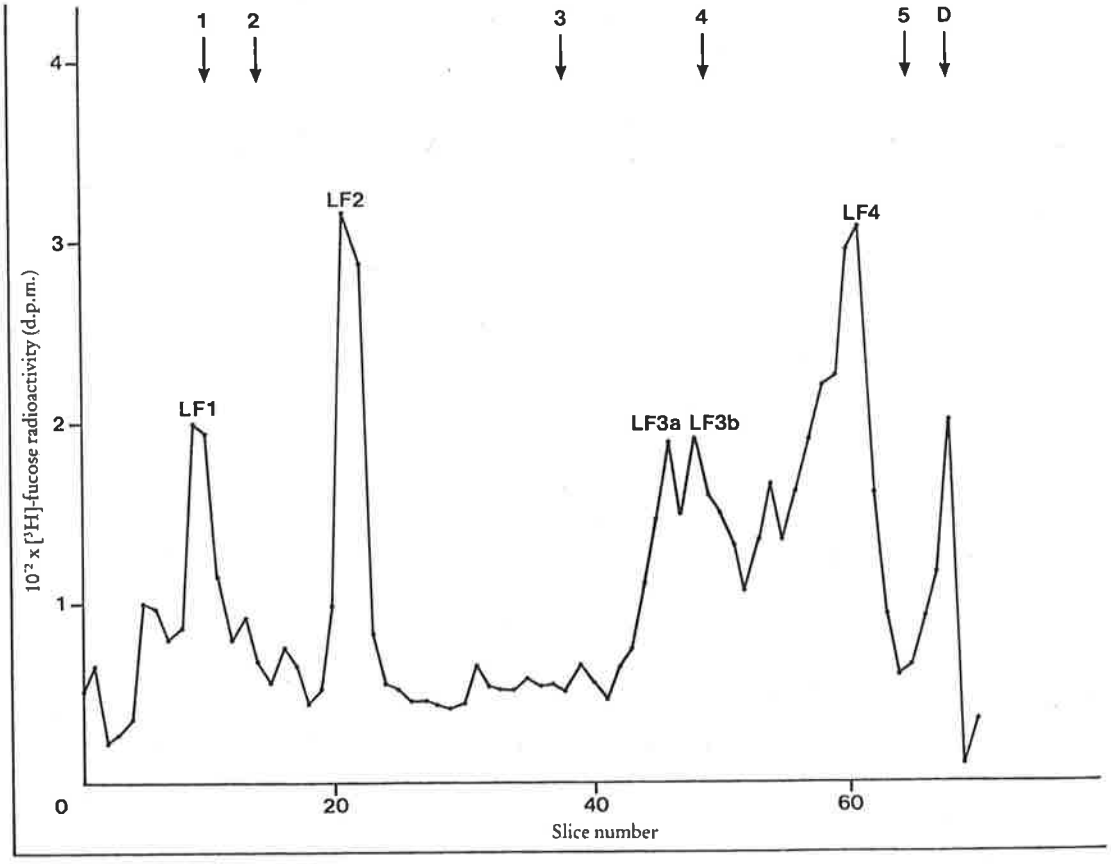

1
2
3
4
5
D
LF1
LF2
LF3a
LF3b
LF4
10^{-2} x [^{3}H]-fucose radioactivity (d.p.m.)
Slice number

Table 4.3

Apparent Molecular Weights of the Major Newly-Synthesized Macromolecules Detected in the Medium of Foetal Calf Ligamentum Nuchae Fibroblast Cultures

[^{3}H]-proline labelled species		[^{3}H]-fucose labelled species	
(Apparent molecular weights x 10^{-3} daltons)			
LP1	225	LF1	225
LP2	180		
LP3	160		
		LF2	155
LP4	135		
LP5	120		
LP6	75	LF3a	75
		LF3b	70
LP7	45	LF4	45

Molecular weight estimates were made by comparisons of mobility with those of co-electrophoresed protein standards (based on the method of Weber and Osborne, 1969).

biosynthetic capabilities of cultured cells. This aspect is discussed further in section 4.4.

4.3.4 Immunoprecipitation Experiments

Immunoprecipitation experiments were performed in an effort to detect the presence of macromolecules immunochemically related to MAg in the labelled medium from ligamentum nuchae fibroblast cultures. The method utilized is described in section 4.2.6 and incorporated several measures intended to maximize the probability of detecting such macromolecules. These included:

i) Use of high titre, highly-specific anti-MAg (ADS) IgG, shown by immunodiffusion to contain precipitating antibody.

ii) A second antibody precipitation step to ensure precipitation of any soluble antigen-antibody complexes.

iii) Preliminary experiments were performed to ensure that maximum precipitation at equivalence was achieved with the second precipitating antiserum.

However, despite repeated attempts, no significant quantity of labelled material could be precipitated. On each occasion the amount of radioactivity measured in a 100µl aliquot of dissolved precipitate (750µl total volume) was only minimally above background. PAGE was performed using large sample loads (300µl) but gel-slicing followed by assay for radioactivity (as described in section 4.2.4) confirmed the absence of labelled species.

This result is surprising in view of the findings of Sear et al. (1981a,b), who used a similar system; possible reasons for such a discrepancy are considered in section 4.4.

4.4 DISCUSSION

Explant cultures of foetal calf ligamentum nuchae fibroblasts were readily established. Cellular morphology and culture characteristics were similar to those described by other workers (Sear et al., 1978; Jones et al., 1980; Lamberg et al., 1980; Mecham et al., 1981).

It was found that fibroblasts grown under scorbutic conditions could be maintained in culture as flat, multi-layered cell membranes for very long periods of time. However, ascorbate supplementation of the culture medium rapidly resulted in detachment and retraction of the culture layer to form a dense cell aggregate in the flask. Formation of similar 'cell knots' has previously been noted in ligamentum nuchae fibroblast cultures grown under both scorbutic (Lamberg et al., 1980) and ascorbate-replete conditions (Jones et al., 1980). The latter authors further observed that ascorbate supplementation hastened the appearance of such cell aggregates, suggesting that cell layer retraction might be due to the formation of a predominantly collagenous extracellular matrix. Abraham et al. (1977) have reported a similar phenomenon in cultures of newborn pig aorta smooth muscle cells.

4.4.1 Ultrastructural Studies

Due to the problems encountered in maintaining ascorbate-supplemented cultures long-term, ultrastructural observations of culture morphology were restricted to scorbutic cultures. Foetal calf ligamentum nuchae cells have been reported to become rapidly senescent in culture (Mecham et al., 1981), and for this reason only tertiary subcultures were employed in this study. Gestational age also appears to be an important determinant of the capacity of foetal calf ligamentum nuchae fibroblasts to produce elastin. Elastin synthesis in bovine foetal nuchal ligament has been shown to become maximal in the third trimester, both in vivo (Cleary et al., 1967) and in vitro (Mecham et al., 1981; 1984). Foetal calves of 200-220 days gestation were therefore selected in order to maximize cellular potential for elastogenesis. Cultures obtained from several different foetuses of identical gestational age exhibited qualitatively similar characteristics.

The ultrastructure of cultured fibroblasts corresponded closely to that described by other authors both in intact ligamentum nuchae (Fahrenbach et al., 1966; Greenlee et al., 1966; Kewley et al., 1978) and in cell culture (Jones et al., 1980; Lamberg et al., 1980). Degenerative changes appeared in some cells after prolonged periods in culture, but a typical fibroblastic morphology was maintained throughout the observation period.

Large numbers of microfibrils (which were morphologically identical to elastin-associated microfibrils) accumulated in the extracellular matrix of fibroblast cultures, but amorphous elastin deposition was not observed at any time up to 65 days post-confluence. Sear et al. (1978) and Lamberg et al. (1980) have reported similar findings in their studies of cultured ligamentum nuchae fibroblasts. However Jones et al. (1980) have shown that if cultures which had formed 'cell knots' were maintained in this state for some time, typical elastin fibres would be formed at the periphery of such aggregates. No elastic fibre formation was observed in cultures which had not retracted to form 'cell knots'.

It is of interest to note that Mecham and his co-workers (Mecham 1981b; Mecham et al., 1981; 1984) have shown that ligamentum nuchae fibroblasts maintained under similar matrix-free conditions will synthesize tropoelastin, but not cross-linked elastin. The presence of extracellular matrix, either as a 'killed' ligament substrate or in organ cultures of ligament minces, was found to induce biosynthesis of cross-linked elastin. It is therefore possible that the presence of 'cell knots' in the study of Jones et al. (1980) acted as an inducer of insoluble elastin deposition.

Synthesis of insoluble collagen fibrils appeared to be almost completely suppressed, and no other formed elements could be identified in the matrix, apart from small patches of fibronectin-like material. Ascorbate deficiency has previously been shown to inhibit collagen fibrillogenesis in vitro by rat smooth muscle cells (De Clerck and Jones, 1980) and foetal calf ligament fibroblasts (Jones et al., 1980).

4.4.2 Glycoprotein Biosynthesis

Glycoprotein biosynthesis by ligamentum nuchae fibroblasts in culture was investigated using [^{3}H]-fucose and [^{3}H]-proline to label newly-synthesized macromolecules. Fucose is a useful glycoconjugate label as it is found in most glycoproteins, is not a component of hyaluronic acid or proteoglycans (except for keratan sulfate) and is not metabolized or interconverted by fibroblasts in vitro (Sear et al., 1975). Proline is predominantly incorporated into collagenous molecules, but is present in significant quantities in most proteins, rendering it a useful general protein label. Use of these compounds also allows direct comparison of the present study with the earlier work of Sear et al. (1978; 1981a,b).

Serum depleted 'maintenance medium' was used in labelling experiments in order to minimize difficulties with the characterization of newly-synthesized macromolecules due to the presence of large quantities of serum proteins in the medium to be studied. In addition, this obviated the possibility of 'non-enzymic tight binding' of radioactive precursor molecules to serum proteins (Herrman, 1974) which may give rise to labelled species not synthesized by the cells being studied. However, it has been noted that the use of media containing low concentrations of FCS (less than 5% v/v) may cause a decrease in cell stability (Sear et al., 1975; 1977) and in total protein synthesis (Mecham et al., 1981).

Sear and his co-workers (Sear et al., 1978; 1981a,b) found that foetal calf ligament fibroblasts could be successfully maintained in serum-free Waymouth's medium MAB 87/3 (Gorham and Waymouth, 1965). One of the major differences between DMEM and Waymouth's medium MAB 87/3 is the presence of insulin as a constituent of the latter medium and it has been shown that insulin may partially compensate for the effects of lowered concentrations of serum on cultured smooth muscle cells (Stout et al., 1975). As Waymouth's medium was not readily available in Australia at the time the studies in this thesis were performed, a series of preliminary experiments were undertaken to establish suitable conditions for the labelling of confluent cultures. It was found that supplementation of DMEM with low concentrations of beef insulin (0.4 units/ml) allowed

the use of FCS concentrations as low as 0.2% (v/v) without ill effect on cell stability over a 24 hour incubation period. In the absence of insulin, use of DMEM containing less than 3% (v/v) FCS led to culture instability, as judged by cell detachment and loss of normal fibroblast morphology. The presence of the proteinase inhibitor, aprotinin (Trasylol), at a concentration of 100IU/ml was without discernible effect.

As noted in section 4.3.3, overall incorporation of radio-labelled precursors into newly-synthesized macromolecules was quite low, especially with regard to [^{3}H]-fucose. As fucose usually occurs in glycoconjugates as the terminal unit of oligosaccharide side-chains, only relatively small amounts may be incorporated into macromolecules when compared to 'core' sugars such as mannose (reviewed by Gottschalk, 1972). It is also possible that, despite maintenance of normal morphology, the 'maintenance medium' used for labelling was unable to sustain normal synthetic activity in cultured fibroblasts. Further comparative experiments, utilizing Waymouth's medium and [^{3}H]-labelled mannose, may be of use in elucidating this problem.

A large number of newly-synthesized macromolecules was shown to be released into the medium by cultured foetal calf ligament fibroblasts. As noted above, aprotinin was added during all labelling steps, and enzyme inhibitors were added to the labelled medium immediately after collection. The presence of molecular species corresponding in electrophoretic mobility to native fibronectin and tropoelastin, which are extremely proteinase-sensitive molecules, implies that the newly-synthesized species detected in the culture medium were not the result of non-specific proteolysis of secreted macromolecules. Highly [^{3}H]-proline labelled molecules corresponding in size to type I collagen proα_1, proα_2, α_1, and α_2-chains were also prominent (Figure 4.12).

Although a [^{3}H]-fucose labelled species of apparent molecular weight 150,000 was present, no corresponding [^{3}H]-proline-labelled band could be found and it is uncertain if this species is related to the 'MFPI' glycoprotein isolated from ligament fibroblast culture medium by Sear et al. (1978, 1981a,b). Repeated attempts were made to precipitate immunologically cross-reacting material from labelled culture medium using anti-MAg antibodies (see section 4.3.4), but no labelled species was found. This may in

part be due to differences in specificity between the Kewley anti-'microfibrillar protein' antibody (Kewley et al., 1977a,b) used by Sear and his co-workers in their experiments and the anti-(MAg) antibody used in the present study. It was noted in Chapter Three that sufficient similarity exists between MFPI (Sear et al., 1981b) and CL glycoprotein (Gibson and Cleary, 1982) to imply that they may, in fact, be related proteins and that some of the antibody activity of the Kewley preparation might therefore be directed toward CL glycoprotein. Comparative studies utilizing both anti-'microfibrillar protein' and anti-(CL glycoprotein) antibodies would be necessary to confirm such a relationship. Alternatively, microfibrillar components may be insolubilized very rapidly in the extracellular matrix, so that little or no soluble antigenically-active material appears in the culture medium.

The rapid appearance of large numbers of microfibrils in the matrix elaborated by long-term cultures of ligament fibroblasts suggests that extraction of cell-free matrix preparations might yield microfibrillar components which could be isolated utilizing anti-MAg antibody. A similar approach has been employed by Sear et al. (1978) in their studies of the synthetic capabilities of cultured ligamentum nuchae fibroblasts.

4.4.3 Conclusions

The studies outlined in this chapter have confirmed that, under conventional culture conditions, foetal calf ligamentum nuchae fibroblasts will elaborate ultrastructurally-typical microfibrils, but do not appear to form amorphous elastin, even after prolonged periods in culture. Although large numbers of microfibrils were observed, the extreme diversity of tissues in which morphologically-indistinguishable microfibrils have been described (see section 1.3.5) emphasizes the need for caution in interpreting these structures as precursors of elastic fibre formation. The presence of true elastic fibres (possessing both microfibrils and an amorphous core) is essential if such microfibrils are to be confidently assessed as being 'elastin-associated'.

Because of these difficulties, it was decided not to persist with studies on ligament fibroblasts, but rather to seek a culture system which produces amorphous elastin more

readily, and which would therefore be more suitable for ultrastructural and immunohistochemical studies. Auricular cartilage chondrocytes have been shown to produce elastin readily in culture (Moskalewski, 1976; Thyberg and Hinek, 1977; Starcher and Mecham, 1981), and are technically straightforward to propagate and maintain. Studies on elastogenesis in rabbit and foetal calf ear cartilage were therefore undertaken. These experiments are described in the following chapter.

CHAPTER FIVE

STUDIES OF ELASTOGENESIS IN AURICULAR CARTILAGE

5.1 INTRODUCTION

Although it has been suggested that cartilage elastin may be a different genetic type (Keith et al., 1977b; 1979), more recent evidence tends to favour biochemical and immunological homology between elastins of cartilage and vascular origin (Foster et al., 1980a; Heeger and Rosenbloom, 1980). This evidence was discussed in detail in section 1.7.2. However, there has been speculation that elastin fibrillogenesis in rabbit ear cartilage may proceed without involvement of microfibrils (Quintarelli et al., 1979), an hypothesis which is at variance with the conventional view of elastic fibre formation (see section 1.3.2).

For these reasons a study of elastogenesis in auricular cartilage, both in vivo and in vitro, seemed a logical and potentially rewarding avenue for investigation. As the immunochemical studies detailed in Chapters Two and Three were carried out on foetal calf tissues, the use of foetal calf ear cartilage from animals of similar gestational age was considered desirable. As there were no previous ultrastructural studies of developing bovine ear cartilage in the literature it was necessary to define the normal electron microscopic anatomy of this tissue. Neonatal and young rabbit ear cartilages were included for comparative purposes, having been previously described in detail by several other authors (see section 1.5.1).

The main objectives, and the experimental approach to them, were as follows:

1. To examine the ultrastructural appearance of elastin in native ear cartilage at high magnification in order to demonstrate the presence or absence of elastin-associated microfibrils.
2. To describe the histogenesis of elastin by auricular chondrocytes in tissue culture. In order to promote the most favourable conditions for elastin synthesis, high-density primary cultures of enzymically-dissociated chondrocytes, maintained under scorbutic conditions, were used (see section 1.5.3). Cultures were examined electron microscopically at several time points after seeding. The effect of ascorbic acid supplementation on the ultrastructural characteristics of culture matrix was also studied.

3. To determine the cross-reactivity of ear cartilage elastin-associated microfibrils with the anti-MAg (ADS) antibody preparation described in Chapter Two. An immunofluorescent technique was used, on both intact cartilage and chondrocyte cultures.

It was shown that both foetal calf and neonatal (2-day-old) rabbit ear cartilage contained recognizable elastin-associated microfibrils, but such structures could not be demonstrated in young (16-day-old) rabbit auricular cartilage. In addition, although chondrocyte cultures obtained from all three tissues possessed the ability to synthesize amorphous elastin, typical microfibrils could only be found in the cell layer of cultures derived from foetal calf and neonatal rabbit auricular cartilage.

Immunofluorescent experiments performed on foetal calf chondrocyte cultures demonstrated a fibrous network of material cross-reacting with anti-MAg antibodies within the culture matrix, further supporting the electron microscopic observations.

5.2 MATERIALS AND METHODS

5.2.1 Materials

All of the materials and reagents utilized in the experiments described in this chapter were obtained from the sources previously detailed in sections 2.2.1, 3.2.1 and 4.2.1.

5.2.2 Tissue Sources

Ears taken from foetal calves were obtained within half an hour of maternal death, as previously described. Gestational age was estimated from crown-rump measurements (Bogart, 1959) and only 200- to 220-day foetuses were taken.

Neonatal (2-day-old) and young (16-day-old) semi-lop (Oxford - New Zealand White cross) rabbits were procured from the University of Adelaide Central Animal House. Rabbits were killed by cervical dislocation, decapitated and the ears removed immediately after death.

5.2.3 Histology of Ear Cartilage

Samples for histology and electron microscopy were taken from one ear and placed in fixative at once. The other ear was then processed for tissue culture as described in section 5.2.5.

Full thickness slices, 2-3mm in depth, were taken from the central portion of the middle third of the pinna and fixed with either 3% (w/v) glutaraldehyde (TAAB) in 0.2M sodium cacodylate buffer, pH 7.3, or 2% (w/v) paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. Tissues were allowed to fix overnight at 4^{o}C, then embedded in paraffin and sectioned in the usual way. Sections were stained for elastic fibres with either Verhoeff-Van Gieson (Pearse, 1968) or Miller (Miller, 1971) stains. Light micrographs were taken with a Leitz Orthoplan microscope using Kodak Pan-F film.

5.2.4 Electron Microscopy of Ear Cartilage

Samples from ear cartilage were taken as above from half way along the pinna, the skin was removed and the cartilage sliced into strips approximately 3mm long and 1mm thick. The full thickness of the cartilage was included in each slice. These were fixed overnight at 4°C by immersion in 3% (w/v) glutaraldehyde (TAAB) in 0.2M sodium cacodylate buffer, pH 7.3, containing 2mM calcium chloride. Specimens were then rinsed thoroughly with 0.15M sodium cacodylate buffer, pH 7.3, and post-fixed for one hour in 1% (w/v) osmium tetroxide in 0.15M sodium cacodylate, pH 7.3. They were then routinely dehydrated and embedded in Spurr's resin (TAAB), as previously described (see section 2.2.3).

Blocks were trimmed so that sections were taken from the central and boundary zone of cartilage in each case. Ultrathin sections were cut on a Reichert OmU2 ultramicrotome, using a diamond knife, and were picked up on copper-rhodium grids. These were stained for 15 minutes with saturated uranyl acetate in 70% (v/v) ethanol, followed by lead citrate (Reynolds, 1963) for a further 15 minutes.

Sections were examined in a Philips EM 300 microscope operated at an accelerating voltage of 80kV.

5.2.5 Culture of Auricular Chondrocytes

The ears were swabbed with 70% (v/v) ethanol and removed from the animal. The skin was dissected off under sterile conditions and the perichondrium very carefully removed. This procedure often took more than one hour. The upper and lower thirds of the cartilage were discarded and the central third was finely minced with scissors for use in cell culture. The culture method employed was a modification of that described by Quintarelli et al. (1979). Cells were dissociated by digestion with a 2mg/ml solution of collagenase in 0.2M Tris/HCl buffer, pH 7.3, containing 0.01M calcium acetate at 37°C with stirring. Digestion was judged to be complete when all but the largest pieces of tissue had disappeared; this usually took between 2 and 3 hours. The dissociated cells were then centrifuged for 10 minutes at 2000rpm in an MSE bench centrifuge and

resuspended in warm DMEM containing 10% (v/v) foetal calf serum and antibiotics (as detailed in section 4.2.2).

This procedure was repeated twice to remove contaminating matrix and damaged cells. Cell density was determined with a haemocytometer (Fuchs-Rosenthal) and cells were inoculated into plastic culture flasks (growth area 25cm^2) at a density of 2 x 10^6 cells/flask. The cultures were incubated overnight at 37^{o}C in an atmosphere of 5% CO_2 : 95% air, and the medium changed the next day. Thereafter the medium (5ml) was changed every 2-3 days until the conclusion of experiments, cultures being maintained under scorbutic conditions. Cultures rapidly became confluent, usually within five days of seeding.

Phase contrast micrographs of cultures were taken two days after seeding and immediately after confluence was reached using a Leitz Orthoplan microscope with phase attachment.

5.2.6 Morphology and Electron Microscopy of the Cell Layer

Three groups of cultures were set up, consisting of dissociated chondrocytes obtained from i) foetal calf, ii) neonatal (2-day-old) rabbit, and iii) young (16-day-old) rabbit, auricular cartilage. Cultures were maintained under scorbutic conditions with DMEM containing 10% (v/v) FCS and antibiotics as described previously for ligamentum nuchae fibroblast cultures (see section 4.2.2).

To facilitate light microscopic examination of the cell layer, sterile glass coverslips were placed in two flasks from each group. At 35 days after confluence, the cell layer was rinsed with warm PBS, and fixed for one hour at room temperature with 2.5% (w/v) glutaraldehyde in 0.067M phosphate buffer (pH 7.4) containing 2mM $CaCl_2$. Coverslips were then mounted on glass slides and the cell layer stained with either Verhoeff-Van Gieson or Miller elastic stains. Light micrographs were then taken as before (see section 5.2.3).

One flask from each group was fixed and processed for electron microscopic examination at 15 and 35 days post-confluence. The technique employed was identical to that described in section 4.2.4.

5.2.7 Effects of Ascorbate Supplementation on the Matrix Characteristics of Foetal Calf Chondrocyte Cultures

Several flasks of foetal calf auricular chondrocyte cultures were maintained after confluence with medium supplemented with freshly prepared ascorbic acid (50µg/ml). The medium was changed every 2-3 days. These cultures were fixed and the cell layer processed for electron microscopy at 28 days post-confluence, in order to determine the effect of ascorbate supplementation on the ultrastructural characteristics of the extracellular matrix.

5.2.8 Immunofluorescent Localization of Anti-MAg Antibodies in Foetal Calf Ear Cartilage

a. Tissue sections: 5µm cryostat sections were made from foetal calf ear cartilage specimens and mounted on glass slides as described in section 3.2.2. Sections were then allowed to react with anti-MAg (ADS) IgG. They were then washed and stained with goat anti-(rabbit IgG)/fluorescein conjugate, using the procedure detailed in section 3.2.2. Controls were provided by treatment of some sections with pre-immune rabbit IgG in place of anti-MAg (ADS) IgG.

b. Cell culture: Primary cultures of foetal calf auricular chondrocytes were grown to confluency in 25cm^2 plastic flasks to which had been added sterile glass coverslips (size 1.5 x 1.0cm). At 21 days post-confluence the cell layer was processed for immunofluorescence microscopy as follows. The cell layer was rinsed three times with warm PBS, then fixed for 5 minutes at 4^{o}C with 2% (w/v) paraformaldehyde in PBS, pH 7.4. Following fixation the cell layer was rinsed twice with cold PBS, the coverslips removed and mounted on glass slides. 100µl of a 10% (v/v) solution of pre-immune goat serum was added to each coverslip and the slides incubated in a humidity chamber for 15 minutes at room temperature. The slides were then washed with PBS for 15 minutes in a stirred chamber. The coverslips were covered with 100µl of 1mg/ml solution of anti-MAg (ADS) IgG and reaction allowed to proceed for 45 minutes. Controls were provided as before by treatment of some coverslips with pre-immune rabbit IgG solutions.

After washing for three 15-minute periods with PBS, the coverslips were incubated for one hour with 100µl of goat anti-(rabbit IgG)/fluorescein conjugate, as described in section 3.2.2. The coverslips were then washed for a further three 20-minute periods, mounted and examined with a Leitz Orthoplan microscope equipped with a Ploemopak 2 epi-illumination unit and Leitz filter block H. Fluorescence micrographs were taken as in section 3.2.2.

5.3 RESULTS

5.3.1 Histology of Ear Cartilage

a. Foetal calf ear cartilage (220 days gestation). A well developed elastic fibre network was demonstrated histochemically with Miller and Verhoeff-Van Gieson stains in foetal calf auricular cartilage (Figure 5.1a). This is best seen in the central zone of the cartilage, where elastic fibres branch and anastomose to form a dense network around the cell lacunae (Figure 5.1a). Serial sections showed that the cells were completely surrounded by the elastic fibre meshwork, but there appeared to be a preferred orientation of the larger fibres in that they were arranged across the cartilage, from top to bottom, between the perichondrial surfaces.

Subjacent to the perichondrium was a transitional zone consisting of longitudinally-aligned, flattened chondrocytes which were somewhat smaller than those in the central zone (Figure 5.1b). The elastic fibre network tapered from the central zone toward the perichondrium, and only very fine ramifications approached the perichondrial surface. No elastic fibres could be distinguished within the perichondrium (Figure 5.1a) which consisted mostly of dense collagen bundles and infrequent elongated fibroblasts.

b. Neonatal (2-day-old) rabbit ear cartilage. Neonatal rabbit ear cartilage appeared histologically to be considerably less mature than foetal calf ear cartilage (220 days gestation). The cartilage zones described above were not readily evident, the cartilage consisting mostly of small, rounded chondroblasts with prominent nuclei and meagre cytoplasm (Figure 5.2).

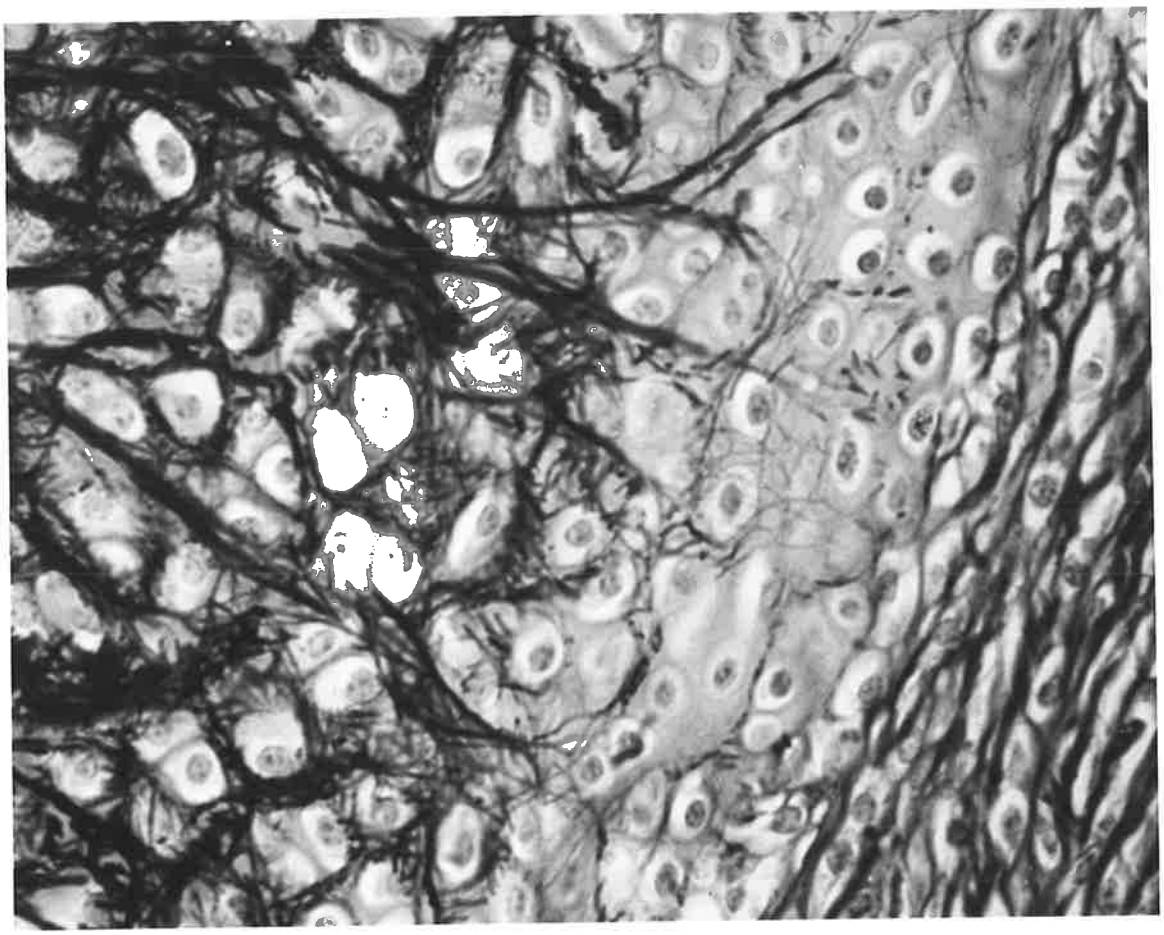
c. Young (16-day-old) rabbit ear cartilage. At this age, the auricular cartilage appeared more differentiated (Figure 5.3), and mature cartilage zones (see section 1.7.1) were easily recognizable. The chondrocytes were larger and, in the central zone, had an ellipsoidal shape similar to that demonstrated in foetal calf ear cartilage. A well-developed transitional zone merging into the perichondrium was evident.

The amount of intercellular matrix was increased, but the cartilage was still relatively cellular. Elastic staining showed a delicate network of interlacing fibres lying

Figure 5.1. Light micrographs of native foetal calf ear cartilage. Sections taken from the mid-portion of the pinna of a 220 day gestation foetus
Miller's elastic stain. Magnification X250.

a. Boundary zone and perichondrial surface. Note the tapering of the elastic fibre network as it approaches the perichondrial surface.

b. Central zone. Note the extremely dense elastic fibre network enclosing cell spaces.



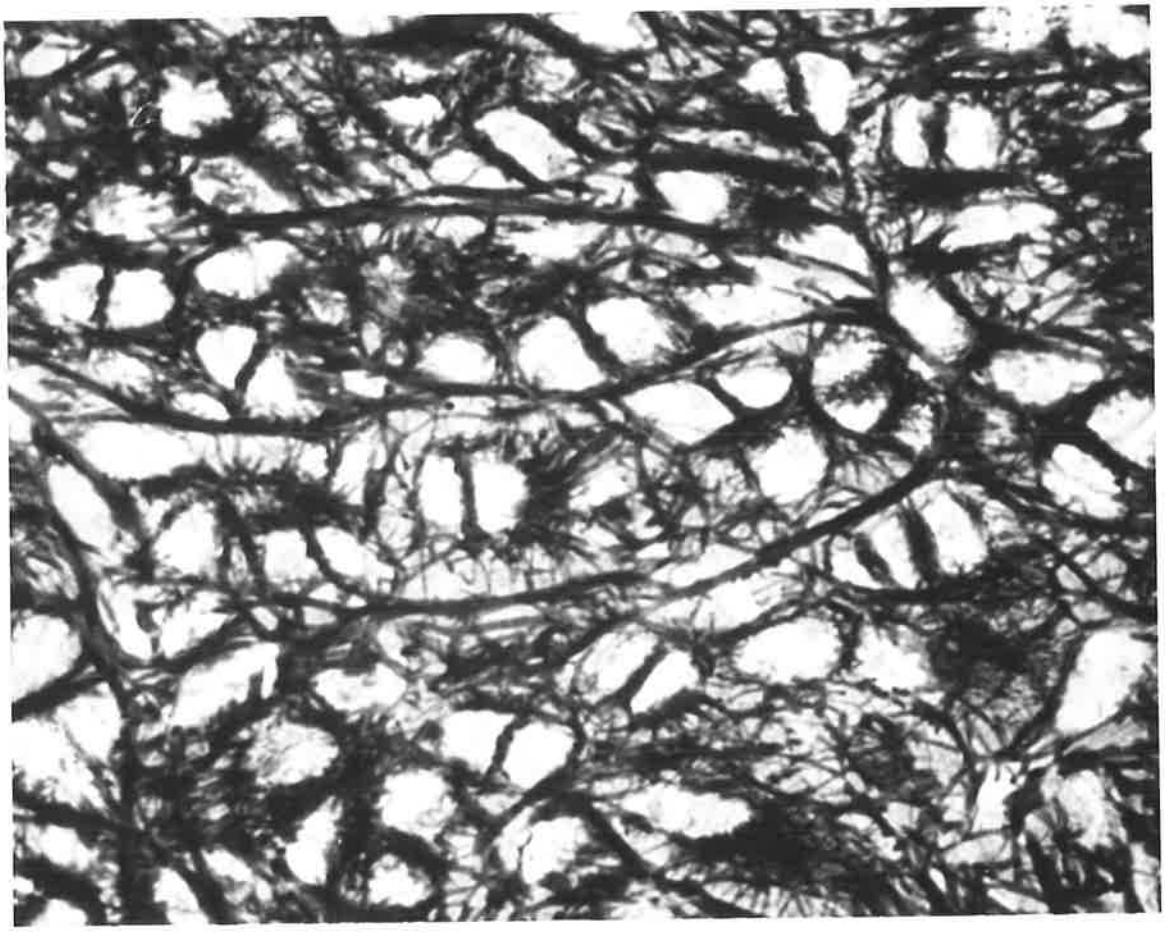
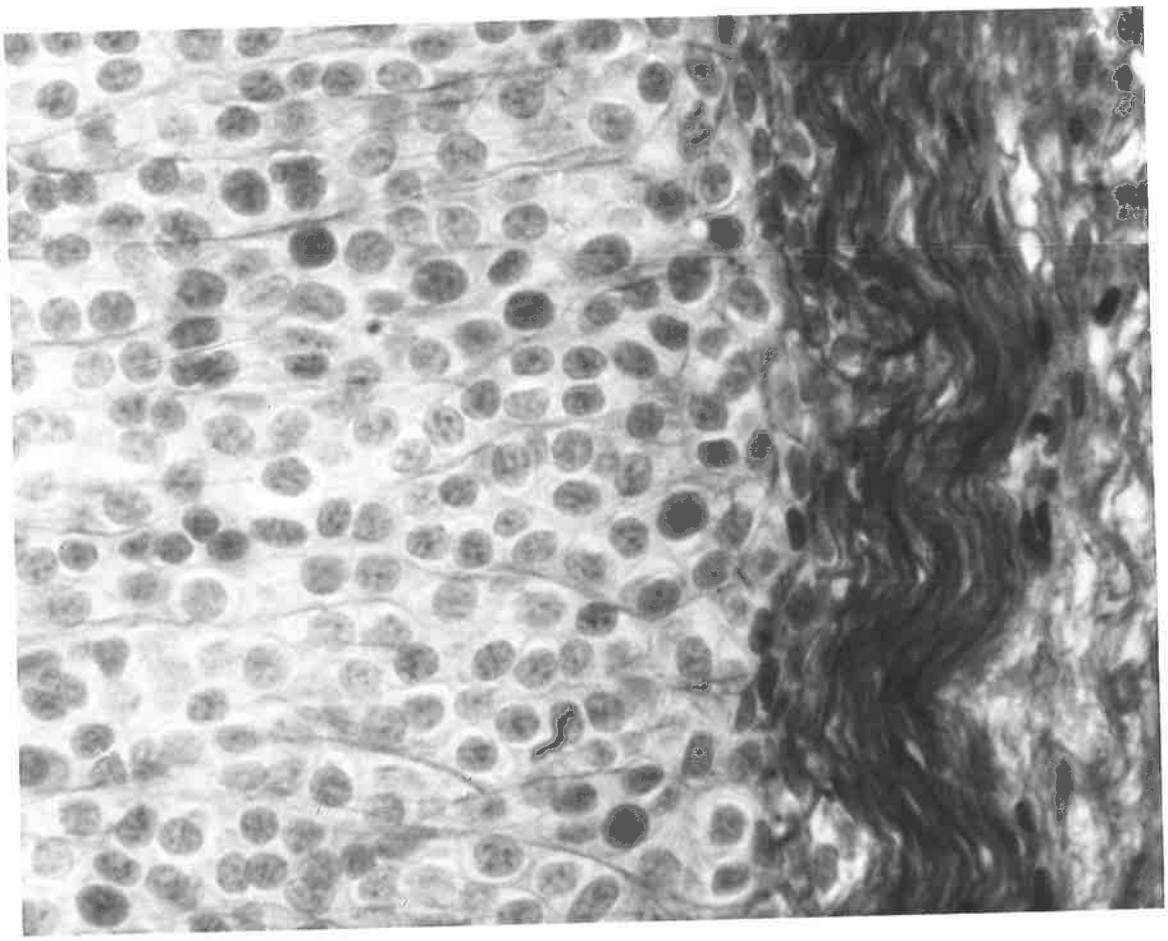
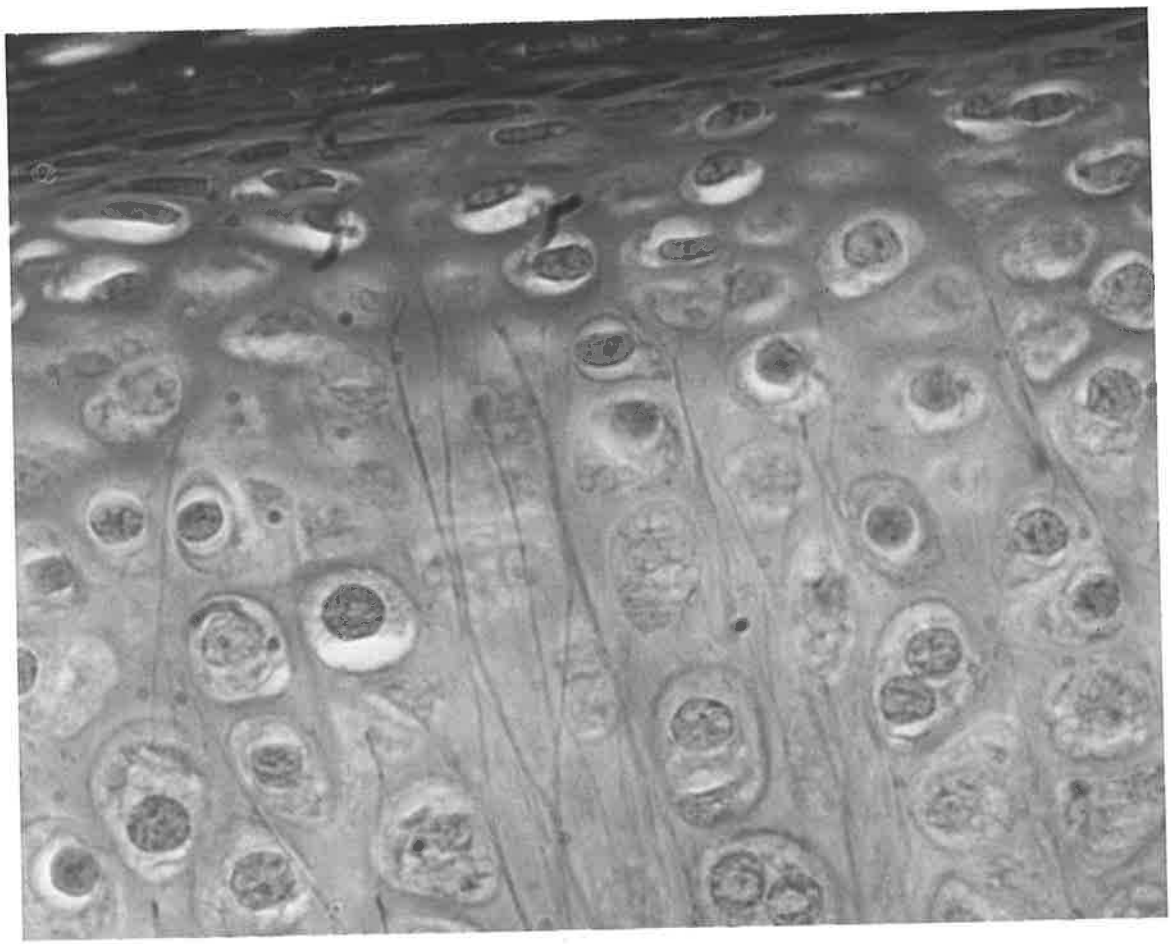


Figure 5.2. Light micrograph of native neonatal (2-day-old) rabbit ear cartilage
The perichondrial surface is at the right of the micrograph. Note the paucity of elastic fibres and poorly developed cartilage zones. Miller's elastic stain. Magnification X400.

Figure 5.3. Light micrograph of native young (16-day-old) rabbit ear cartilage
The periochondrial surface is at the top right-hand corner of the micrograph. Note the considerably better-developed elastic fibre network and larger amount of intercellular matrix compared to the neonatal rabbit ear cartilage. Verhoeff-Van Gieson stain. Magnification X400.





between the cells, in some places appearing to surround them. This network was much less prominent than that of foetal calf ear cartilage (cf. Figure 5.1).

5.3.2 Ultrastructural Characteristics

a. Foetal calf ear cartilage (220 days gestation)

Cellular morphology. The chondrocytes of the **central zone** were approximately elliptical in shape, measuring 7-10μm along the long axis. They had large nuclei (about 3-5μm in diameter) and a moderate amount of cytoplasm, the margins of which were extremely irregular, forming numerous fine, often branched, processes extending into the matrix. These gave the sectioned cell membrane a scalloped appearance (Figure 5.4). The cytoplasm contained a moderately developed rough endoplasmic reticulum with dilated cisternae filled with granular electron-dense material. Free ribosomes and small numbers of oval mitochondria were also seen in the cytoplasm. A Golgi complex could be discerned in most cells close to the nucleus, and coated vesicles were frequently seen. These contained coarsely granular, moderately electron-dense, material. Little glycogen was present in the cytoplasm, but cells frequently contained one or more large lipid droplets which did not appear to be membrane-bound (Figure 5.5). Solitary cilia and centrioles were occasionally observed in interphase cells (Figure 5.6).

Cytoplasmic microfilaments were a prominent feature, running in randomly-oriented bundles. These microfilaments occupied a substantial proportion of the cell volume, the filament-containing areas being sharply demarcated from the surrounding cytoplasm. Although ubiquitously distributed throughout the cell, microfilaments were particularly well seen at the periphery of lipid droplets, and around the nucleus (Figure 5.5).

Chondrocytes of the **boundary zone** corresponded to this general description, but were usually more elongated and had a slightly greater amount of cytoplasm which contained more rough endoplasmic reticulum and fewer microfilaments. They rarely contained lipid droplets.

Matrix characteristics. An extensive and well-developed extracellular matrix was present at 220 days gestation (Figures 5.4 and 5.5), widely separating the chondrocytes from each other. The 'stellate reticulum' (Smith, 1970) formed an overall background, consisting of rounded or polygonal electron-dense granules of variable size (approximately 20-80nm), frequently associated with thin (approximately 3-5nm) filaments interconnecting them with each other and the fibrous elements of the matrix (Figure 5.7).

Fine, straight collagen fibrils, ranging from about 20-100nm, traversed the matrix, seemingly in random orientations. Collagen fibril diameter appeared to increase with distance from the cell, and only in the larger fibrils could indistinct cross-striations be identified (Figure 5.8).

Large numbers of elastic fibres were apparent, predominantly situated near the centre of the intercellular space. These fibres varied considerably in size and shape (the largest being of the order of 1μm in diameter), were irregularly branched and appeared to be discontinuous. At low magnifications, such fibres could be seen to consist of a central amorphous, lightly-stained core with a dark-staining mantle of apparently fibrillar material (Figure 5.8). At higher resolution, in longitudinal section, peripheral microfibrils, approximately 12nm in diameter, could be discerned. Some of these showed an indistinct, beaded periodicity (Figure 5.9). Microfibrillar structures were more difficult to demonstrate in cross-sectioned elastic fibres and were clearly seen only in the smaller elastic fibres (Figure 5.10). It was also found that the amount of identifiable microfibrillar material decreased rapidly as elastic fibre size increased. In addition, the intimate association of the stellate reticulum and collagen fibrils with the surface of the elastic fibres rendered positive identification of microfibrillar structures difficult in some sections (Figure 5.11).

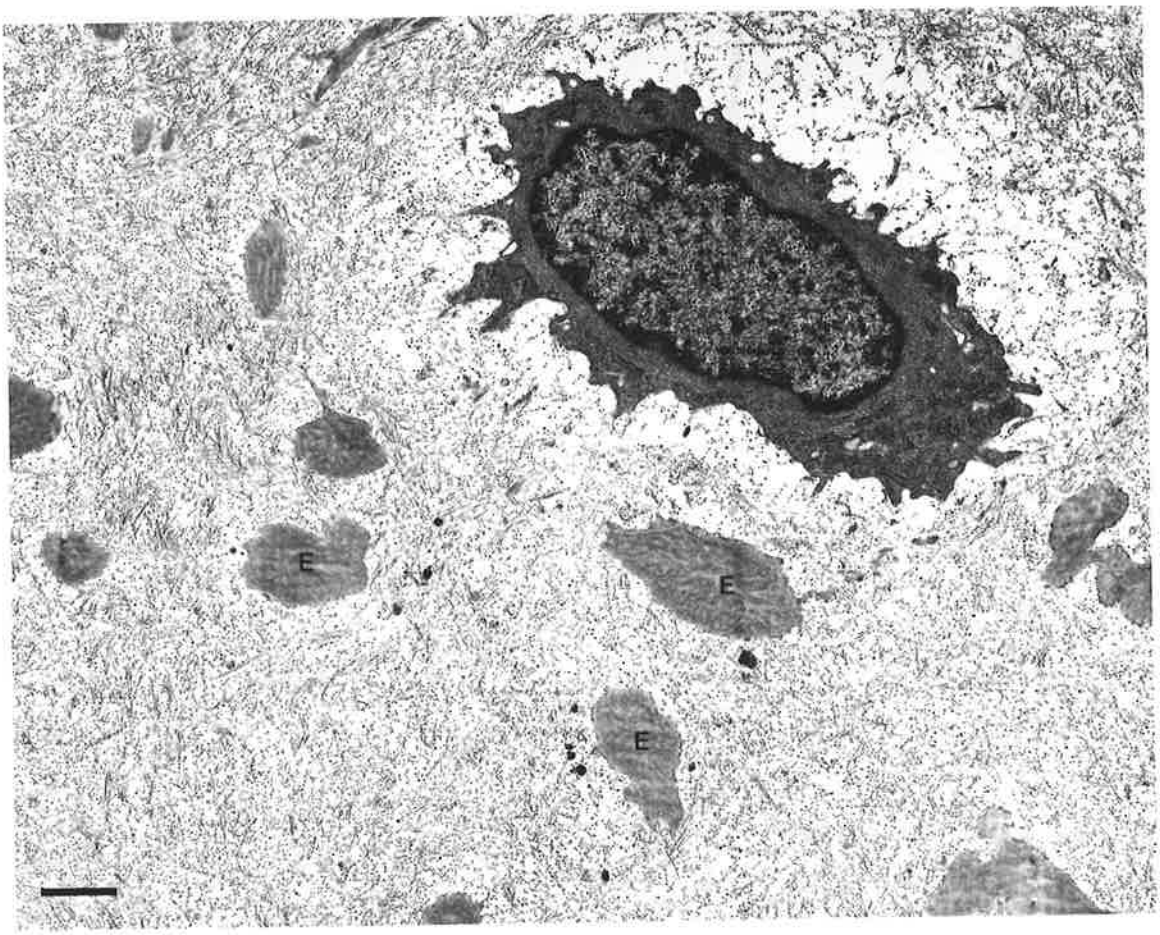
The matrix in the immediate pericellular region appeared attenuated, being almost entirely composed of much looser 'stellate reticulum' than was seen in areas located further from the cells. The electron-dense matrix granules found in this pericellular area were larger than in other areas of the matrix (see Figure 5.7). Small developing

Figure 5.4. Transmission electron micrograph of native foetal calf ear cartilage showing general architecture

Note the predominance of collagen and proteoglycan in the extracellular matrix, which is rarefied in the immediate pericellular region. Large elastic fibres (E) are scattered in the matrix. The chondrocyte shows the typical irregular cellular margins and ellipsoidal shape. Section taken from the mid-portion of the pinna. 220 days gestation. X 10,000. Scale = 1μm.

Figure 5.5. Transmission electron micrograph of native foetal calf ear cartilage

Similar section to Figure 5.4. Note the large lipid droplets (L) within the cytoplasm of the chondrocyte. These are not membrane-bound, but are surrounded by a dense aggregation of microfilaments. 220 days gestation. X10,000. Scale = 1μm.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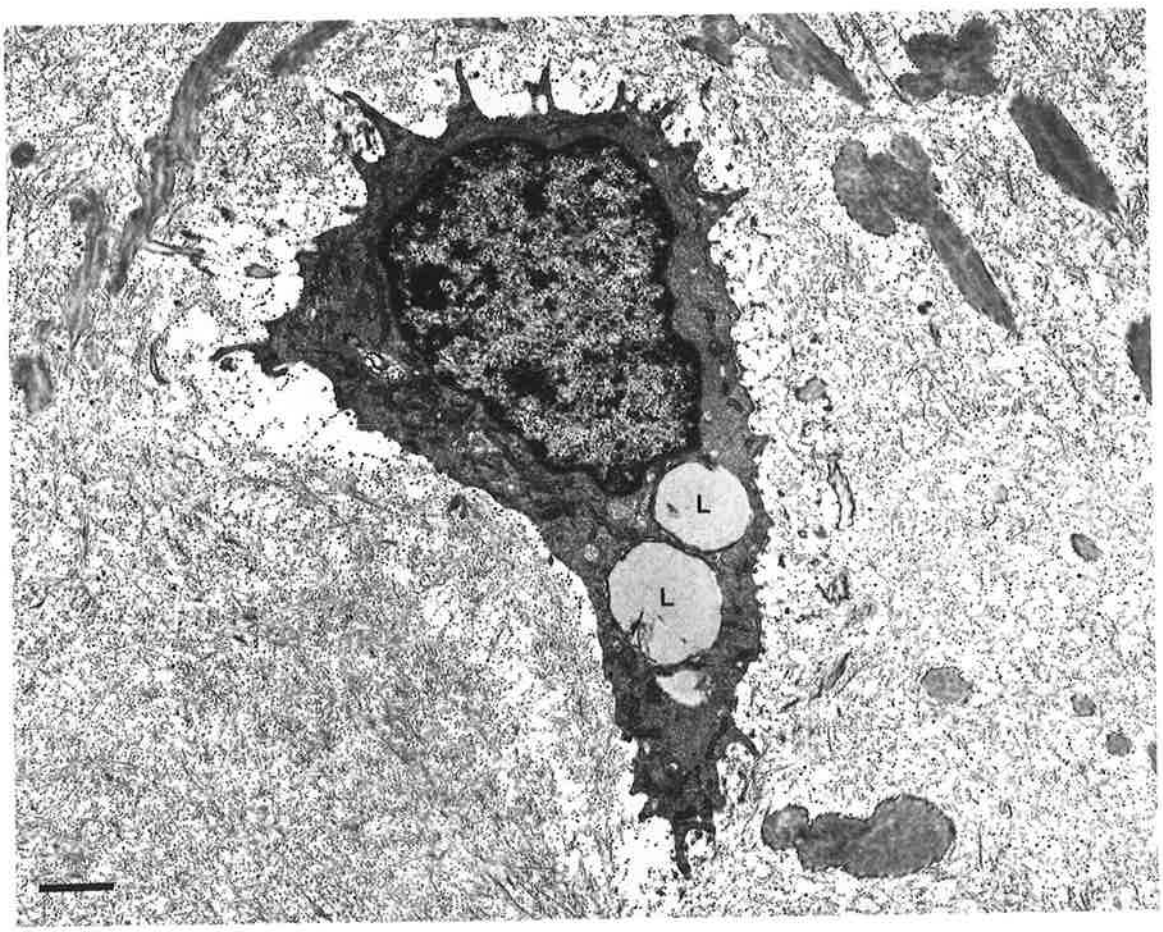
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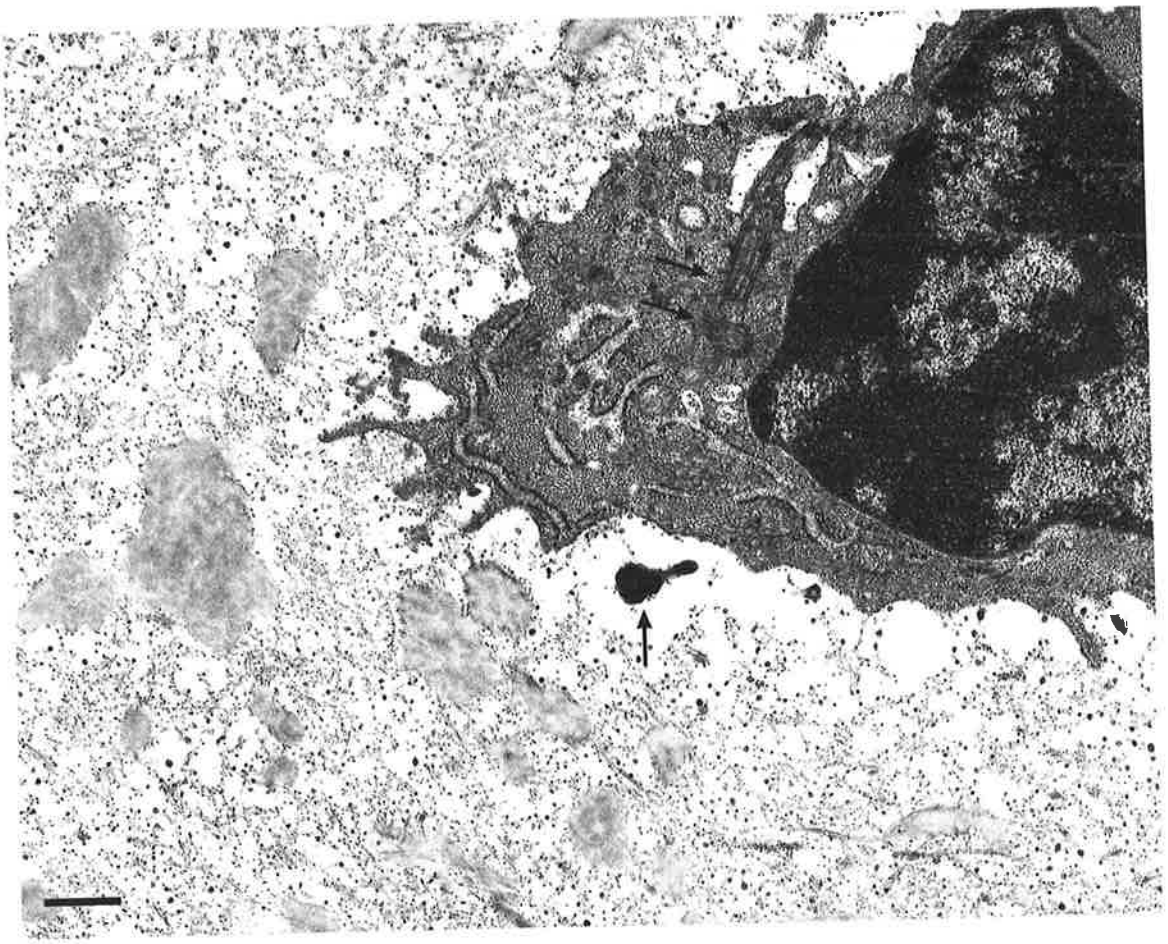
Figure 5.6. Transmission electron micrograph of a native foetal calf ear cartilage chondrocyte showing a centriole and solitary cilium

A centriole and solitary cilium (↑↑) can be seen within the cytoplasm. Note large numbers of cytoplasmic microfilaments and dilated cisternae of endoplasmic reticulum. An electron-dense matrix vesicle (↑) can be seen adjacent to the plasma membrane. Section taken from the mid-portion of the pinna. 220 days gestation. X20,000. Scale = 0.5μm.

Figure 5.7. Transmission electron micrograph of native foetal calf ear cartilage showing details of the pericellular matrix

Note the decrease in density of the 'stellate reticulum' near the cell membrane. Several small elastic fibres can be seen in the matrix. A few profiles of rough endoplasmic reticulum and cytoplasmic microfilaments are seen within the cell. Note the patch of amorphous, granular material adjacent to the plasma membrane. The nature of this material is uncertain. 220 days gestation. X40,000. Scale = 200nm



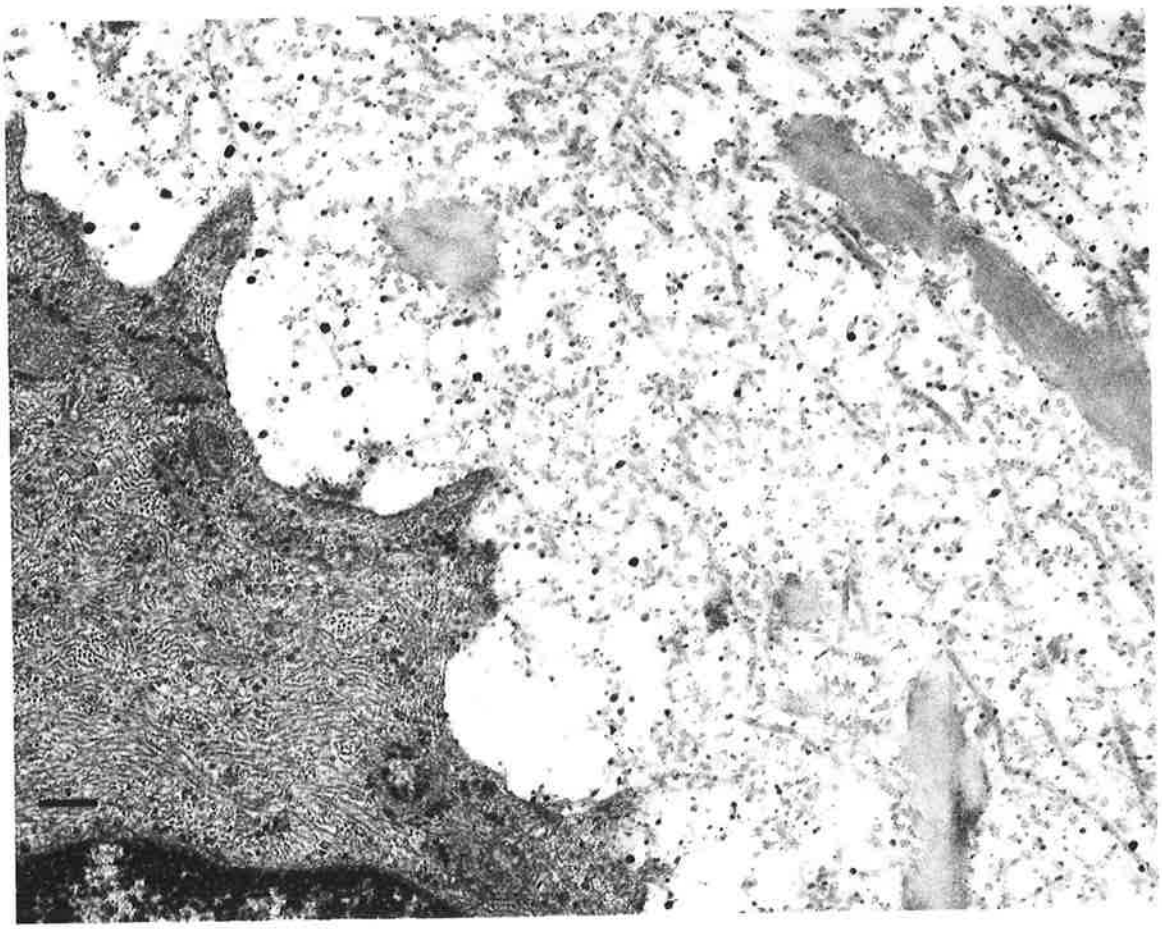
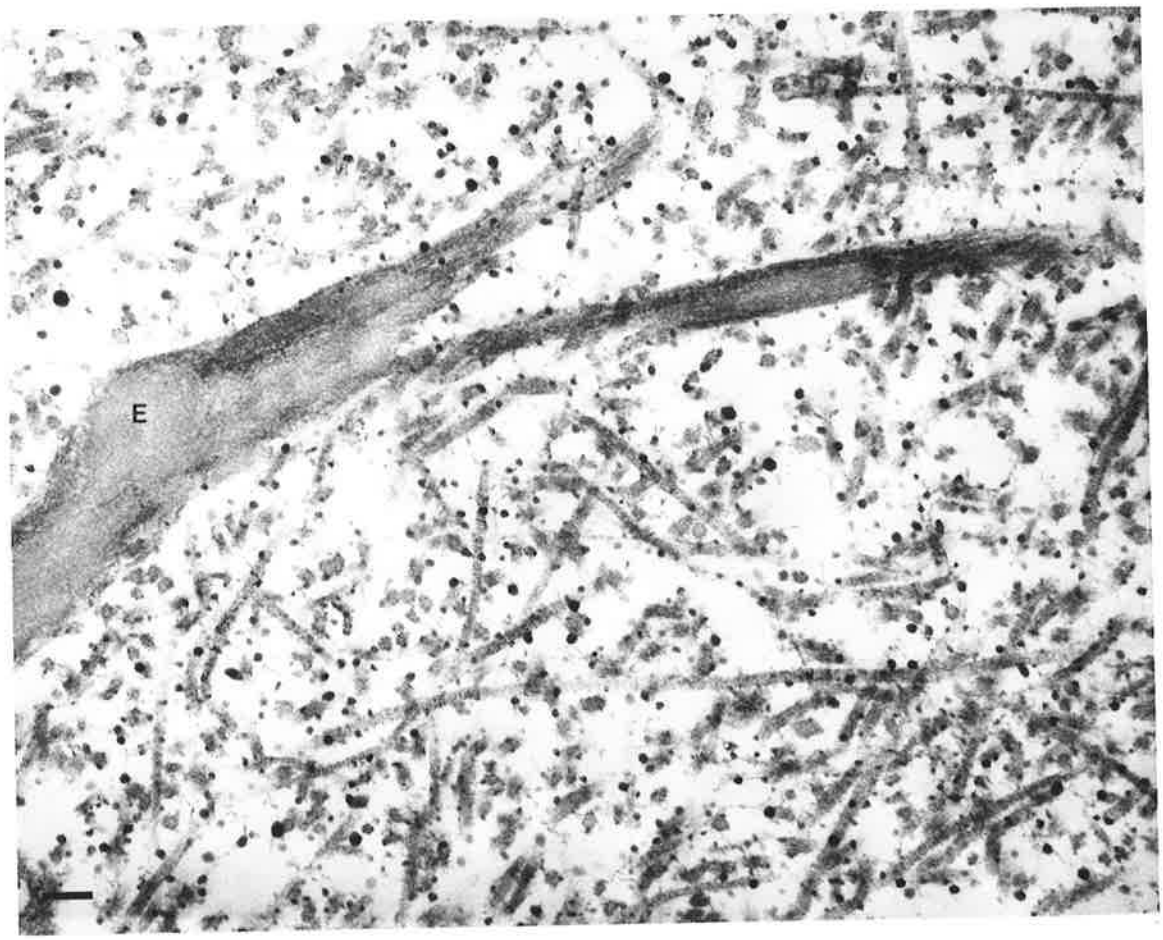


Figure 5.8. Transmission electron micrograph of native foetal calf ear cartilage showing detail of the 'stellate reticulum' of the intercellular matrix

Note the close association of collagen fibrils with proteoglycan granules, and their interconnection by thin matrix filaments. Some of the larger collagen fibrils show indistinct cross-striations. Part of a small longitudinally-sectioned elastic fibre (E) can also be seen. This appears to have an electron-dense fibrillar coat which is difficult to clearly resolve. Section taken from the mid-portion of the pinna. 220 days gestation. X65,000. Scale = 100nm.

Figure 5.9. Transmission electron micrograph of native foetal calf ear cartilage showing microfibrils in association with a small elastic fibre

High-power micrograph of a tangentially-sectioned small elastic fibre (E). Note the short microfibrils at the end of this fibre. Those which can be clearly resolved (↑) have an apparent diameter of 10-14nm and can be clearly delineated from adjacent collagen fibrils (C). Proteoglycan granules encrust the surface of the fibre. 220 days gestation. X160,000. Scale = 100nm.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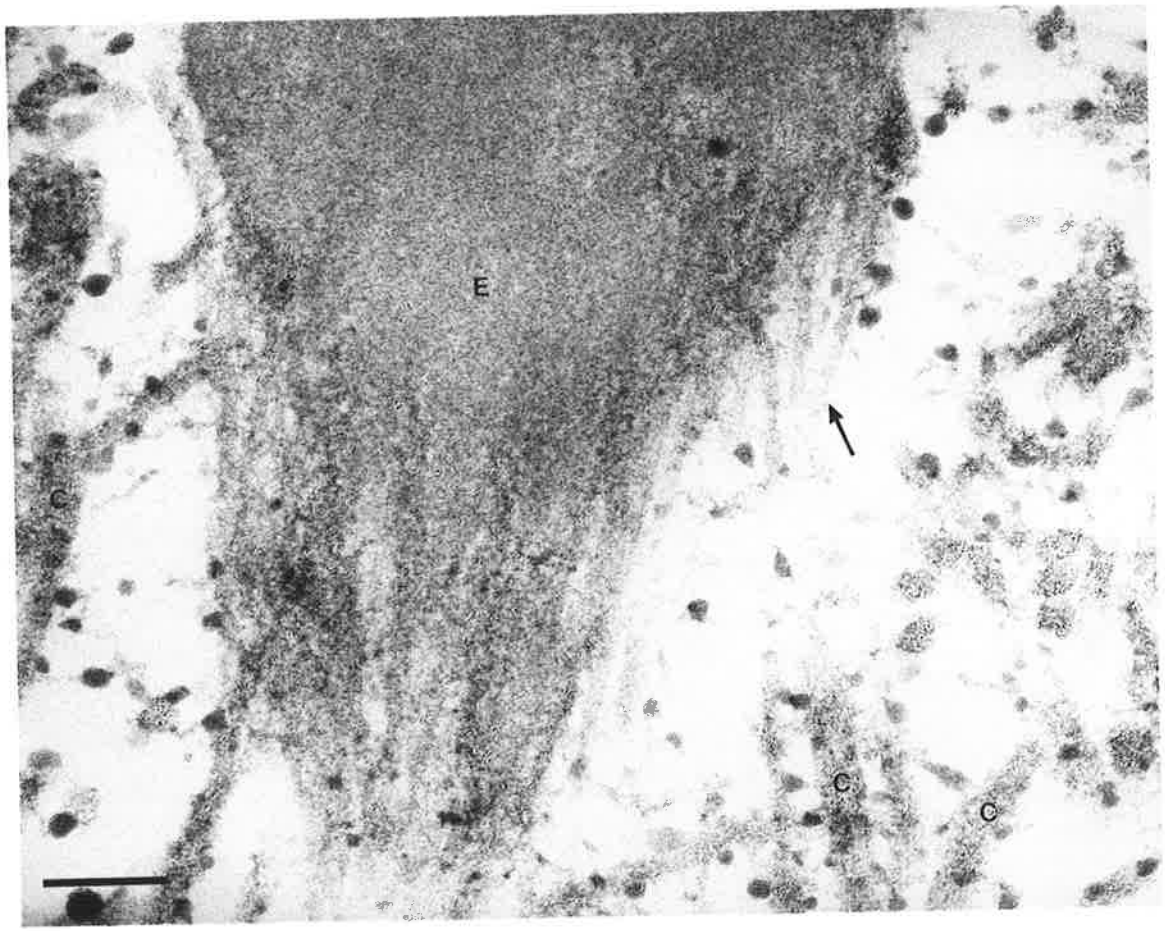
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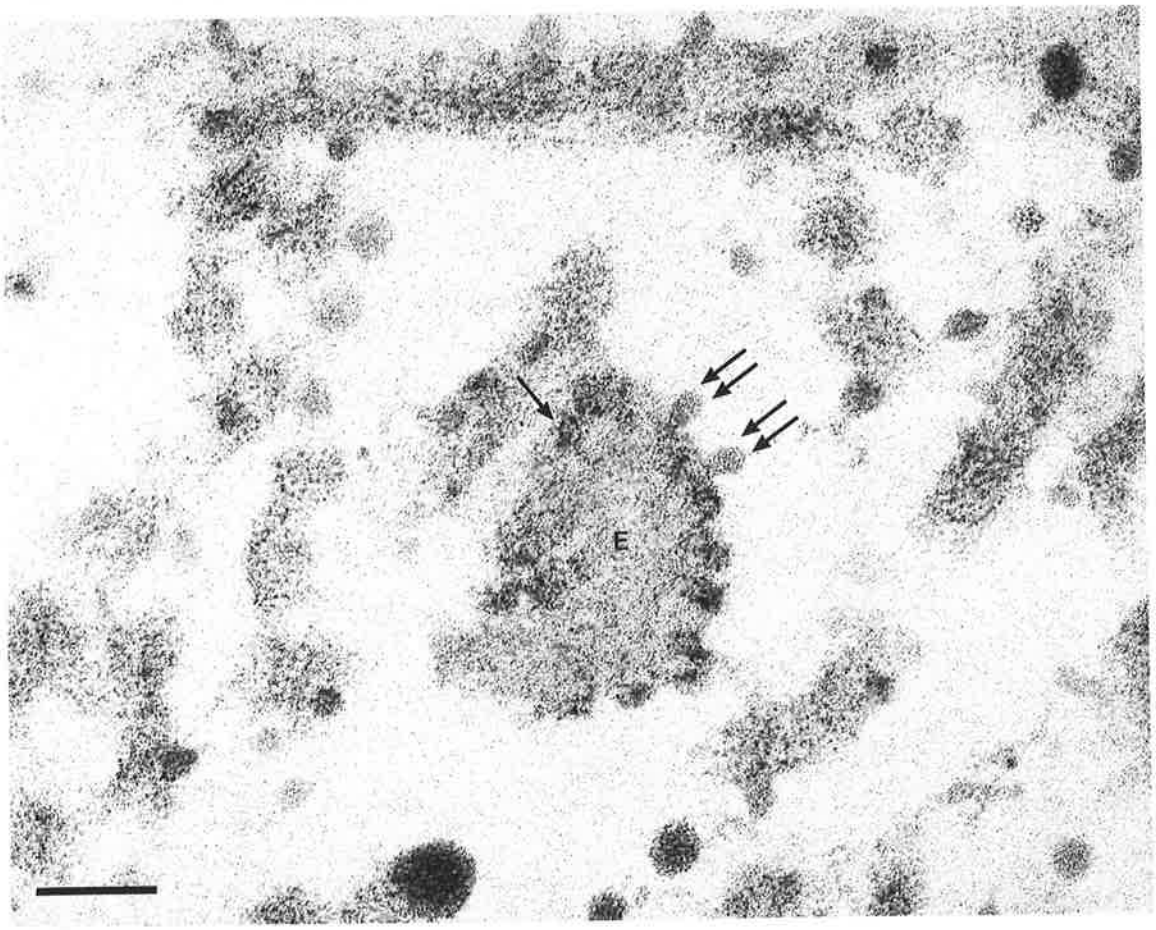
Figure 5.10. Transmission electron micrograph of native foetal calf ear cartilage showing the appearance of elastin-associated microfibrils in cross-section
High-resolution micrograph of a small elastic fibre (E) in the pericellular matrix, seen in cross-section. Note the approximately circular, electron-dense structures seen at the periphery of the fibre. One of these can be clearly resolved as a tubular microfibril (↑). Several proteoglycan granules (↑↑) can be seen apparently attached to this elastic fibre. 220 days gestation. X310,000. Scale = 50nm.

Figure 5.11. Transmission electron micrograph of native foetal calf ear cartilage showing close association of proteoglycan with the surface of an elastic fibre
Part of a longitudinally-sectioned elastic fibre is shown. Short elastin-associated microfibrils (↑) can be seen at the end of the fibre, but are obscured in some places by dense surface coating of proteoglycan granules. 220 days gestation. X65,000. Scale = 200nm.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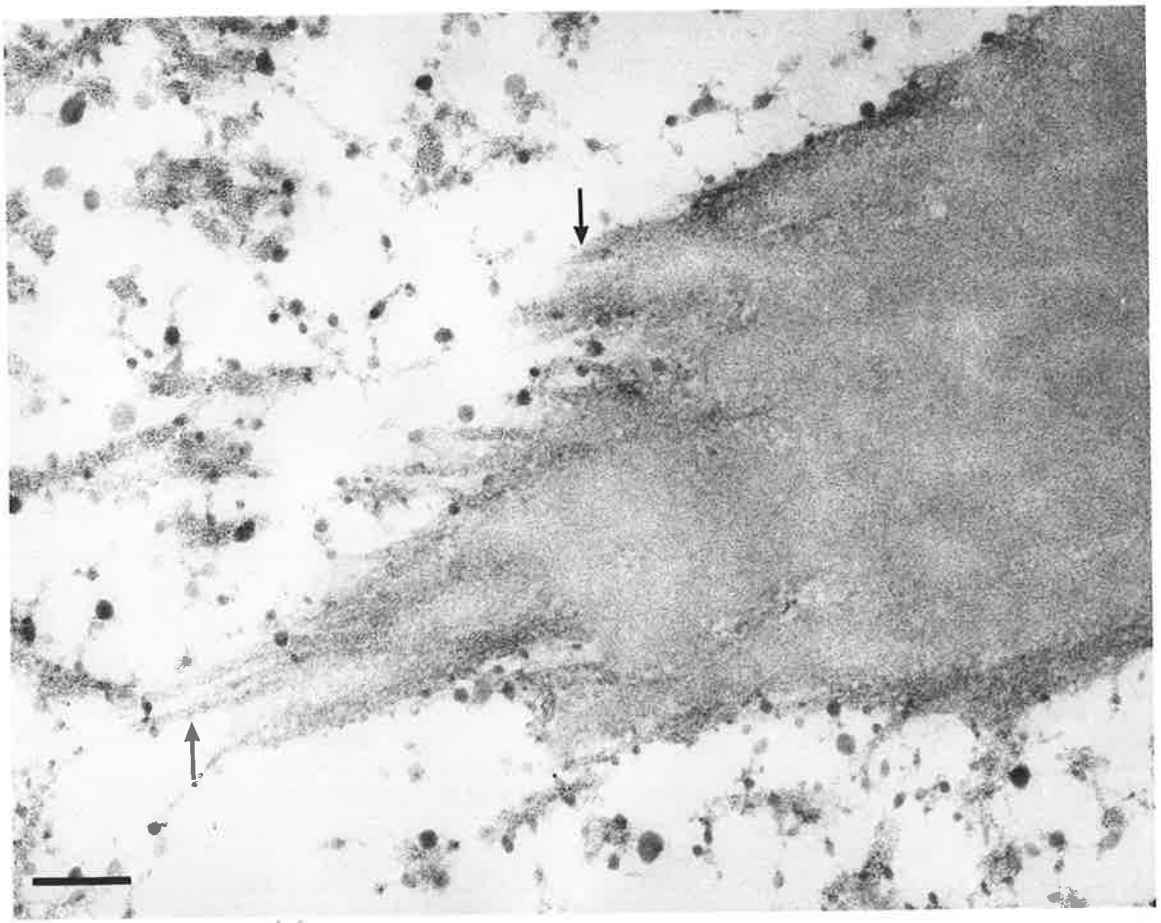
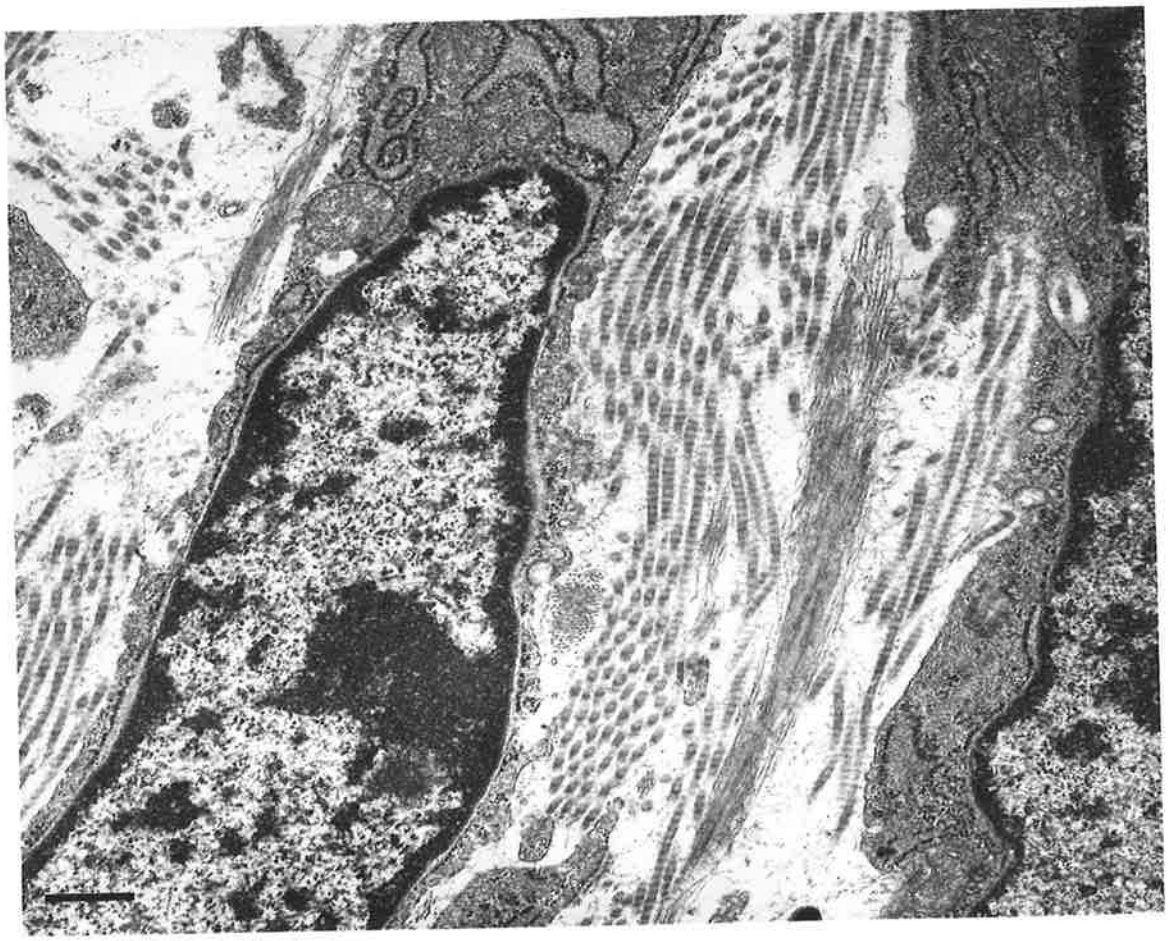


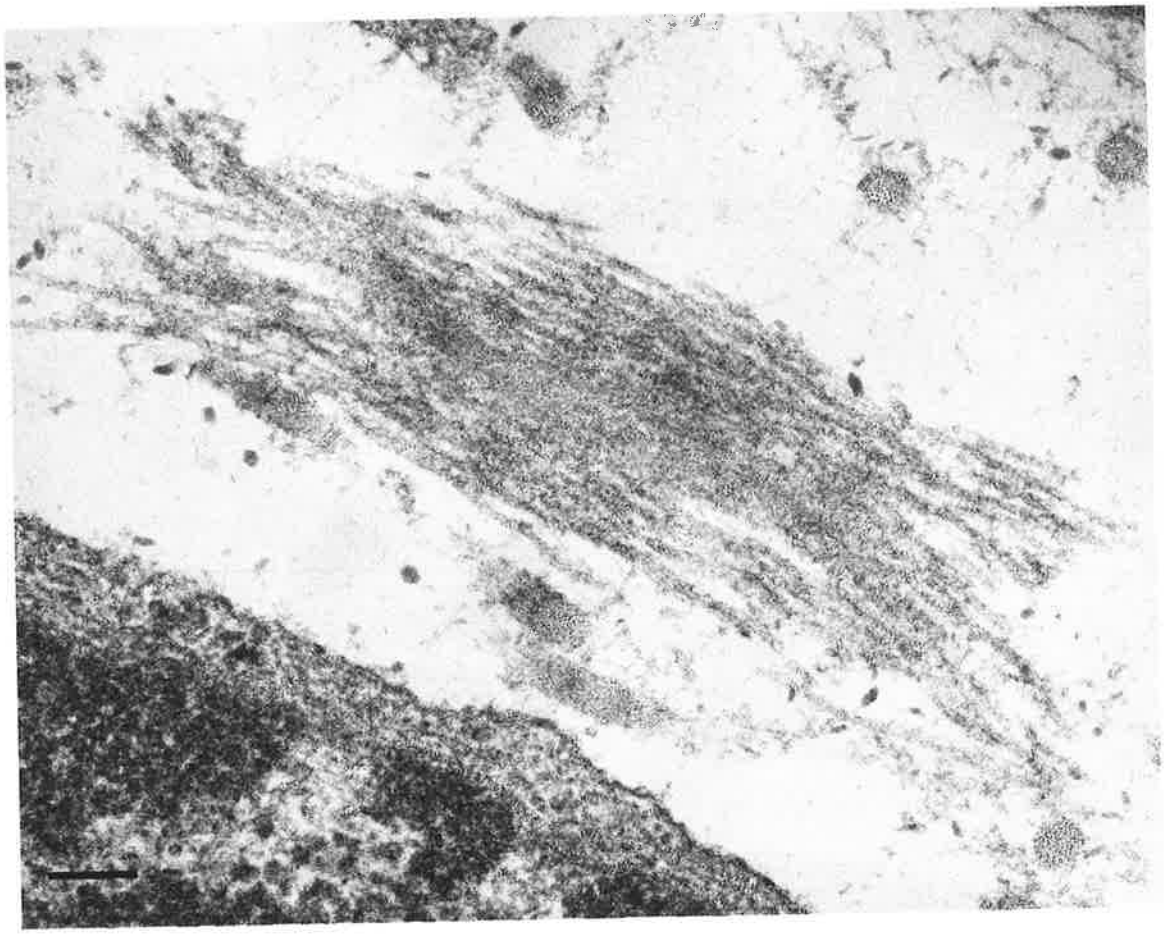
Figure 5.12. Transmission electron micrograph of the perichondrium of native foetal calf ear cartilage showing general architecture

Longitudinal sections of several fibroblast-like perichondrial cells are shown. The extracellular matrix consists primarily of bundles of collagen fibrils interspersed with small elastic fibres, which possess typical elastin-associated microfibrils. 220 days gestation. X23,000. Scale = 0.5μm.

Figure 5.13. Transmission electron micrograph of the perichondrium of native foetal calf ear cartilage showing detail of a developing elastic fibre

High-resolution micrograph of a longitudinally-sectioned small elastic fibre adjacent to a perichondrial cell. Note the patches of amorphous elastin forming within a parallel array of elastin-associated microfibrils. 220 days gestation. X115,000. Scale = 100nm.





elastic fibres were occasionally noted close to the cell, but few collagen fibrils were present. Patches of an amorphous granular material were often seen close to the plasmalemma. The nature of this material is uncertain (Figure 5.7).

Perichondrial morphology. Ultrastructurally, as well as histologically, the perichondrium appeared quite distinct from the underlying elastic cartilage. The cells of the perichondrium had a typical fibroblastic morphology (Figure 5.12), similar to that seen in foetal calf ligamentum nuchae. The perichondrial matrix consisted almost entirely of bundles of heavily cross-striated collagen fibres, interspersed with immature elastic fibres. These developing elastic fibres were usually found in close apposition to the cell membrane and at high resolution appeared as bundles of microfibrils in parallel array, containing irregular patches of amorphous elastin (Figure 5.13).

b. Neonatal (2-day-old) rabbit ear cartilage

Neonatal rabbit auricular cartilage chondrocytes closely resembled those of foetal calf ear cartilage, but were approximately half the size. Cellular outline was rather variable but most cells were roughly elliptical, with large nuclei and scanty cytoplasm (Figure 5.14). Microfilaments were relatively inconspicuous at this age, being present as small discrete bundles near the nucleus. Lipid droplets were infrequently observed, and were usually very small in size. A moderate amount of rough endoplasmic reticulum was present, and numerous free ribosomes were scattered throughout the cytoplasm. Other cytoplasmic components included mitochondria and a small Golgi complex. Solitary cilia and centrioles were rarely seen.

Matrix characteristics. Ear cartilage at this age was found to be a very cellular tissue with short intercellular distances and a poorly developed matrix (Figure 5.15). The 'stellate reticulum' was very sparse and there was a relative paucity of fine collagen fibrils.

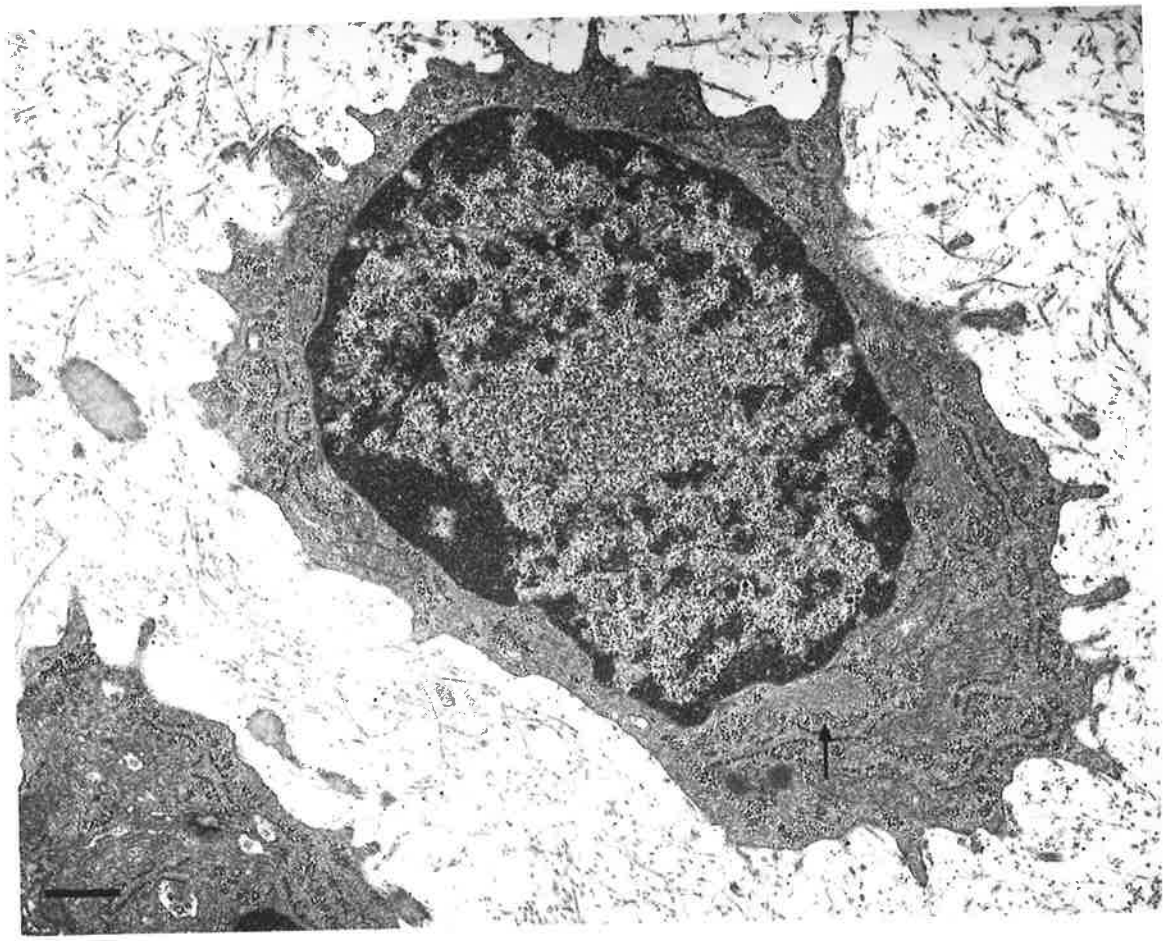
Immature elastic fibres were seen scattered throughout the matrix, the larger fibres tending to lie equidistant from cells. Many small fibres were seen in close association with the cell surface, often lying in infoldings of the cell membrane, closely applied to the plasmalemmal surface (Figure 5.18). Low power examination of these fibres showed

Figure 5.14. Transmission electron micrograph of native neonatal (2-day-old) rabbit ear cartilage showing details of chondrocyte morphology

Chondrocytes show the typical irregular cell margins and ellipsoidal shape. Note the cisternae of rough endoplasmic reticulum and numerous free cytoplasmic ribosomes. Small aggregate of microfilaments (↑) can be seen near the nucleus. Sections taken from the mid-portion of the pinna. X20,000. Scale = 0.5μm.

Figure 5.15. Transmission electron micrograph of native neonatal (2-day-old) rabbit ear cartilage showing details of the intercellular matrix

The collagen and proteoglycan components of the matrix appear very sparse in comparison with foetal calf ear cartilage (cf. Figures 5.6 and 5.8). Several elastic fibres are seen in longitudinal section. These fibres appear to have a peripheral coat of microfibrils which can be clearly differentiated from the amorphous component. X25,000. Scale = 0.5μm.



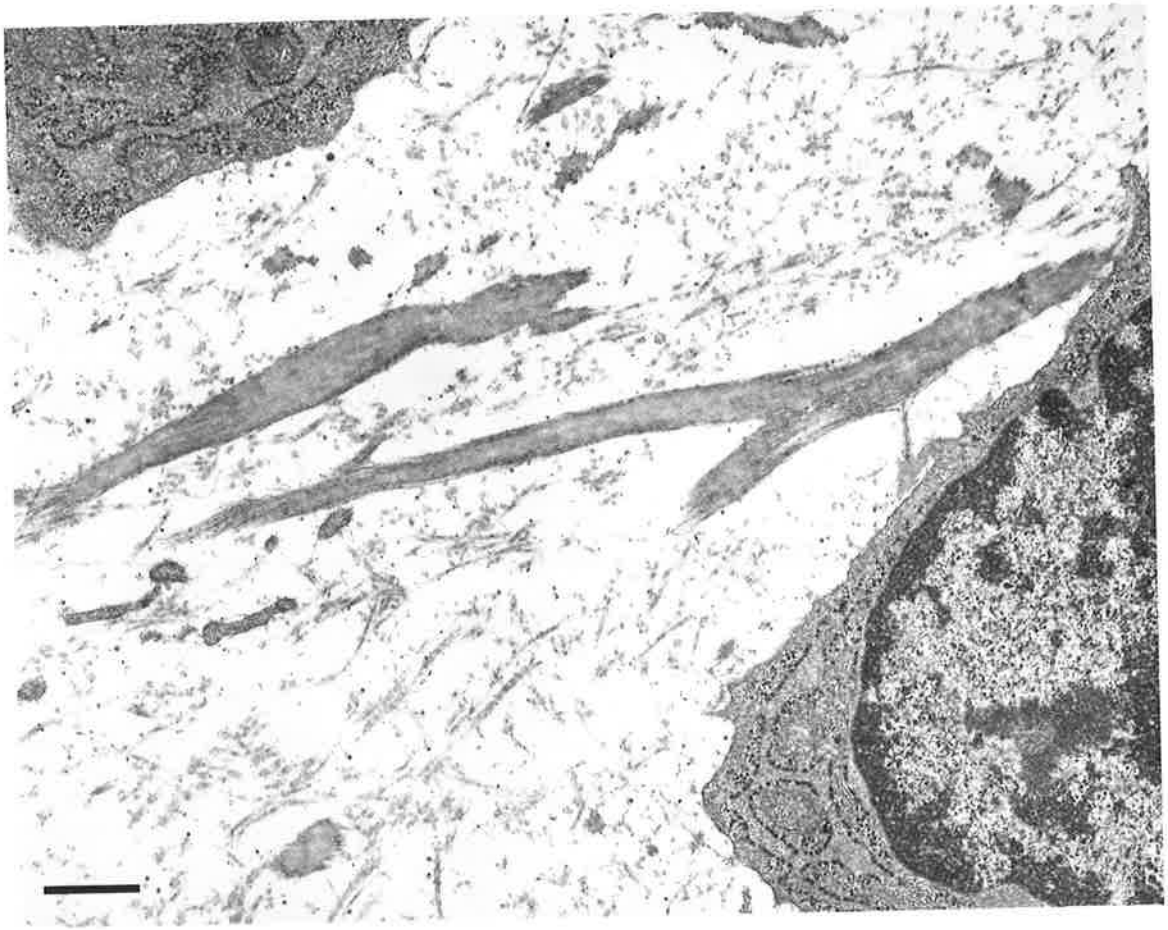
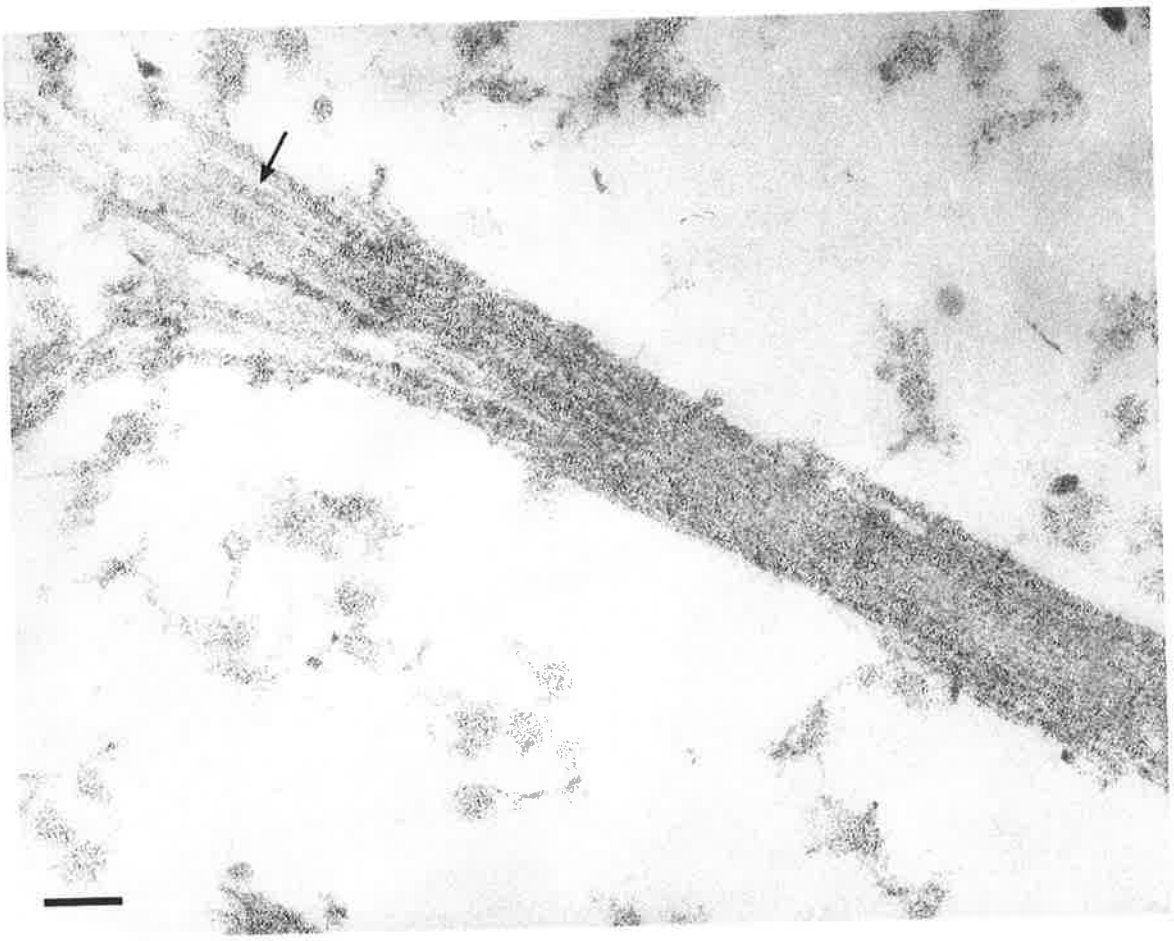


Figure 5.16. Transmission electron micrograph showing high-power detail of an elastic fibre from native neonatal (2-day-old) rabbit ear cartilage

A longitudinally-sectioned small elastic fibre can be seen to splay out into microfibrils at its left-hand end. Some microfibrils (↑) can be seen to consist of parallel rows of stained particles. X200,000. Scale = 50nm.

Figure 5.17. Transmission electron micrograph of native neonatal (2-day-old) rabbit ear cartilage showing high-power detail of elastin-associated microfibrils

A cross-section of a small elastic fibre (**E**) is shown. Note the relatively electron-dense material arranged around the periphery of this fibre. Several circular arrangements of stain particles (↑) can be seen. These have an approximate diameter of 12nm and are interpreted as microfibrils seen in cross-section. X320,000. Scale = 50nm.



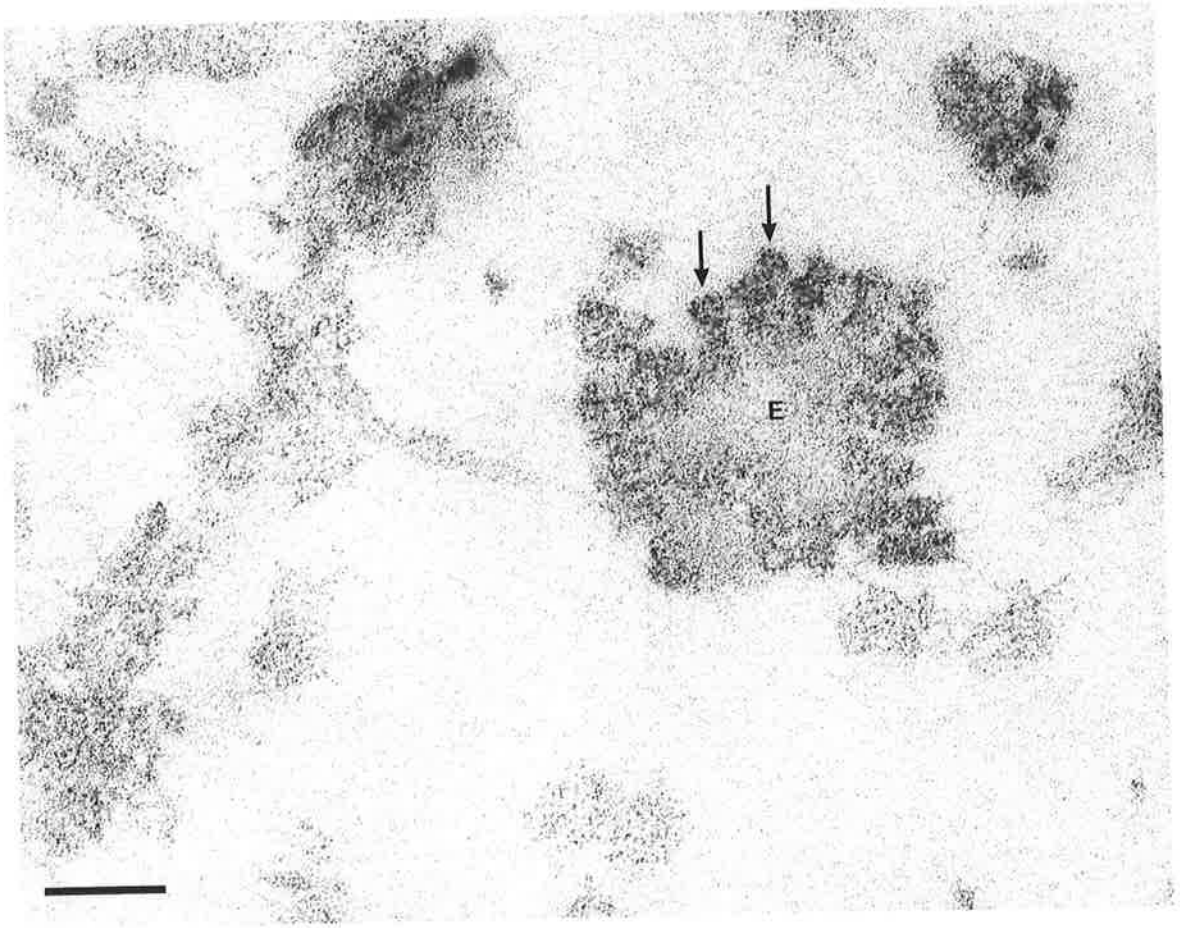

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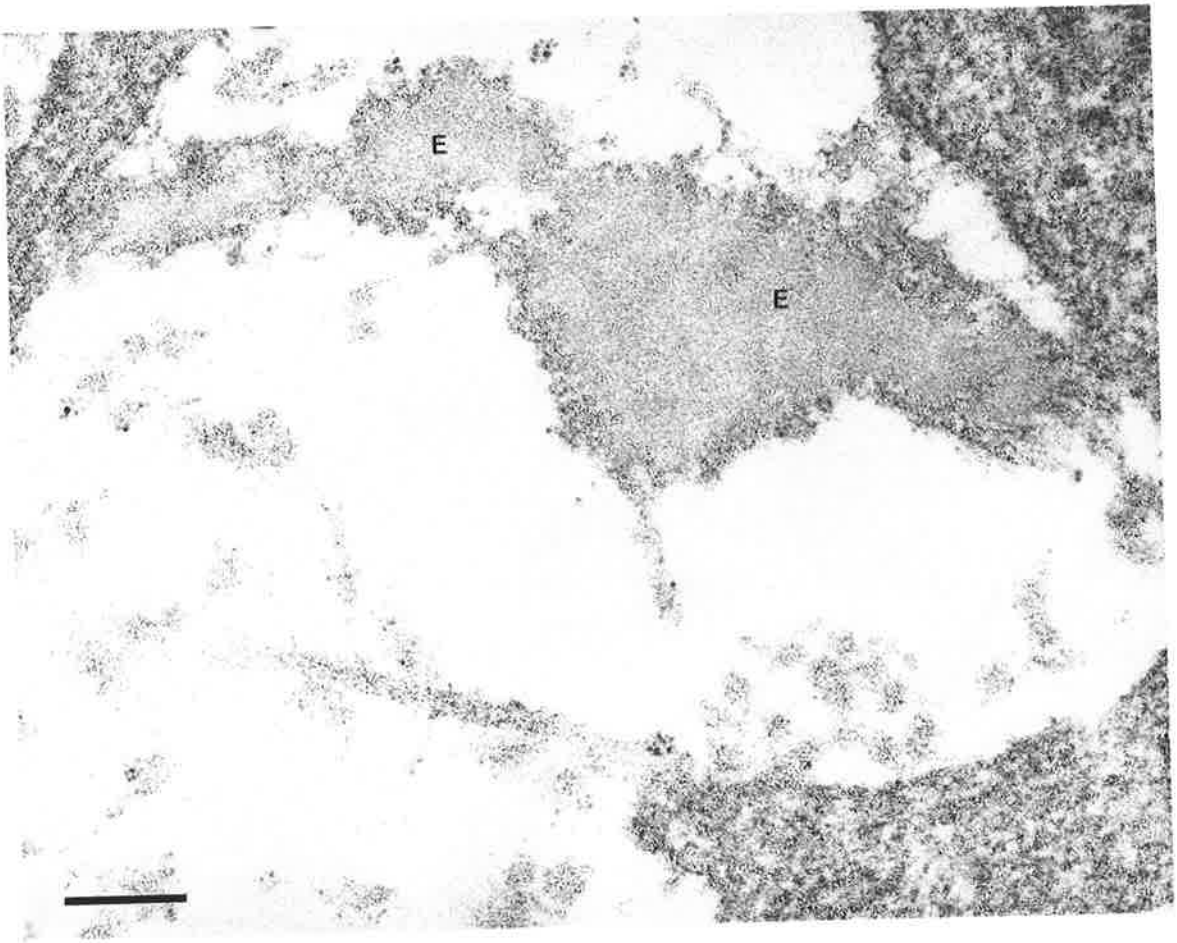
Figure 5.18. Transmission electron micrograph of native neonatal (2-day-old) rabbit ear cartilage

Cross-sections of 2 small elastic fibres (**E**) are seen lying within an embayment formed by the cell membrane of a chondrocyte. The peripheries of these fibres are fringed with electron-dense structures, which in some places appear to form circles of dark-staining particles (↑). These structures probably represent elastin-associated microfibrils seen in cross-section. X160,000. Scale = 100nm.

Figure 5.19. Transmission electron micrograph of native young (16-day-old) rabbit ear cartilage showing details of chondrocyte morphology

Note the proportionately greater cytoplasmic volume and overall size in comparison to young (2-day-old) rabbit ear cartilage (cf. Figure 5.14). Numerous profiles of distended rough endoplasmic reticulum are seen and there are increased numbers of mitochondria. A tract of microfilaments (**M**) can be seen within the cytoplasm. A number of small, electron-dense elastic fibres (↑) can be seen embedded in a predominantly proteoglycan matrix. Sections from the central zone of cartilage taken from the mid-portion of the pinna. X15,000. Scale = 1μm.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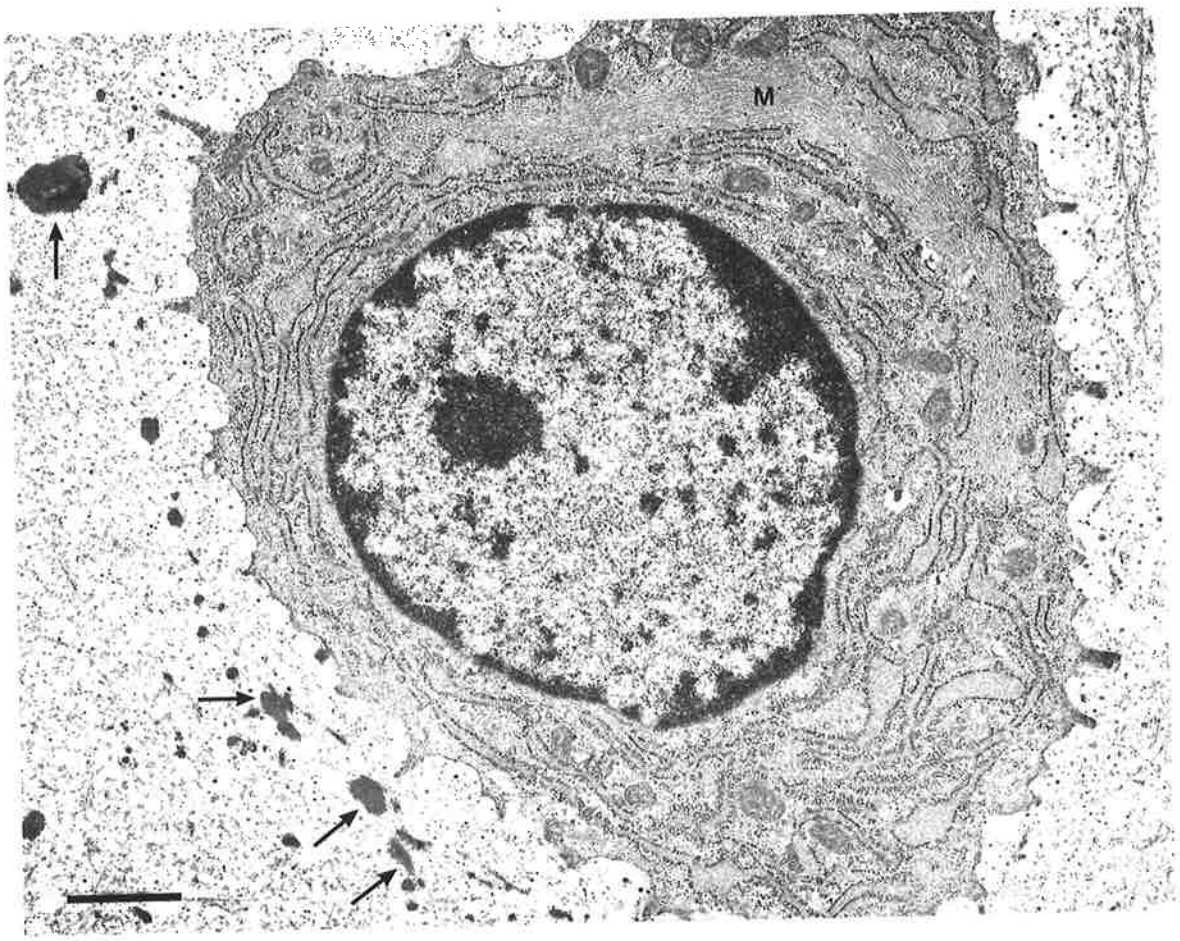

M

them to consist of a moderately electron-dense core surrounded by a darker-staining fibrillar coat (Figure 5.15). Higher resolution micrographs of small elastic fibres in longitudinal section showed that they possessed microfibrils of typical morphology, often best displayed at the end of the fibre. An indistinct beaded periodicity could be discerned, and in some places the microfibrils appeared to consist of a poorly-staining central region with particles of stain aligned in parallel array over their surface, reminiscent of tram-tracks (Figure 5.16). Cross-sectional appearances were more variable, microfibrils being seen as darker-staining, rounded structures surrounding, and within the interstices of, the amorphous component of the fibre. In some sections they could be seen to consist of a lightly-stained central area surrounded by a circular rim of dark-staining material (Figure 5.17). The diameter of these microfibrils was approximately 12nm.

c. Young (16-day-old) rabbit ear cartilage

Cellular morphology. The ultrastructural characteristics of young rabbit auricular chondrocytes were qualitatively similar to those observed in neonatal cartilage, but cell size was considerably increased, measuring between 8μm and 14μm at the long axis (Figure 5.19). Cell nuclei were also larger than in neonatal cartilage, but there was a proportionally greater increase in cytoplasmic volume (due primarily to expansion of the rough endoplasmic reticulum), resulting in a lowered nucleo-cytoplasmic ratio. The composition of organelles within the interphase cells had changed somewhat. Abundant RER with widely dilated cisternae containing a moderately electron-dense, coarsely granular, amorphous substance was present, and there were increased numbers of mitochondria, suggesting an intensification of the synthetic activity of the cell. Aggregates of microfilaments were prominent in the cytoplasm, but neither lipid droplets nor cilia were evident at this age.

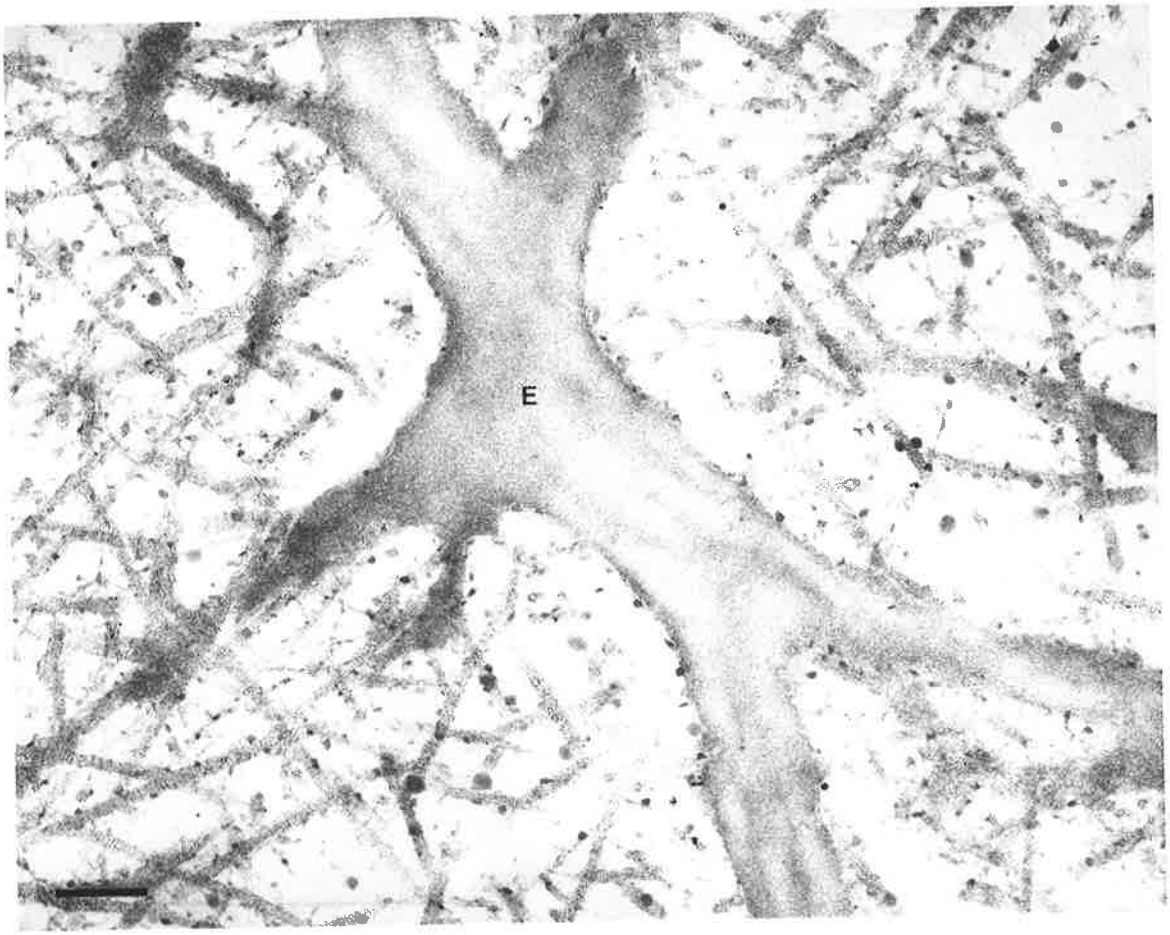
Matrix characteristics. The extracellular matrix of young rabbit ear cartilage displayed an overall increase in the amount and density of cartilage matrix granules and collagen fibrils compared to that of neonatal cartilage. Small electron-dense elastic fibres tended to lie further from the cell, forming an irregular network of branching elastic

Figure 5.20. Transmission electron micrograph of the intercellular matrix of native young (16-day-old) rabbit ear cartilage

Note the dense collagen and proteoglycan matrix surrounding a large, branching elastic fibre (E) which lies approximately equidistant between adjacent cells (not shown). No recognizable microfibrillar material can be seen around this fibre. The surface of the fibre appears heavily coated with proteoglycan granules. Section taken from the central zone of the cartilage. X60,000. Scale = 200nm.

Figure 5.21. Transmission electron micrograph showing high-power detail of an elastic fibre in native young (16-day-old) rabbit ear cartilage

Longitudinal section of a small elastic fibre (E). No microfibrillar material can be identified. Note the encrustation of the surface of the fibre with proteoglycan granules and thin matrix filaments. X160,000. Scale = 100nm.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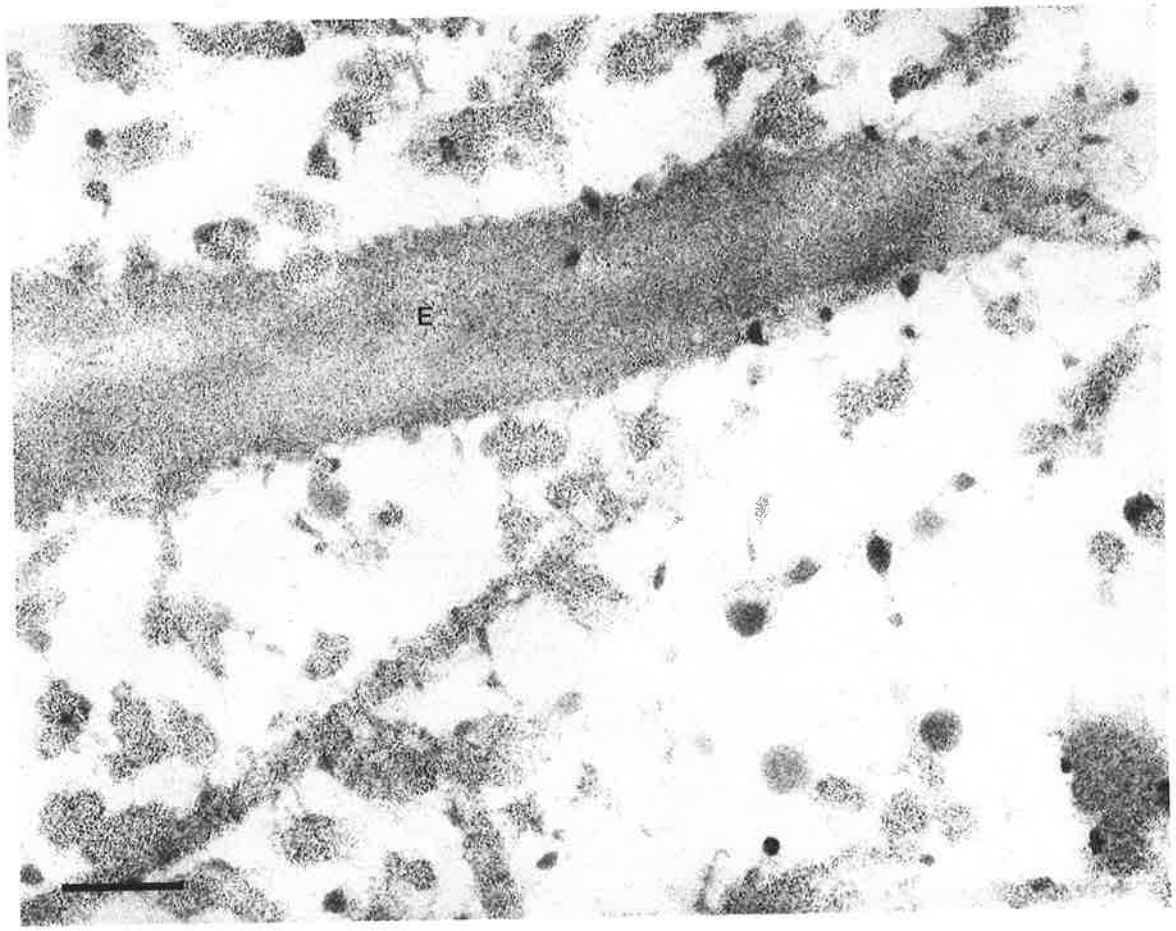
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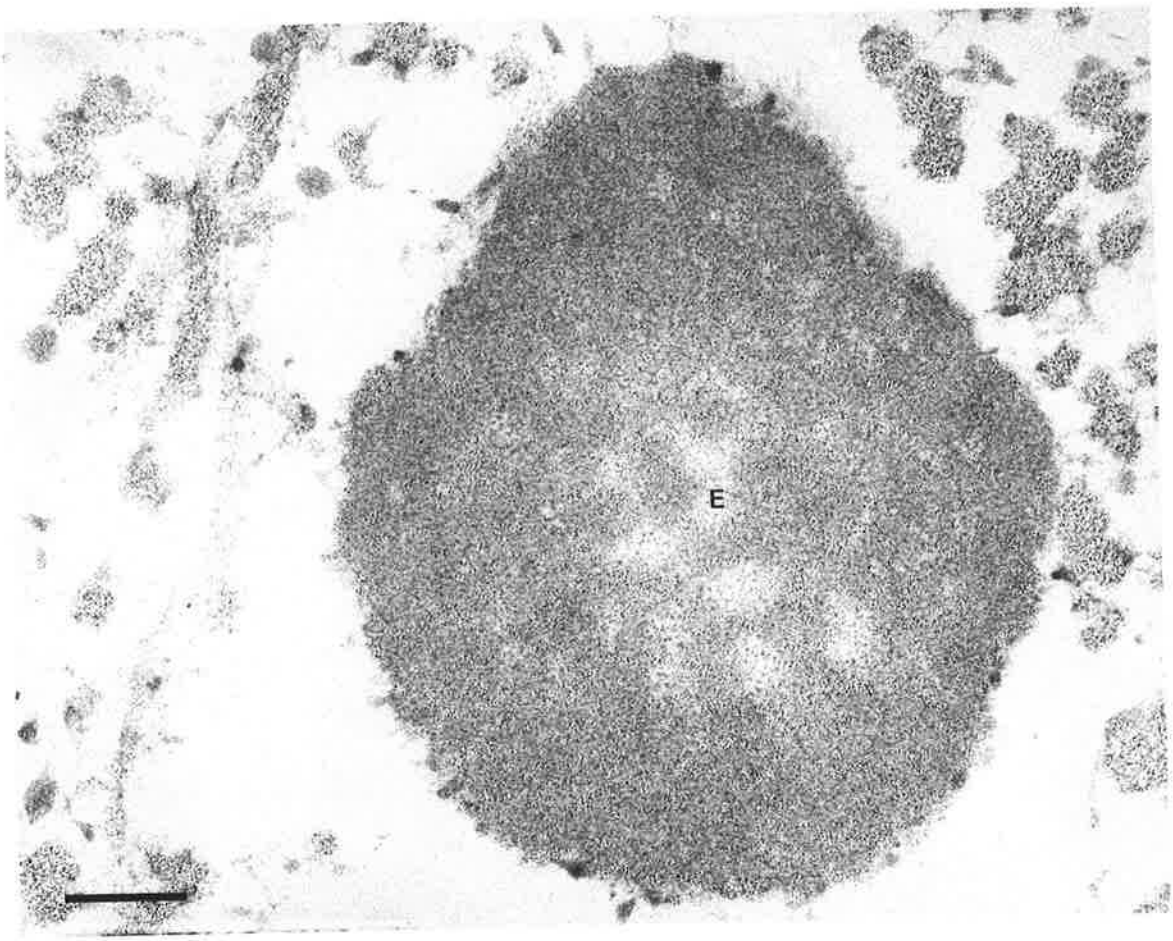
Figure 5.22. Transmission electron micrograph showing high-power detail of a cross-sectioned elastic fibre in native young (16-day-old) rabbit ear cartilage

Note the complete absence of any identifiable microfibrillar material at the periphery of the small elastic fibre (**E**) seen in centre field. Coating of the surface with proteoglycan and matrix filaments is again evident. X160,000. Scale = 100nm.

Figure 5.23. Transmission electron micrograph of an elastic fibre in native young (16-day-old) rabbit ear cartilage showing proteoglycan coating

Note the dense encrustation along the surface of the branching elastic fibre (**E**) seen in this section. Proteoglycan granules and matrix filaments appear to extend into the interstices of the fibre (↑). X160,000. Scale = 100nm.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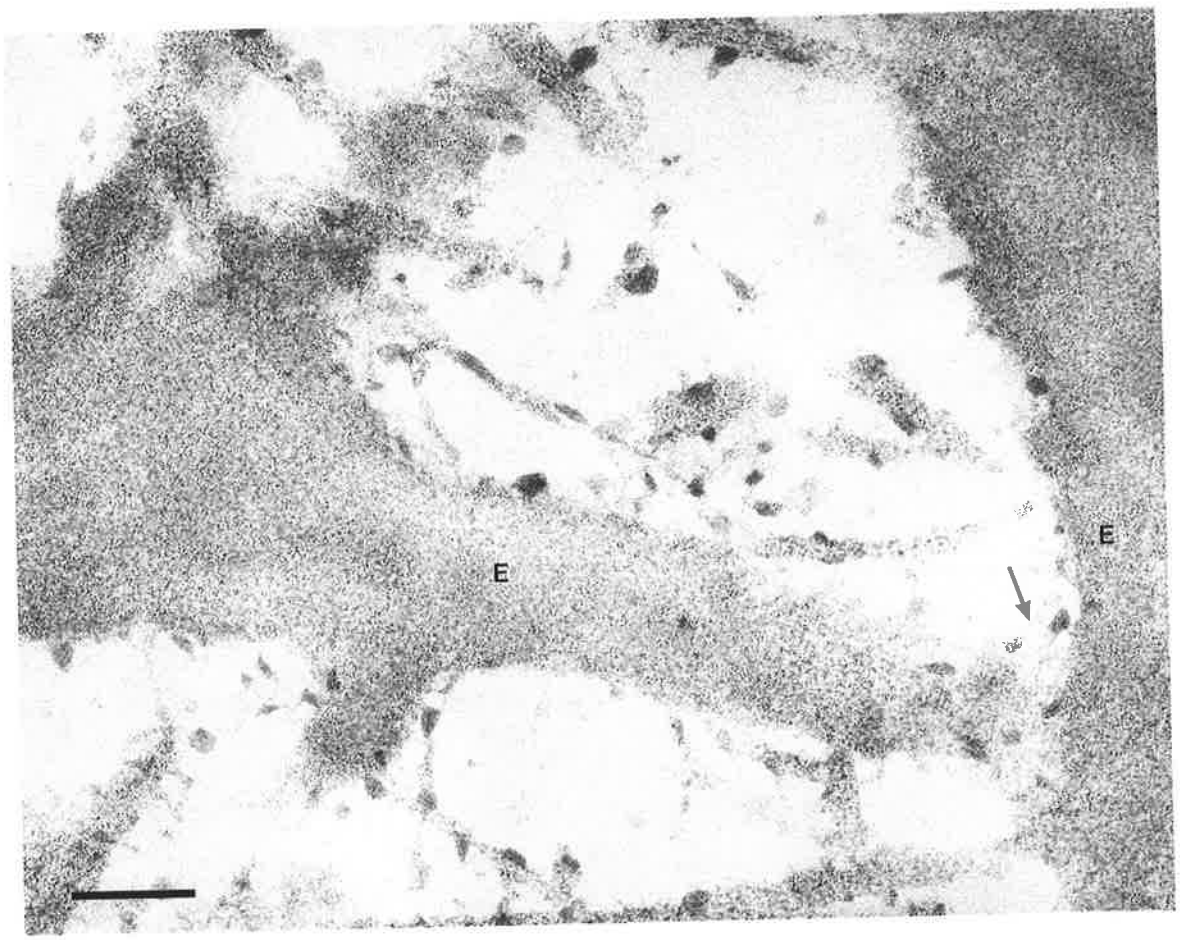
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plates around the cells (Figure 5.20). These large fibres varied in electron density, the central region being of lower density than the edges. Linear densities were often seen within the fibre, creating a marbled effect when examined at low power (Figure 5.20).

The most striking feature, however, was the complete lack of identifiable microfibrillar material in association with the elastic fibres. This was a consistent observation in both large and small elastic fibres. High resolution studies of small elastic fibres confirmed the absence of microfibrils in both longitudinal (Figure 5.21) and transverse (Figure 5.22) sections. The surface of such fibres was heavily coated with matrix granules and thin filaments, which penetrated into the interstices of the fibre (Figure 5.23). This surface alignment of proteoglycan-like material was much denser and more uniform than the seemingly random contact between elastic fibres and other matrix components found in foetal calf and neonatal rabbit ear cartilage.

5.3.3 Macroscopic Appearance of Auricular Chondrocyte Cultures

Dissociated auricular chondrocytes from foetal calf and neonatal and young rabbit ear cartilages were cultured as described in section 5.2.5. Chondrocytes from all three tissues attached readily to the flask surface. Phase contrast microscopy two days after seeding revealed a varied morphology, ranging from elongated spindle-shaped cells to plump polygonal cells with short cytoplasmic processes (Figure 5.24). Cells had large nuclei, often with one or two nucleoli, and binucleate cells were frequently observed. Numerous mitoses were evident. Within five days of seeding, confluent monolayers of rounded or polygonal cells had formed in the flask (Figure 5.25). Cells were closely applied to each other, and only minute quantities of intercellular material were observed. The pattern of growth to this stage was very similar for cells derived from all three tissues, but diverged thereafter.

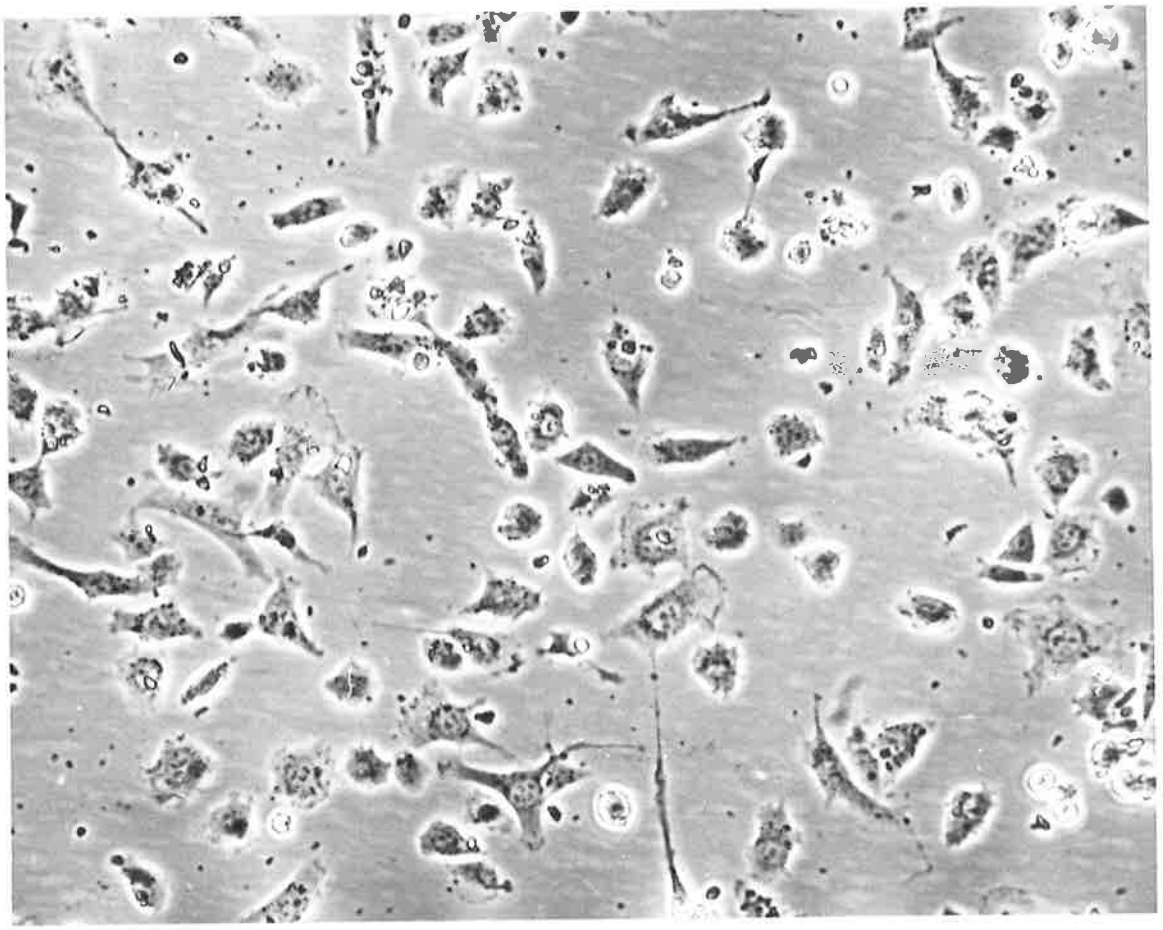
Foetal calf and neonatal rabbit auricular chondrocytes formed multiple overlapping layers of cells, giving rise to a cell layer up to 5 cells in thickness, with small amounts of intercellular matrix visible on light microscopy. Cultures maintained this appearance over long periods of time in culture, showing no tendency to retract or form cell ridges.

Figure 5.24. Phase contrast micrographs of cultures of enzymically-dissociated chondrocytes one day after seeding

Magnification X100. Cultures maintained under scorbutic conditions.

a. Foetal calf auricular chondrocytes.

b. Young (16-day-old) rabbit auricular chondrocytes.



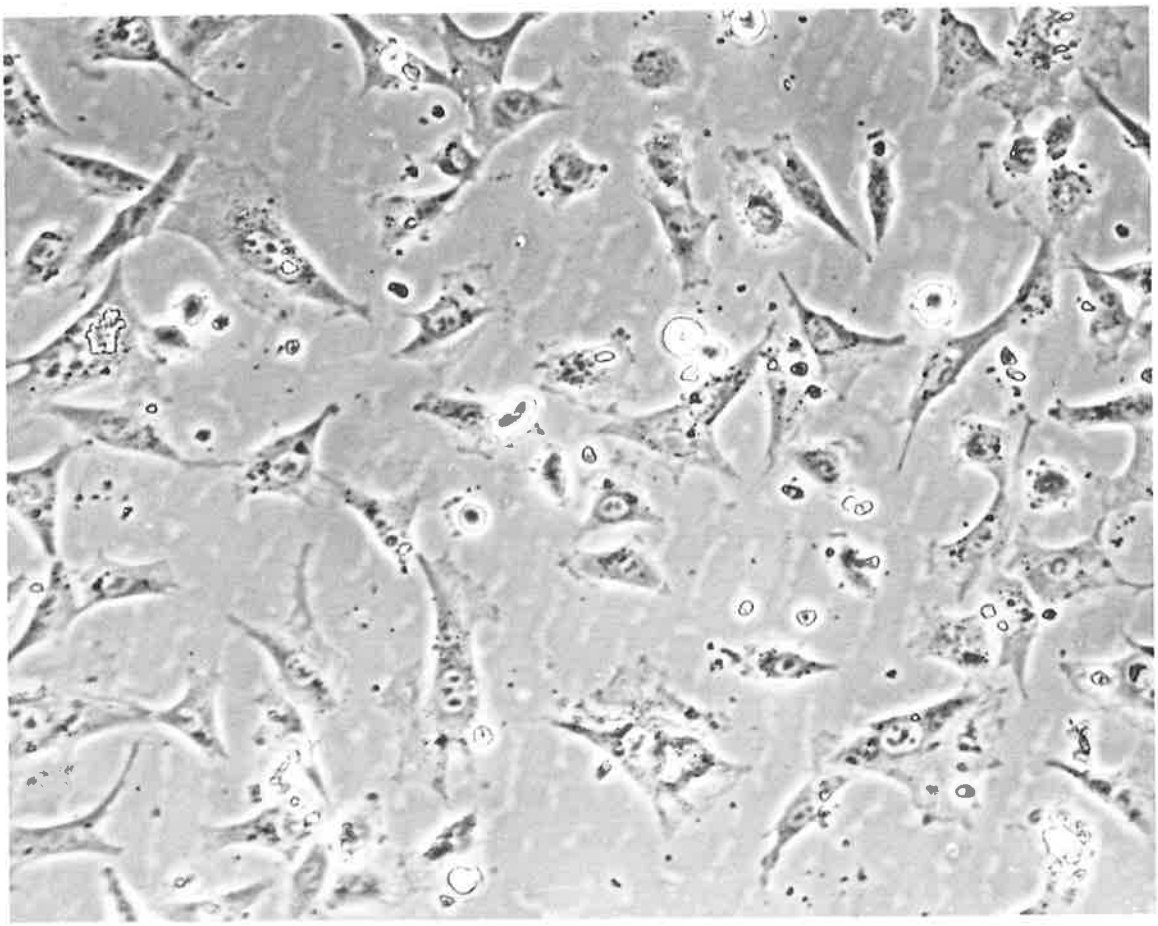
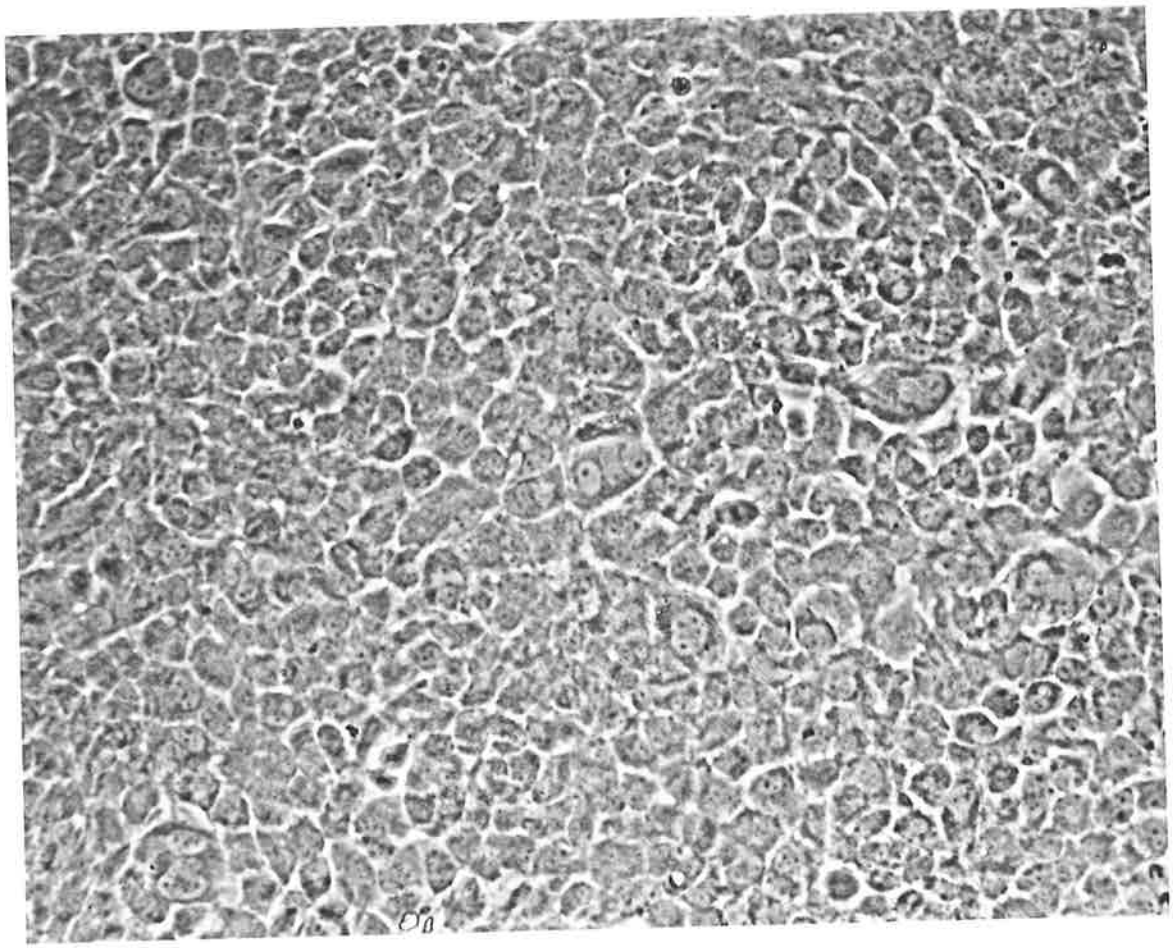


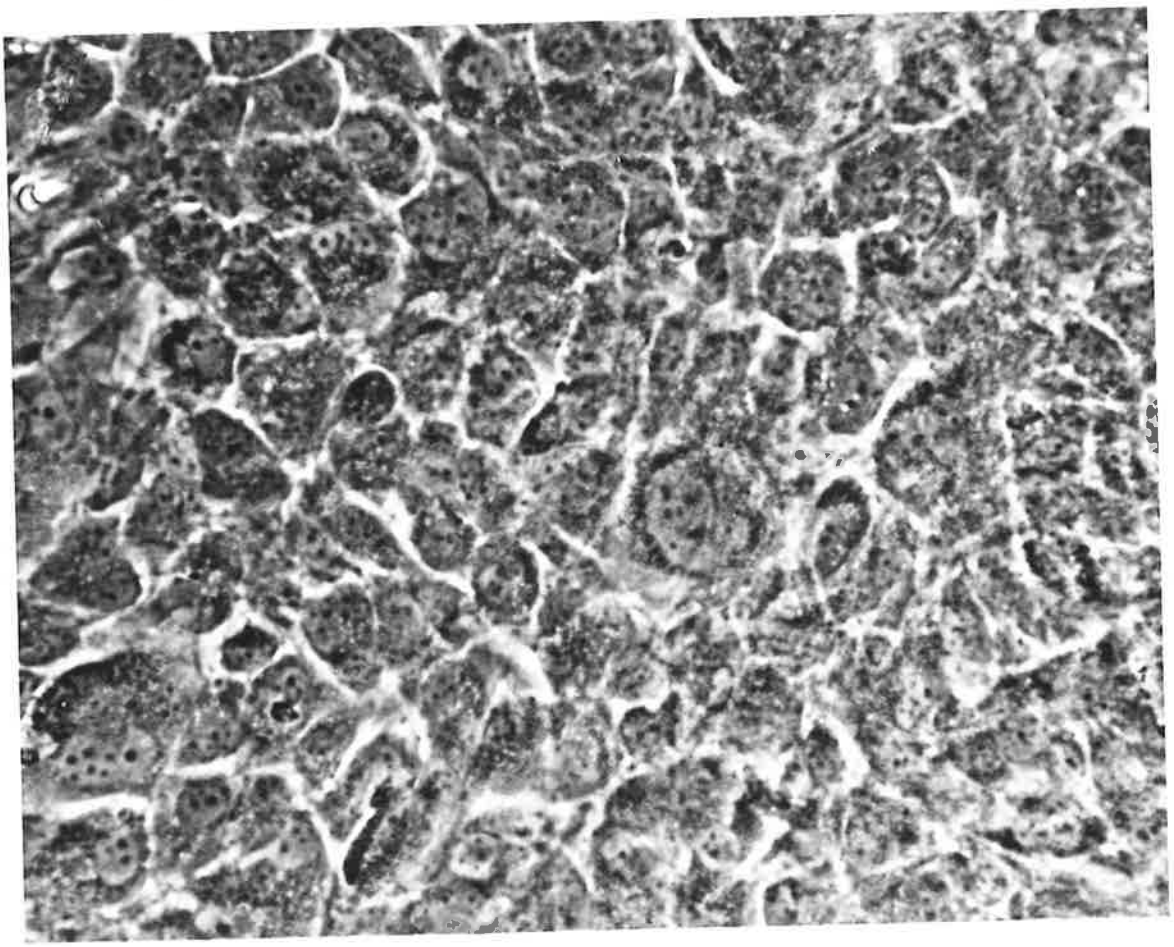
Figure 5.25. Phase contrast micrographs of cultures of enzymically-dissociated chondrocytes 2 days after confluence

Magnification X100. Cultures maintained under scorbutic conditions.

a. Neonatal (2-day-old) rabbit auricular chondrocytes.

b. Young (16-day-old) rabbit auricular chondrocytes.





Young rabbit chondrocytes in culture displayed quite a different pattern of growth, forming dense polylayers of cells surrounded by an extensive extracellular matrix which separated them widely from each other. In some places cells seemed to lie in spaces resembling the lacunae of intact cartilage. At 35 days post-confluence the cells had produced a thick cartilaginous membrane 1-2mm in thickness in the culture flask.

5.3.4 Histology of Auricular Chondrocyte Cultures

Chondrocytes from all three tissues described above were cultured in plastic flasks containing glass coverslips, as outlined in section 5.2.6. Thirty-five days after confluence, the cultures were fixed, coverslips removed, and the cell layer stained for elastic fibres with Miller stain (see section 5.2.3). The light microscopic appearances were as follows:

a. Foetal calf auricular chondrocyte cultures (Figure 5.26). Cultures consisted of several layers of small cells with rounded nuclei, separated by small amounts of intercellular matrix containing elastic fibres. These thin fibres formed a delicate, branching network between the cells, often looping around them to encircle the cell space. Some larger calibre, irregular elastic fibres were incorporated in this network. No other formed elements were identified in the matrix.

b. Neonatal rabbit auricular chondrocyte cultures (Figure 5.27). The cell layer was composed of rather smaller cells than those seen in foetal calf cultures. They tended to pile over each other, obscuring matrix detail somewhat. A modest amount of intercellular material was present, which contained a network of tiny elastic fibres randomly dispersed throughout the matrix. In addition a few larger fibres could be seen coursing between the cells for a short distance. The overall amount of stainable elastin was much less than in foetal calf cultures.

c. Young rabbit auricular chondrocyte cultures (Figure 5.28). The histological appearance of young rabbit cultures differed strikingly from both foetal calf and neonatal rabbit cultures. As described in section 5.3.3, young rabbit auricular chondrocytes formed a thick cartilaginous membrane, in which cells were embedded, often

Figure 5.26. Light micrograph of a culture of enzymically-dissociated foetal calf auricular chondrocytes 35 days after confluence

Chondrocytes were cultured in flasks containing glass coverslips. 35 days after confluence, the cultures were fixed with 2.5% (w/v) glutaraldehyde, the coverslips removed, and mounted on glass slides. The cell layer was then stained for elastic fibres with Miller's elastic stain. Note the sparse extracellular matrix, which contains delicate elastic fibres forming a branching network surrounding the cell spaces. Some of these fibres appear to be composed of punctate globules of elastic-staining material. Culture maintained under scorbutic conditions. Magnification X500.

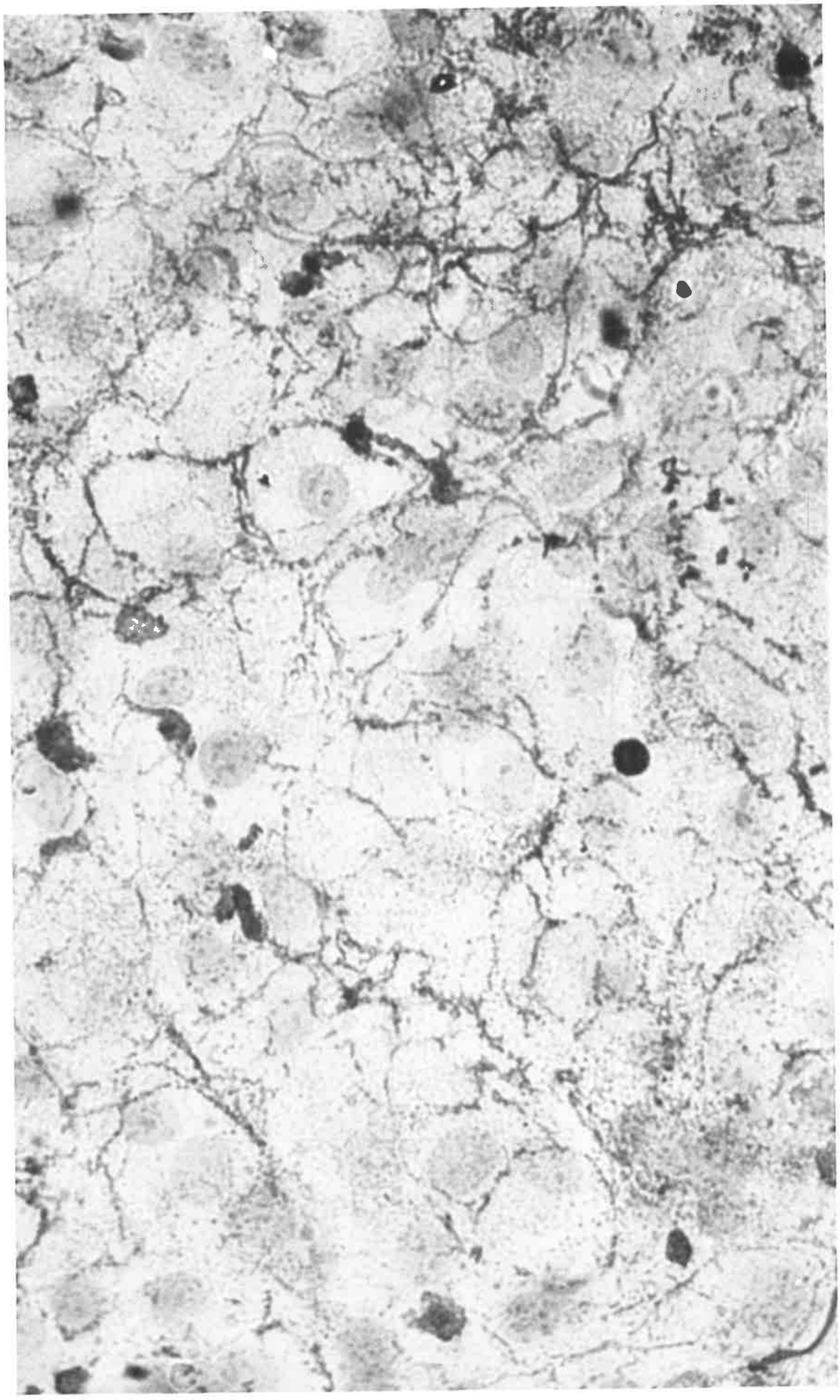
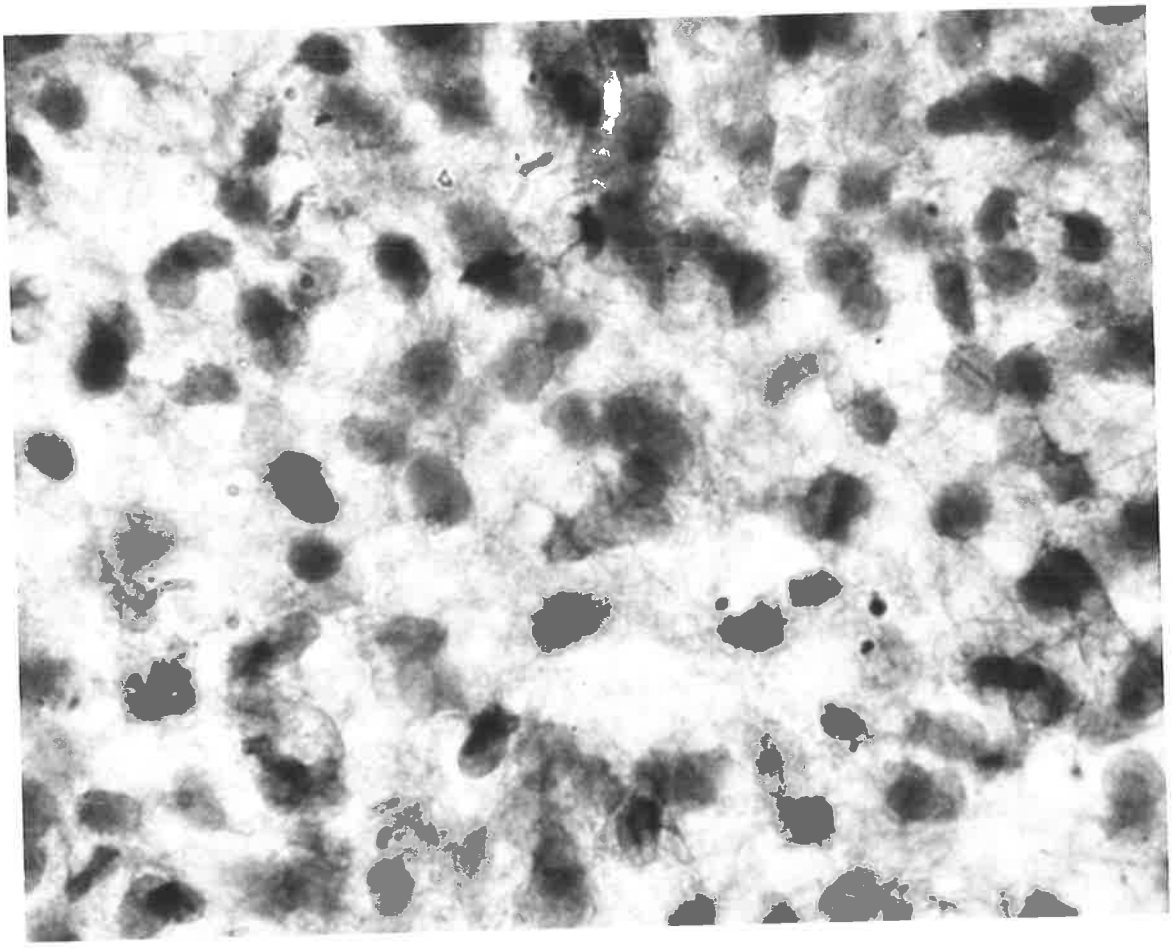


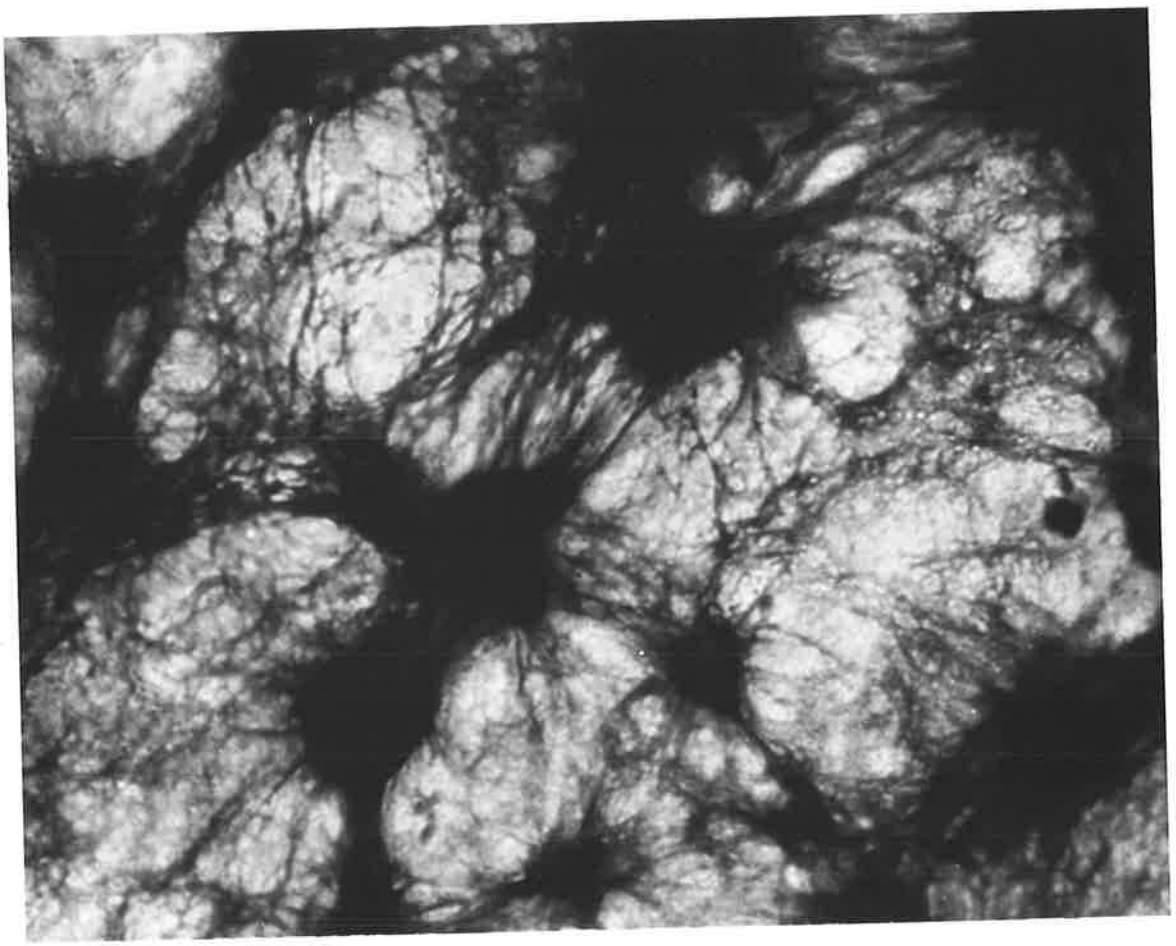
Figure 5.27. Light micrograph of a culture of enzymically-dissociated neonatal (2-day-old) rabbit auricular chondrocytes 35 days after confluence

Prepared by an identical technique to that outlined in Figure 5.26. The cells appear smaller than those seen in foetal calf auricular chondrocyte cultures (cf. Figure 5.26) and there is little apparent extracellular matrix present. Note the fine feltwork of elastic-staining fibres which are dispersed throughout the matrix. Culture maintained under scorbutic conditions. Miller's elastic stain. Magnification X250.

Figure 5.28. Light micrograph of a culture of enzymically-dissociated young (16-day-old) rabbit auricular chondrocytes 35 days after confluence

Prepared by an identical technique to that outlined in Figure 5.26. Note the extensive extracellular matrix with large intercellular spaces and dense network of elastic-staining fibres. These fibres appeared concentrated in the pericellular region, obscuring cell detail. Culture maintained under scorbutic conditions. Miller's elastic stain. Magnification X250.





separated by very large intercellular spaces. After elastic staining, cells were obscured by a dense capsule of elastic fibres occupying the pericellular space, from which smaller fibres radiated out into the intercellular matrix, ramifying to form a complex, tangled network of fibres interconnecting cells at all levels. So dense was the proliferation of elastic fibres that no cell or other matrix details could be seen.

5.3.5 Ultrastructure of Auricular Chondrocyte Cultures

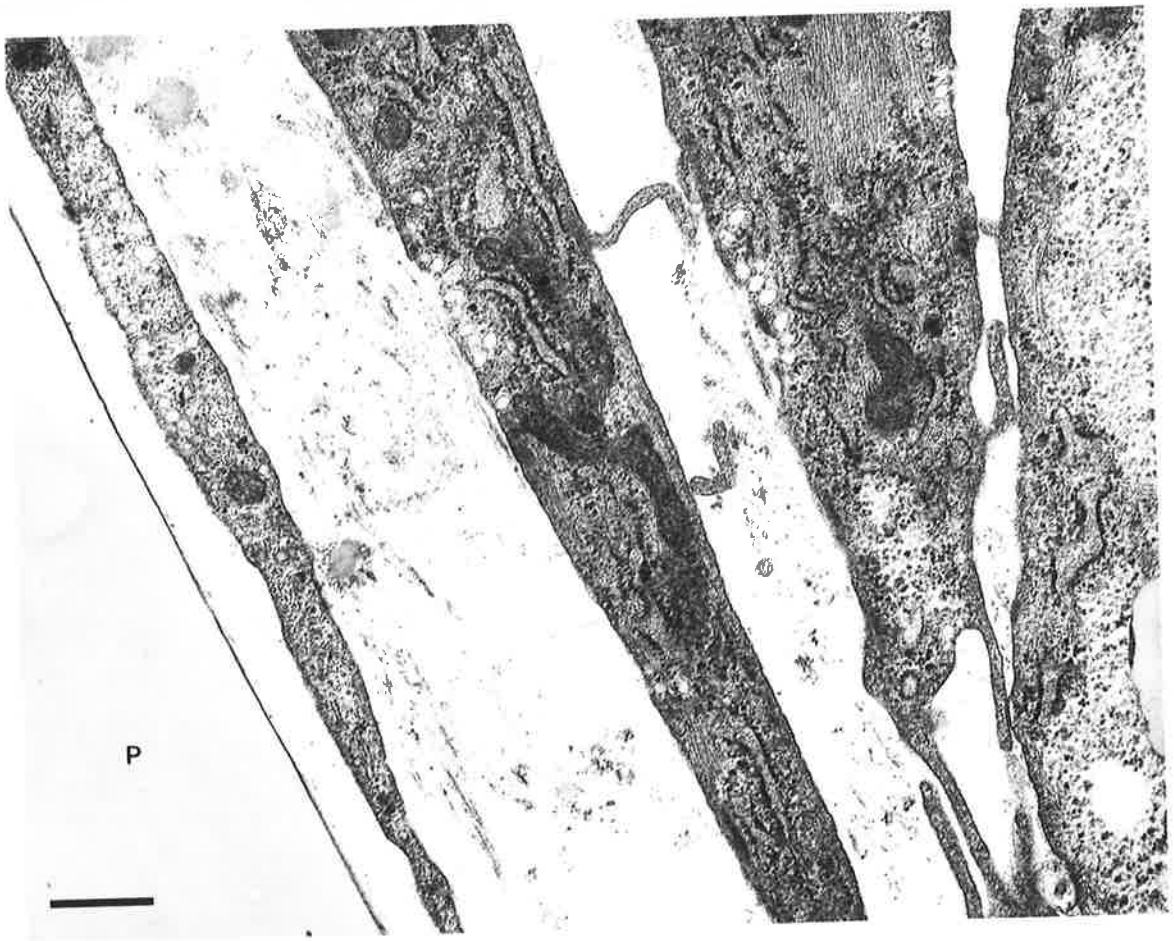
Auricular chondrocyte cultures derived from foetal calf and neonatal and young rabbit ear cartilage were maintained in plastic culture flasks as described in section 5.2.5. Cultures were fixed and processed in situ for electron microscopy at 15 and 35 days post-confluence (see sections 4.2.4 and 5.2.6).

a. Foetal calf auricular chondrocyte cultures

15 days post-confluence. The cultures consisted of multiple overlapping layers of elongated cells (Figure 5.29), the organelle complement of which corresponded closely to that observed in native foetal calf ear cartilage (see section 5.3.2a). In the superficial parts of the culture, the cells were closely apposed, but in the basal layers cells were separated from each other and the plastic culture surface by a scanty intercellular matrix. The plasma membrane was often irregular in outline and frequent pseudopodia and phago/exocytic inpouchings were observed (Figure 5.30). The cells appeared metabolically active with a well-developed RER and prominent Golgi complex (Figure 5.30) from which numerous smooth and coated vesicles were seen to arise. Well developed tracts of cytoplasmic microfilaments were seen (Figure 5.31), usually lying close to the plasma membrane and often surrounding dilated cisternae of RER and associated mitochondria. Superficially situated cells often contained large amounts of glycogen granules, forming cytoplasmic 'lakes'. Lipid droplets were frequently observed lying within these glycogen 'lakes'. Such droplets were never membrane-bound and were not surrounded by microfilament 'capsules' as were observed in native ear cartilage (see Figure 5.5).

Figure 5.29. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 15 days after confluence, showing general characteristics
The cell layer consists of overlapping, elongated cells which have numerous pseudopodia. Note the well-developed rough endoplasmic reticulum, numerous mitochondria and large amounts of cytoplasmic glycogen. The plastic culture surface (**P**) is to the left of the micrograph. Culture X27,000. Scale = 0.5μm.

Figure 5.30. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 15 days after confluence, showing cellular details
Note the Golgi complex (**G**) and numerous cytoplasmic smooth and coated vesicles, some of which approach and appear to merge with the cell membrane (**↓**). The cell on the right of the micrograph contains a large intracellular accumulation of granular material, probably glycogen. A small elastic fibre can be seen attached to the plastic culture surface (**P**). Culture maintained under scorbutic conditions. X27,000.
Scale = 0.5μm.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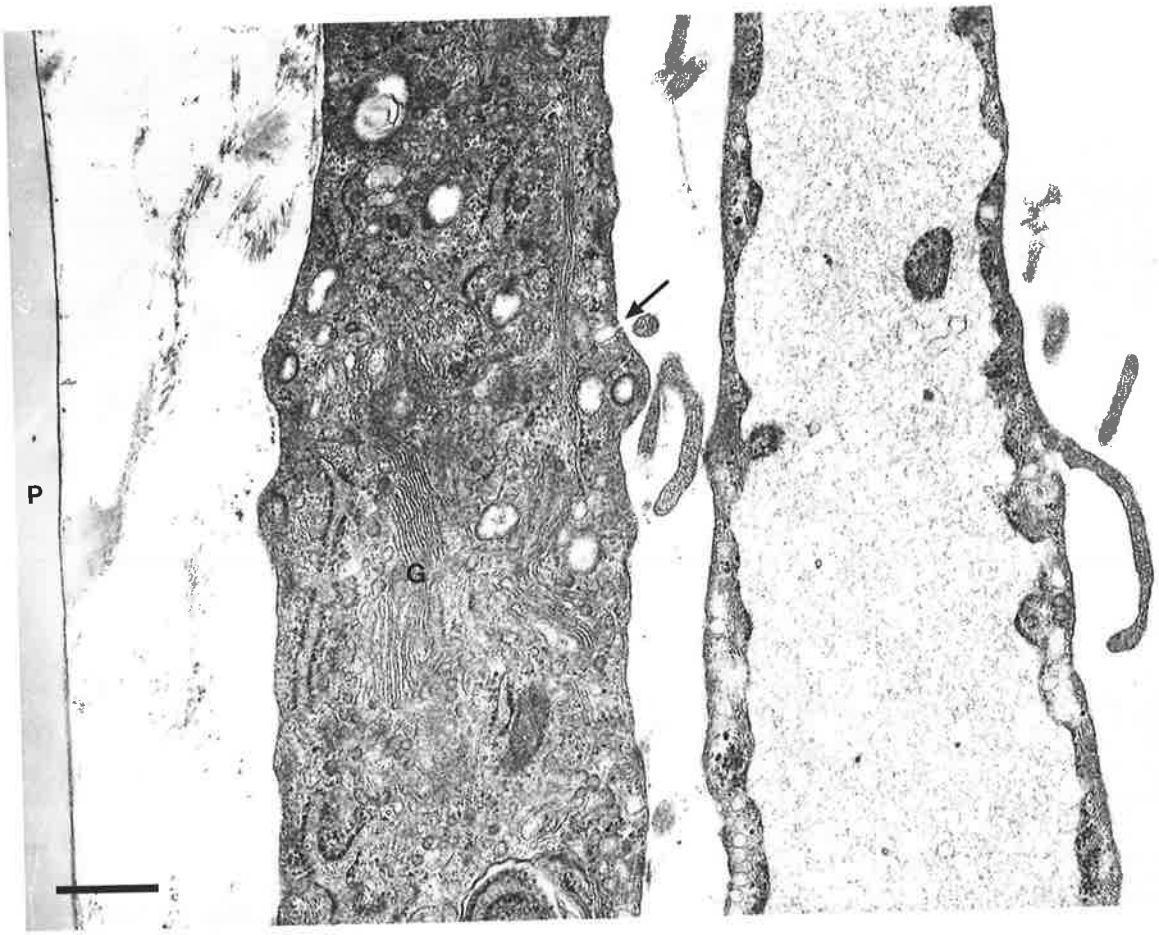
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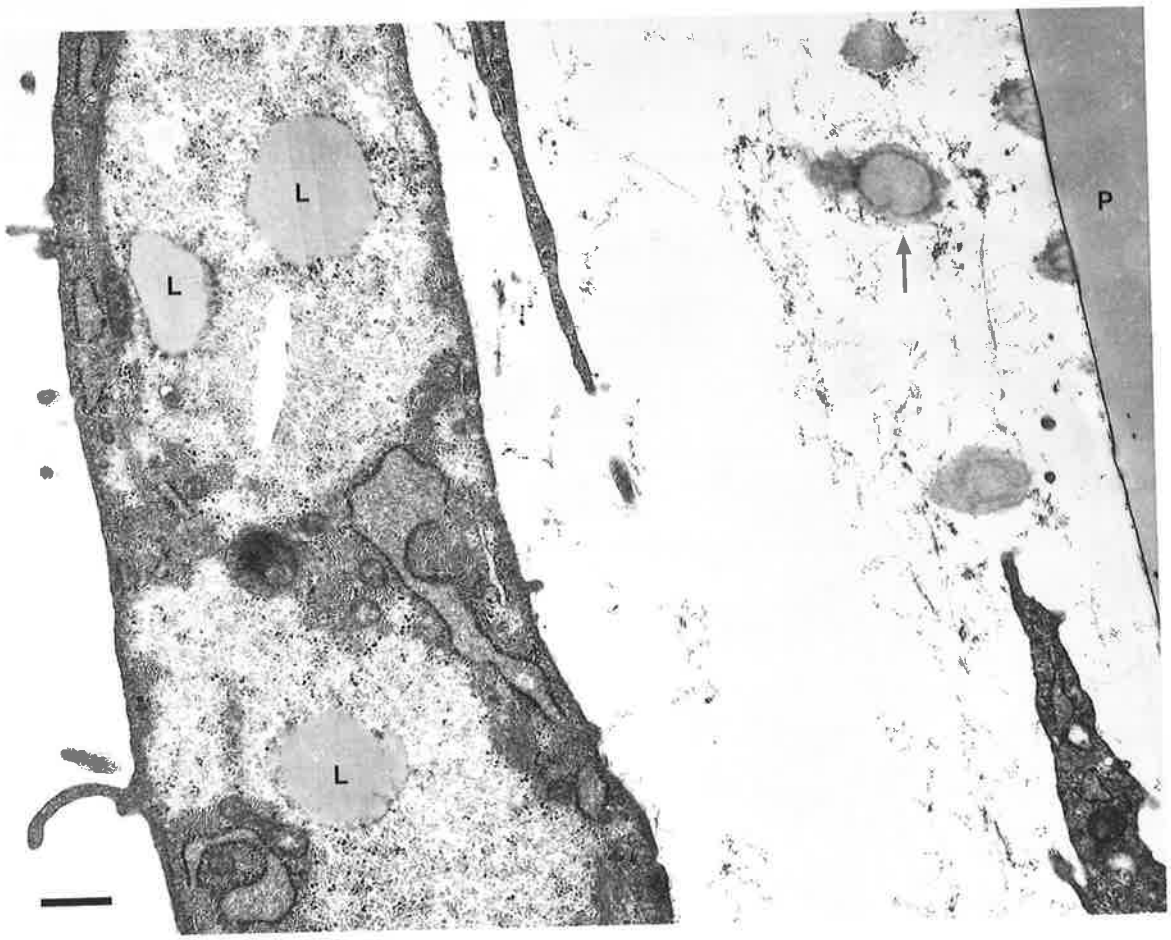
Figure 5.31 Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 15 days after confluence, showing details of a cell and adjacent matrix
Several lipid droplets (**L**) can be seen lying within a cytoplasmic 'lake' of glycogen granules. Cell organelles, including rough endoplasmic reticulum and mitochondria, which border this 'lake' are surrounded by a network of microfilaments. Several small elastic fibres are seen in the extracellular matrix, lying close to the plastic culture surface (**P**). Some of these fibres appear to have a central lighter-staining core, surrounded by a rim of lighter staining elastin (↑). Small numbers of elastin-associated microfibrils can be seen in association with these elastic fibres. Culture maintained under scorbutic conditions. X18,000. Scale = 0.5μm.

Figure 5.32. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 15 days after confluence, showing high-power detail of elastic fibres
Several small elastic fibres (**E**) can be seen lying between 2 adjacent cells. These fibres are surrounded by randomly-oriented microfibrils. Microfibrils cut in cross-section have a typical tubular appearance (↑). Culture maintained under scorbutic conditions. X160,000. Scale = 100nm.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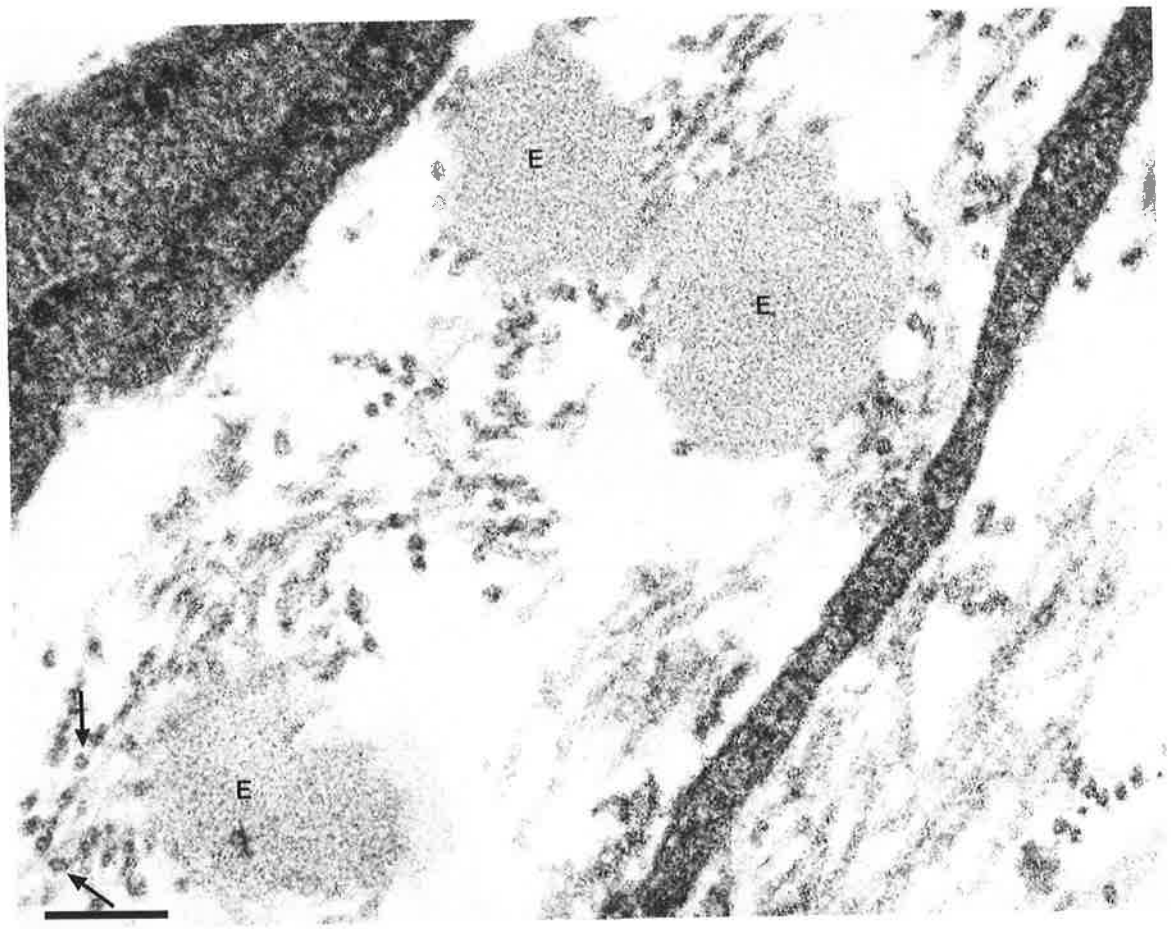
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The intercellular matrix consisted primarily of bundles of randomly oriented microfibrils interspersed with isolated clumps of amorphous material which had the staining characteristics of amorphous elastin (Figure 5.32). Only minute amounts of matrix material were observed between cells lying superficially in the cultures, and identifiable elastic fibres were confined to the deepest parts of the cell layer, sometimes apparently attached to the plastic culture surface. In some sections the amorphous component appeared to consist of a central lightly-stained core surrounded by a thin rim of more electron-dense material (Figure 5.31). Such elastic fibres had only a very sparse mantle of microfibrils.

Occasional larger fibres, some with a hint of cross-striation, were seen, but no typical collagen fibrils could be identified. Small amounts of material resembling the 'stellate reticulum' of native cartilage was scattered throughout the matrix, often intimately associated with the plasma membrane and formed elements of the matrix (Figure 5.31).

35 days post-confluence. Although culture morphology was similar to that described above, cells appeared more attenuated in outline, and occasional degenerate cells were evident, especially in the basal layer of the culture (Figure 5.33).

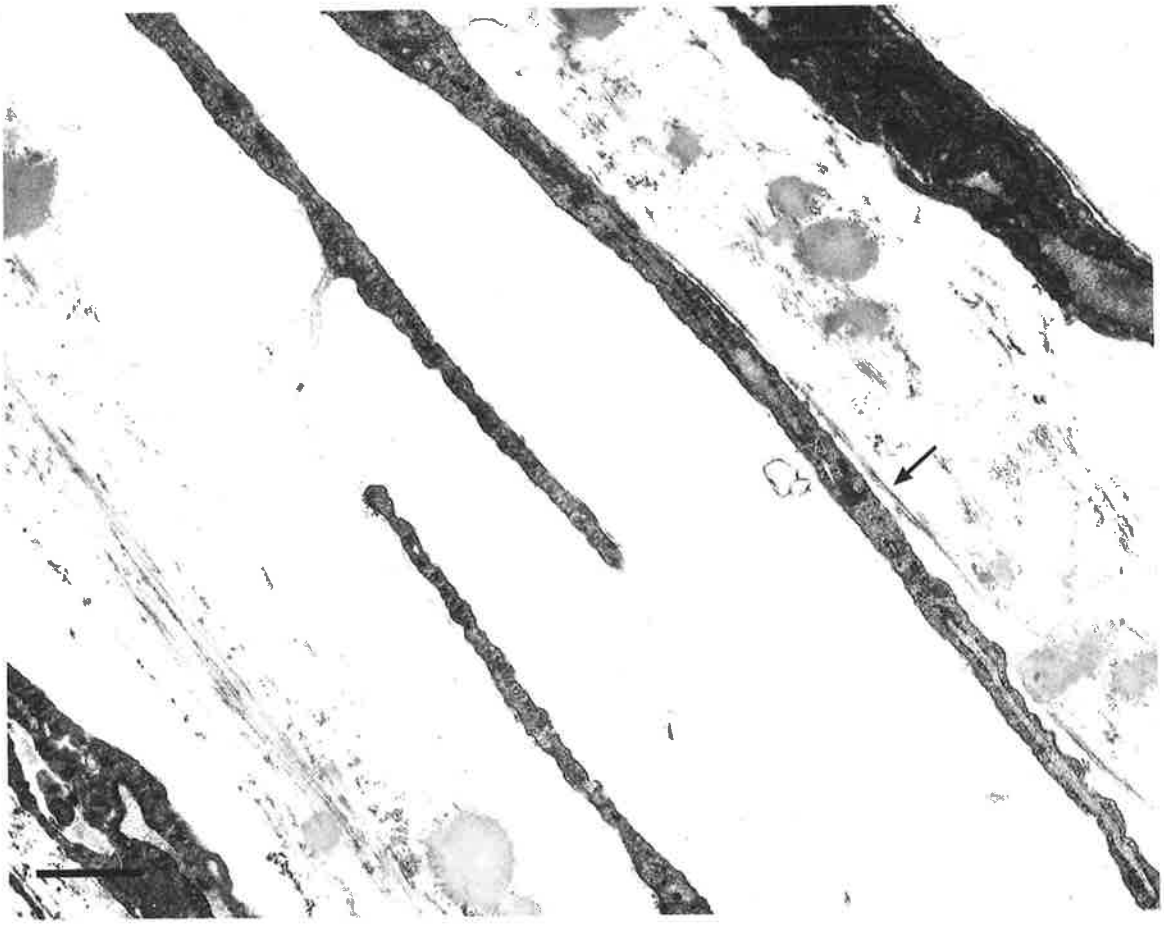
Elastic fibres were the dominant feature of the extracellular matrix, usually associated with arrays of microfibrils. These fibres appeared better developed than in cultures examined at 15 days post-confluence, and usually lay about mid-way between the cells. At high power, microfibrils of typical morphology could be distinguished at the periphery of such fibres (Figure 5.34).

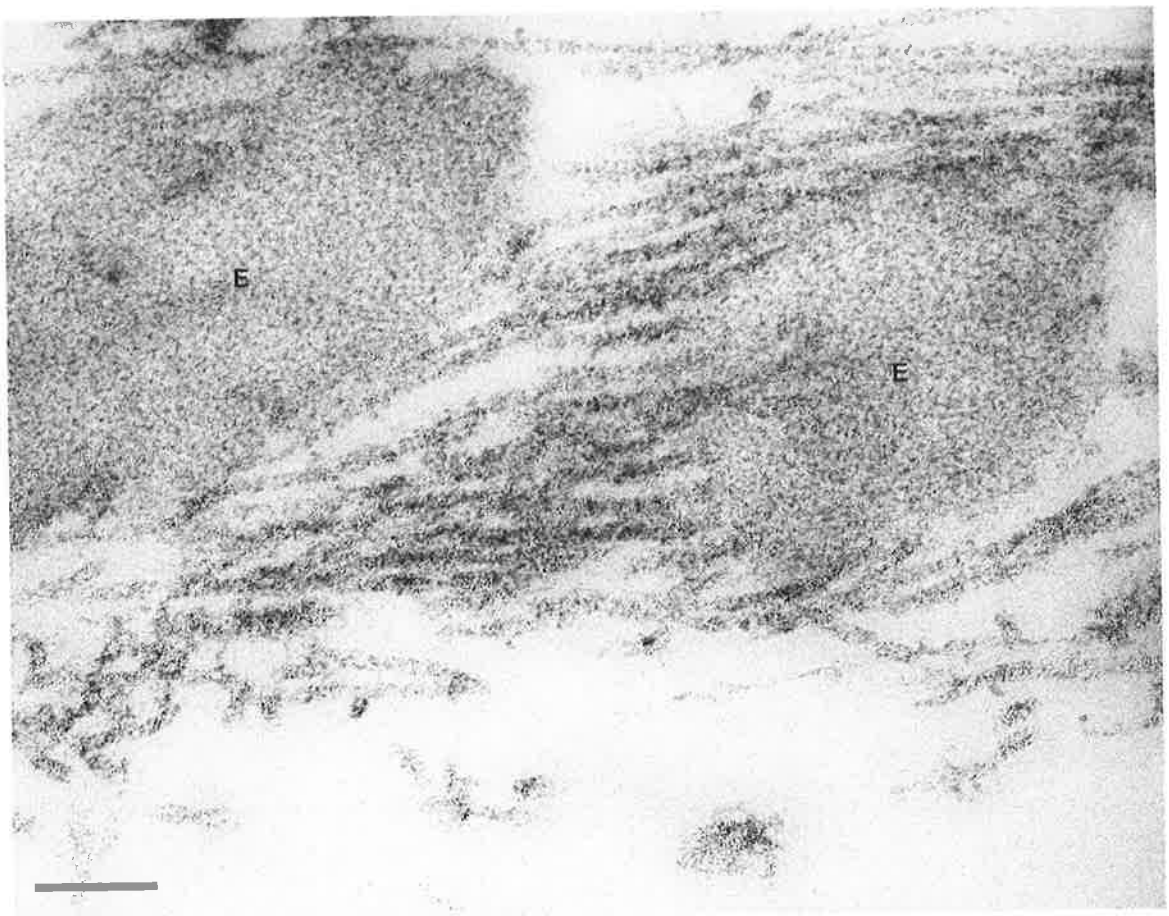
A few larger fibres possessing indistinct cross-striations were observed, often in close proximity to the cell membrane. Sparse amounts of proteoglycan-like material was scattered amongst the other formed elements of the matrix.

At both stages much smaller amounts of intercellular matrix were seen in culture than in native foetal calf ear cartilage of comparable donor age, and there was a very marked reduction particularly of recognizable proteoglycan and collagen. As these cultures were maintained under scorbutic conditions, parallel cultures were

Figure 5.33. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 35 days after confluence, showing general characteristics
Note the very attenuated cell profiles and large intercellular distances. The extracellular matrix can be seen to consist predominantly of small elastic fibres associated with arrays of microfibrils. A few larger fibres, some with indistinct cross-striations, are seen closely apposed to the cell membrane (↑). The plastic culture surface (not seen in the micrograph) is toward the top right. Culture maintained under scorbutic conditions. X27,000. Scale = 0.5µm.

Figure 5.34. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes at 35 days after confluence, showing high-power detail of an elastic fibre
Parts of 2 small elastic fibres (E) can be seen, surrounded by microfibrils. These microfibrils have an indistinct periodicity when seen in longitudinal section. Culture maintained under scorbutic conditions. X160,000. Scale = 100nm.

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supplemented with ascorbate to assess the qualitative effects on matrix production. The results of this study are presented in section 5.3.6.

b. Neonatal (2-day-old) rabbit auricular chondrocyte cultures

15 days post-confluence. At this stage the cultures were composed of flattened chondroblasts arranged in overlapping layers some 2-3 cells thick. Unfortunately the embedding medium did not adhere strongly to the surface of the plastic culture dish, resulting in tearing of ultrathin sections on the knife edge. Thus only rather thicker sections could be obtained (silver-gold interference colour), with consequent impairment of the resolution of cell detail (Figure 5.35). However such cells had a well-developed RER, with dilated cisternae, large, often lobulated nuclei and frequent matrix vacuoles. No lipid droplets were seen within the cytoplasm.

The intercellular matrix of such cultures was qualitatively similar to that observed in cultures of foetal calf auricular chondrocytes, consisting predominantly of small elastic fibres interspersed with haphazardly-oriented tracts of microfibrils (Figure 5.36). Transversely sectioned microfibrils were seen to consist of a darkly staining periphery and a less electron-dense core. They had an estimated diameter of 12-14nm. Occasional larger fibres were seen, but these were not clearly cross-striated. No typical collagen fibrils were observed. Very little proteoglycan-like material was in evidence, but thin, wispy fibrils were scattered through the matrix, sometimes forming small accretions.

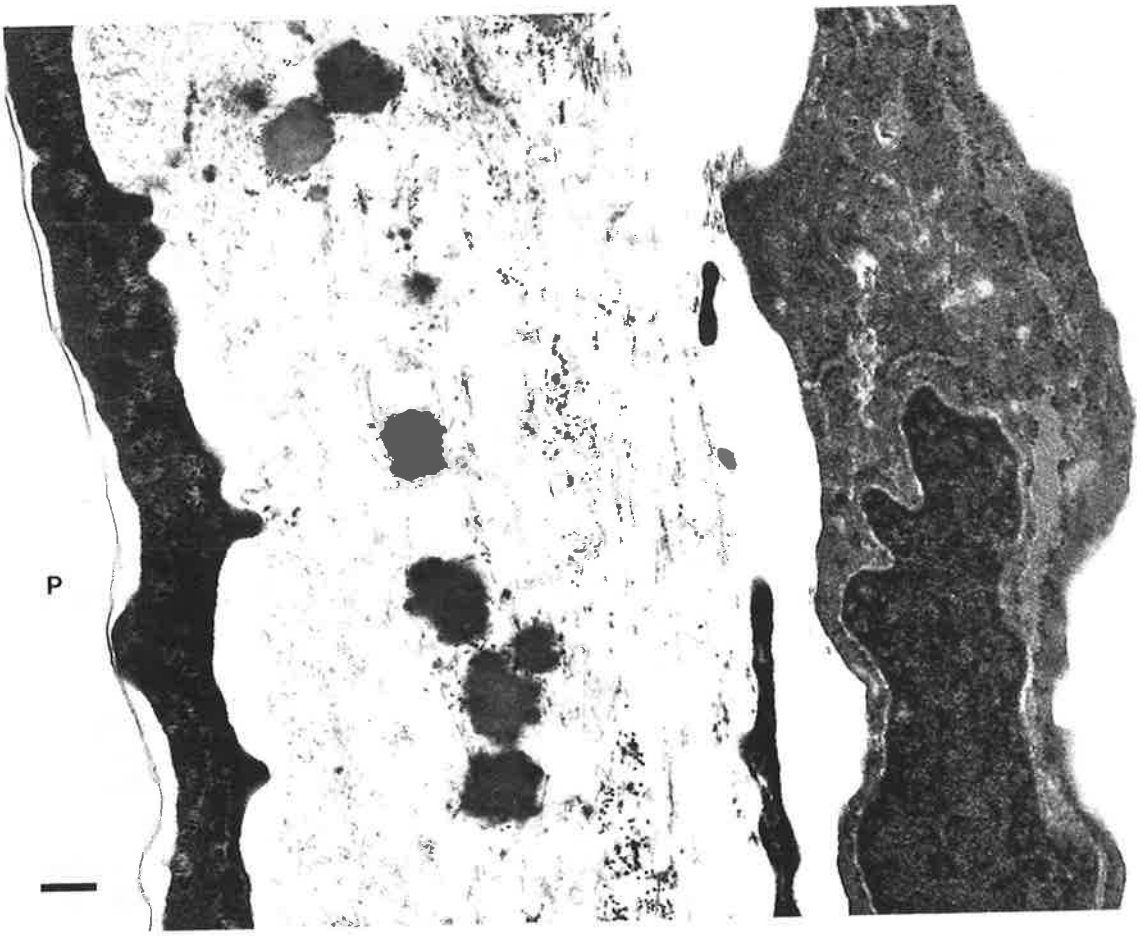
35 days post-confluence. The cell layer had become much thicker (about 15µm in depth) and degenerate cells were frequently observed in deeper parts of the culture (Figure 5.37). Technical difficulties were again encountered due to differences in the hardness of the plastic culture flask and the embedding medium, resulting in compression of thin sections at the interface of their surfaces. Consequently, cells situated near the plastic surface appeared very dark due to the increased thickness of the section in this area. Superficially situated cells appeared viable, with a well-developed RER, frequent mitochondria and cytoplasmic collections of glycogen granules.

Figure 5.35. Transmission electron micrograph of a culture of neonatal (2-day-old) rabbit auricular chondrocytes at 15 days after confluence, showing general characteristics

Cells appear very dark due to compression of the section at the plastic-resin interface. Several longitudinally-flattened cells can be seen, but cellular details cannot be clearly distinguished. The extracellular matrix consists predominantly of small, electon-dense elastic fibres and associated microfibrils. The plastic culture surface (P) is at the left of the micrograph. Culture maintained under scorbutic conditions. X36,000. Scale = 200nm.

Figure 5.36. Transmission electron micrograph of a culture of neonatal (2-day-old) rabbit auricular chondrocytes at 15 days after confluence, showing high-power detail of an elastic fibre

A small elastic fibre (E) is shown with associated microfibrils. Cross-sectioned microfibrils (↑) show typical morphology. Culture maintained under scorbutic conditions. X155,000. Scale = 100nm.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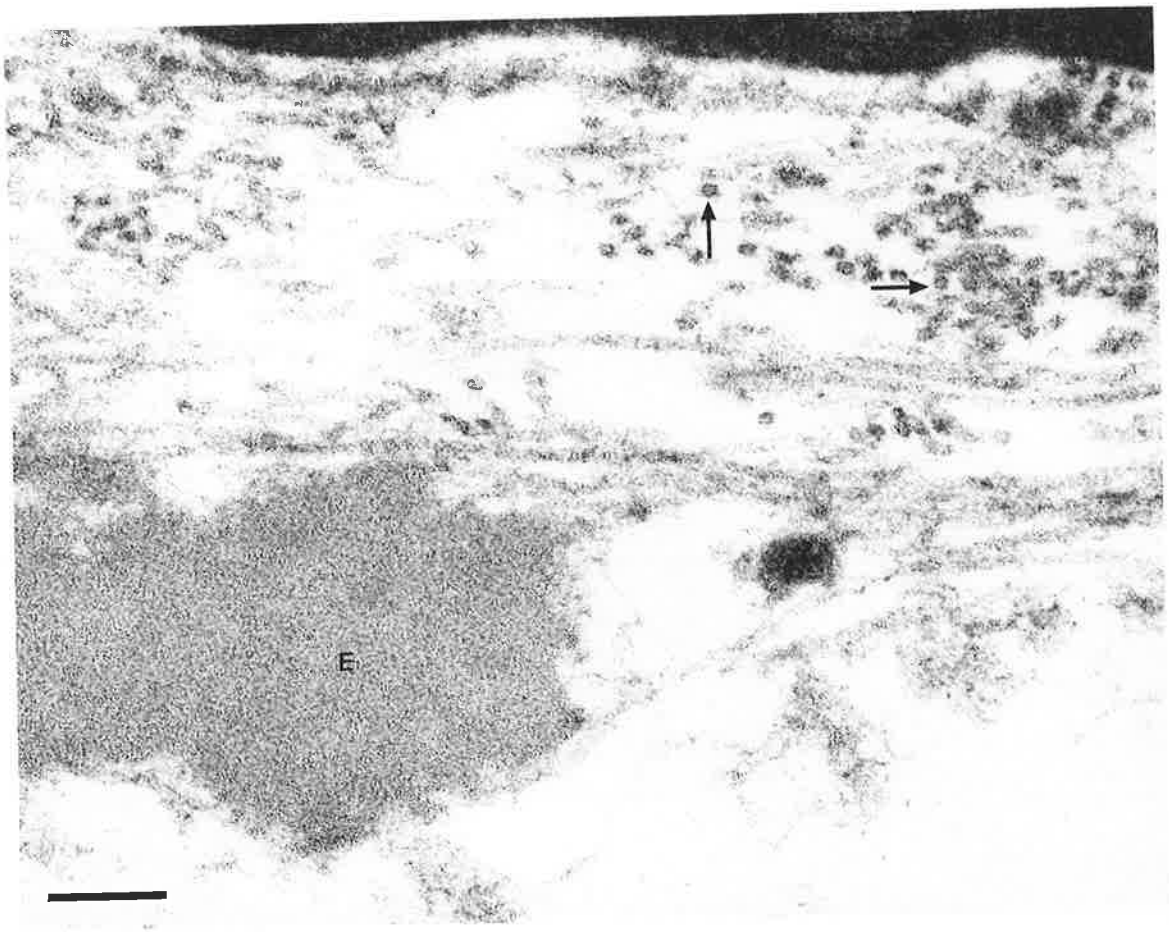
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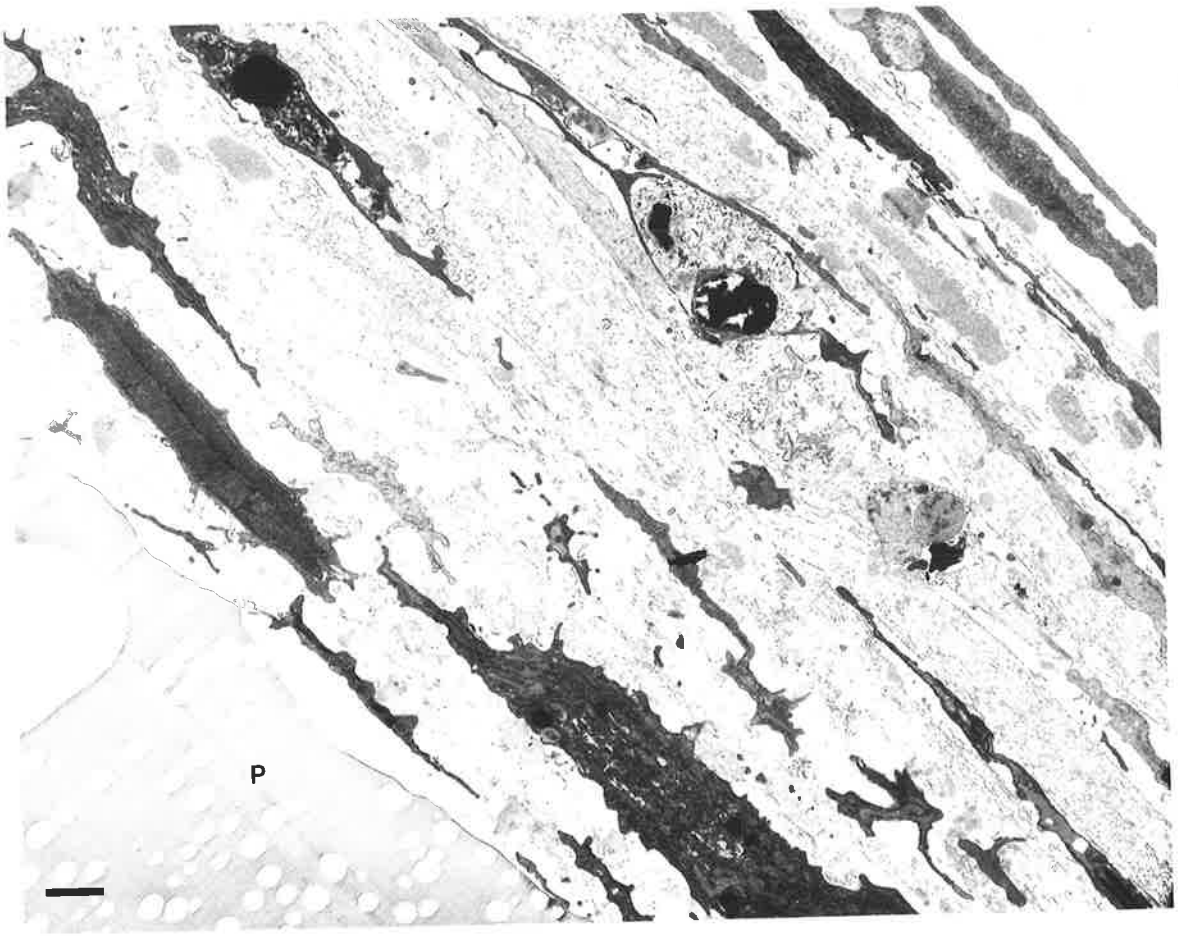
Figure 5.37. Transmission electron micrograph of a culture of neonatal (2-day-old) rabbit auricular chondrocytes at 35 days after confluence

The cell layer can be seen to be much deeper than at 15 days after confluence (cf. Figure 5.35) and several degenerate cells are evident. The extracellular matrix is also more extensive. The plastic culture surface (**P**) is at the left of the micrograph. Culture maintained under scorbutic conditions. X7,500. Scale = 1µm.

Figure 5.38. Transmission electron micrograph of a culture of neonatal (2-day-old) rabbit auricular chondrocytes at 35 days after confluence, showing details of the matrix

Several small elastic fibres (**E**) with peripheral microfibrils can be seen. Collections of granular amorphous material are also seen in the matrix (↟). Matrix granules and fine filaments are present, but do not appear to form an organized 'stellate reticulum'. The plastic culture surface (**P**) is to the right of the micrograph. Culture maintained under scorbutic conditions. X22,000. Scale = 0.5µm.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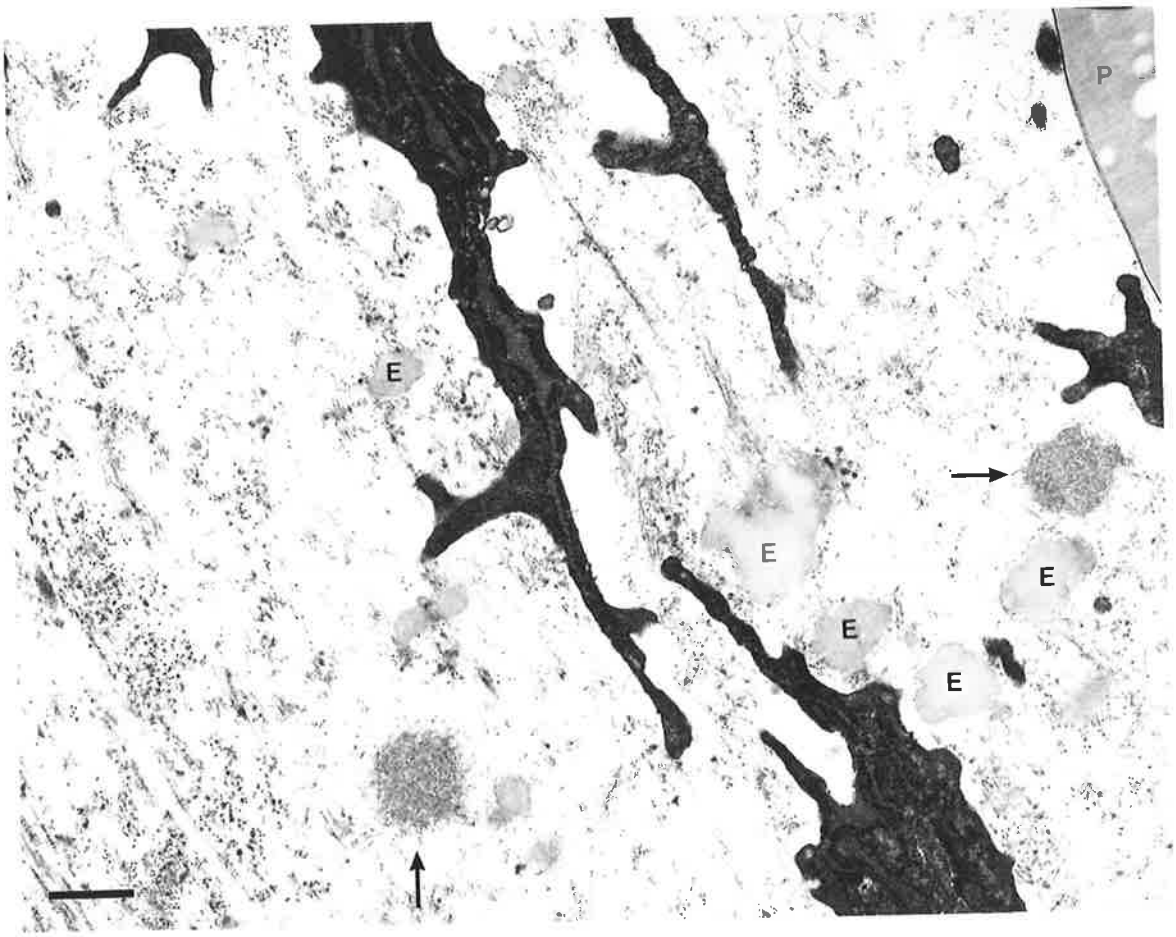
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The intercellular matrix was considerably more abundant, and although elastic fibres were still present, they were less prominent than at 15 days post-confluence (Figure 5.38). Few microfibrils of typical appearance were seen in association with the elastic fibres, and these appeared only very loosely organized. Tracts of larger fibrils could be seen, again not clearly cross-striated. Proportionately larger amounts of matrix granules and fine filaments were observed, but these did not form a typical 'stellate reticulum' such as that observed in the native tissue.

Large collections of granular, electron-dense material were observed (Figure 5.38). This material was not seen in cultures at 15 days post-confluence, and was frequently found in the region of degenerating cells, suggesting that it is probably cell debris, perhaps of nuclear origin.

c. Young (16-day-old) rabbit auricular chondrocyte cultures

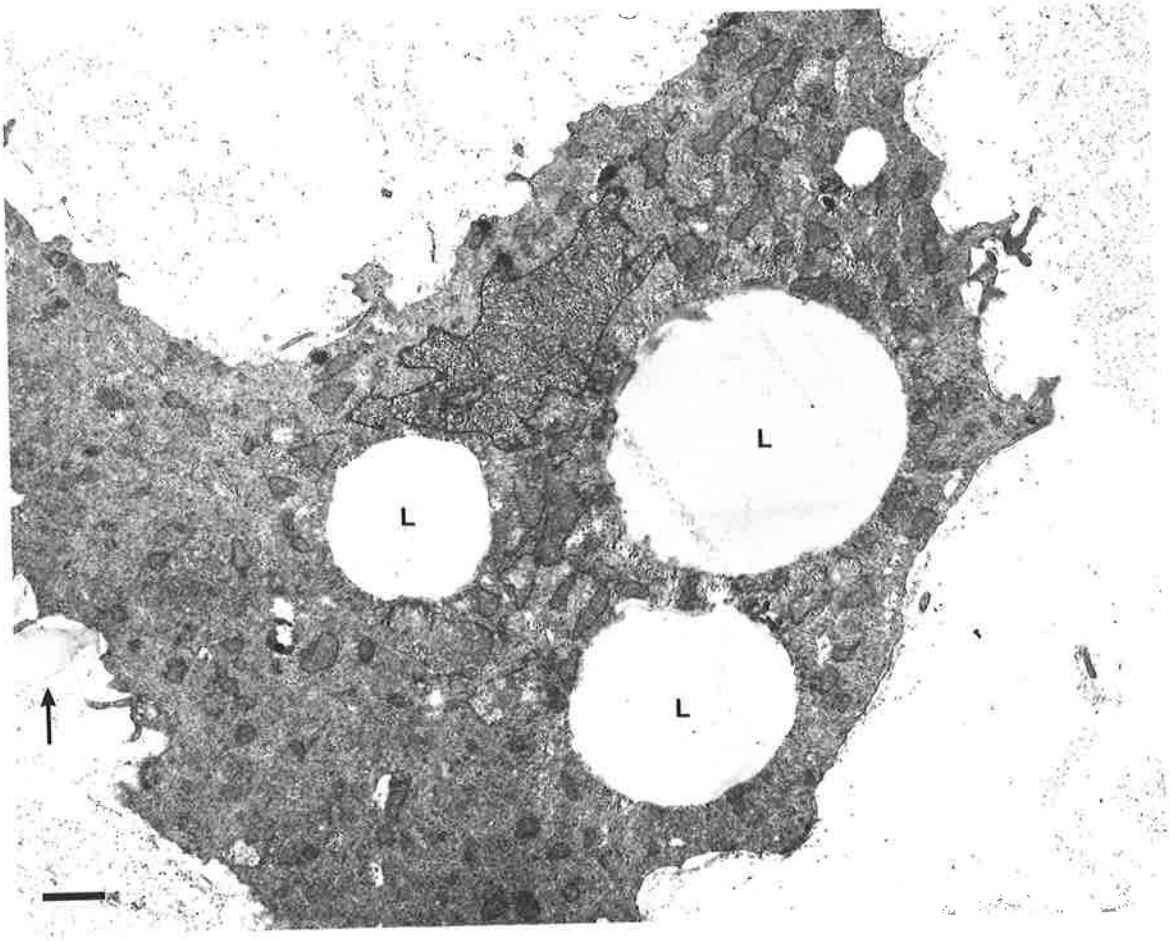
As described in section 5.3.3, auricular chondrocytes from young rabbit ear cartilage quickly formed a thick, membranous culture layer, which by 35 days post-confluence was some 1-2mm in thickness. This pattern of growth was markedly different from that observed in cultures of foetal calf and neonatal rabbit auricular chondrocytes.

15 days post-confluence. The culture layer was composed mostly of an extensive loose extracellular matrix, in which individual cells or small groups of cells were embedded. These cells were separated by proportionately larger amounts of matrix than was observed in intact ear cartilage (Figure 5.39). Although similar in morphology to native ear cartilage chondrocytes, cultured cells had a lowered nucleo-cytoplasmic ratio, and usually contained one or more large lipid droplets. Large amounts of RER with widely dilated cisternae were observed, and cytoplasmic microfilaments were again a prominent feature.

The intercellular matrix consisted primarily of proteoglycan granules and microfilaments, forming an extensive, loose 'stellate reticulum', which was somewhat rarified in the immediate pericellular region. Elastic fibres were relatively inconspicuous, often being found midway between cells. Such fibres were heavily coated with proteoglycan-like material, and no peripheral microfibrils could be identified, either in longitudinal or

Figure 5.39. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 15 days after confluence
Cells were embedded in a thick proteoglycan matrix, and were much larger in comparison to those seen in cultures of neonatal rabbit auricular chondrocytes (cf. Figures 5.35 and 5.37). The cell shown here is approximately 15μm in diameter. It contains large quantities of rough endoplasmic reticulum and several lipid droplets (L). A small elastic fibre (↑) abuts the cell at the left of the micrograph. The extensive extracellular matrix is composed almost entirely of a loose proteoglycan 'stellate reticulum'. Culture maintained under scorbutic conditions. X8,000. Scale = 1μm.

Figure 5.40. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 15 days after confluence, showing details of the matrix
Several small elastic fibres (E) are seen, surrounded by proteoglycan granules and thin matrix filaments. Several indistinctly cross-striated collagen fibrils (↑) can be seen. Note the absence of identifiable microfibrillar material and dense surface coating of the elastic fibre with proteoglycan granules. Culture maintained under scorbutic conditions. X65,000. Scale = 200nm.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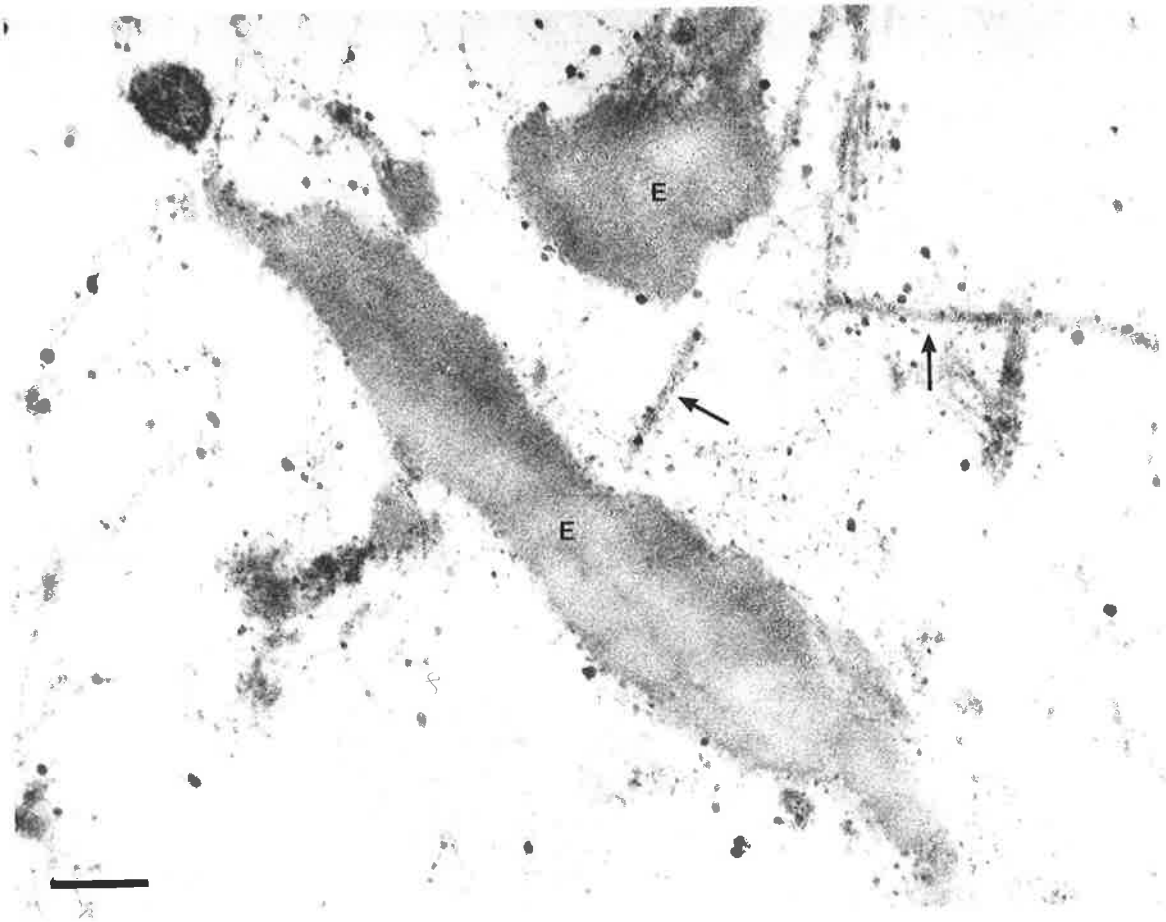

E
E

cross-sections (Figures 5.40 and 5.41). This appearance was similar to that observed in intact cartilage.

Few collagen fibrils were seen (Figure 5.40), and these, too, showed condensation of proteoglycan-like material on to their surfaces. Large fibres possessed typical cross-striations. The paucity of collagen fibrils compared to native cartilage may be due to the scorbutic conditions under which cells were cultured. No other formed extracellular elements could be identified.

35 days post-confluence. The general morphology of cultures closely resembled that observed at 15 days post-confluence. Cells (individually and in small groups) were scattered sparsely in a predominantly proteoglycan matrix which formed the vast bulk of the culture. In places the membrane so formed was about 2mm in thickness, and some grid-spaces (of a 200-mesh grid) were occupied only by extracellular material. Cells appeared even more widely separated by matrix than at 15 days post-confluence. Cell organelles and cytoplasmic inclusions were identical to those described above (Figure 5.42) and cell size was similar to that found in intact cartilage, usually between approximately 8µm and 12µm at the long axis.

The intercellular matrix was composed almost entirely of a loose 'stellate reticulum' of proteoglycan-like material, in which occasional patches of elastic fibres and collagen fibrils could be found. It was again impossible to demonstrate elastin-associated microfibrils on the edges of elastic fibres, even at high resolution, due to the very dense coating of the surface of such fibres with granular proteoglycan-like material (Figure 5.43). This appearance corresponded closely to that observed in 15 day post-confluence cultures and in native 16-day-old rabbit ear cartilage. It is of interest to note that peripheral elastin-associated microfibrils were clearly seen in native neonatal ear cartilage, and in elastic fibres formed in vitro by cultures of neonatal auricular chondrocytes (see sections 5.3.2ii and 5.3.5ii), in which the extracellular matrix was observed to contain much less proteoglycan-like material.

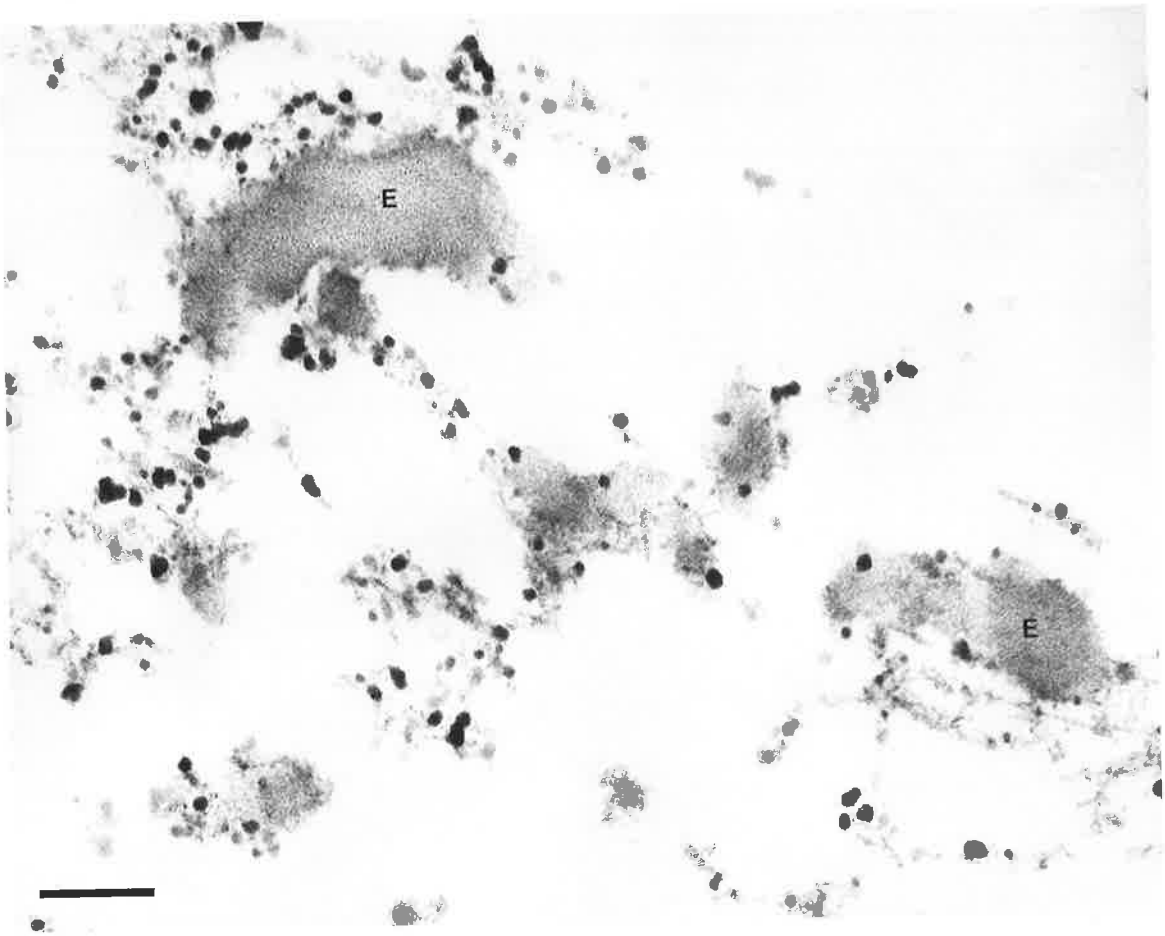
Collagen fibrils were found only occasionally and were also usually heavily encrusted with proteoglycan-like material (Figure 5.44). Few distinctly cross-striated fibrils could be identified.

Figure 5.41. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 15 days after confluence. High-power detail of a small elastic fibre

Similar section to Figure 5.40, to emphasize the very dense association of proteoglycan granules with elastic fibres. No microfibrils can be seen. X75,000. Scale = 200nm.

Figure 5.42. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 35 days after confluence. Low-power view showing general characteristics

Note the similar cellular and matrix morphology to that observed in cultures at 15 days after confluence (Figure 5.39). Culture maintained under scorbutic conditions. X9,000. Scale = 1μm.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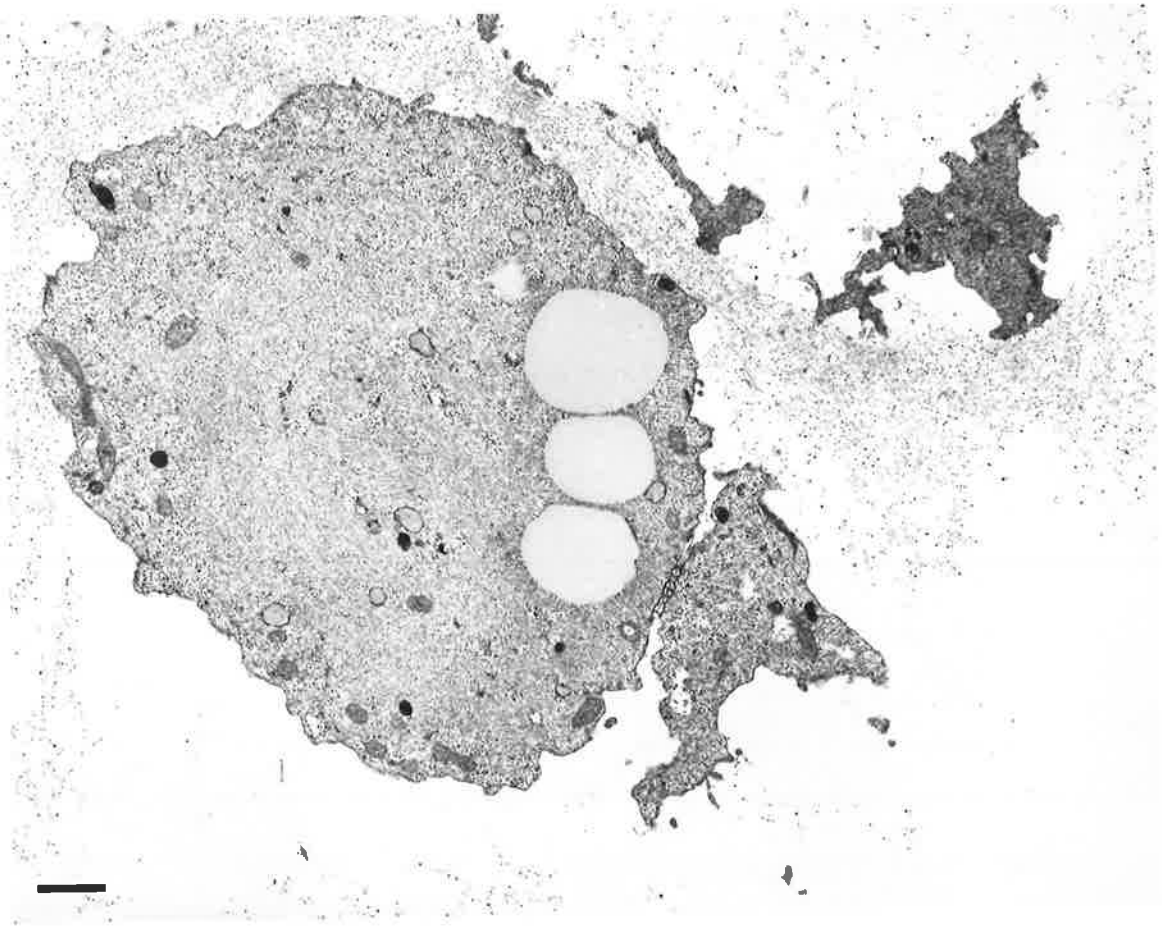
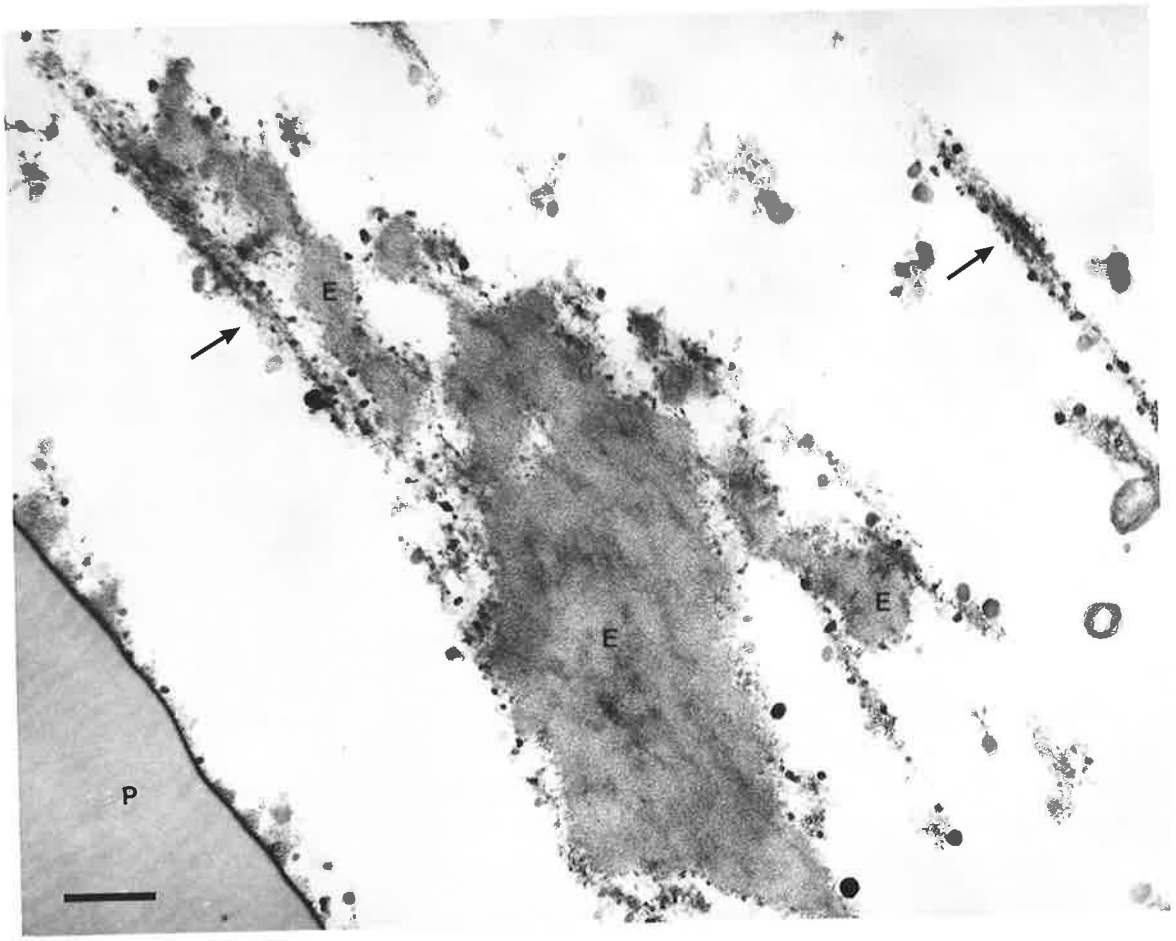


Figure 5.43. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 35 days after confluence. High power detail of elastic fibres.
Several elastic fibres (**E**) are shown, which have a dense surface coating of proteoglycan. Adjacent collagen fibrils (↑) are also densely coated. Elastin-associated microfibrils cannot be discerned. The plastic culture surface (**P**) is at the left of the micrograph. Culture maintained under scorbutic conditions. X60,000. Scale = 200nm.

Figure 5.44. Transmission electron micrograph of a culture of young (16-day-old) rabbit auricular chondrocytes at 35 days after confluence. Medium power view showing matrix details.
Similar section to Figure 5.43. Heavy encrustation of elastic fibres (**E**) and collagen fibrils is apparent. The plastic culture surface (**P**) is at the right of the micrograph. X23,000. Scale = 0.5μm.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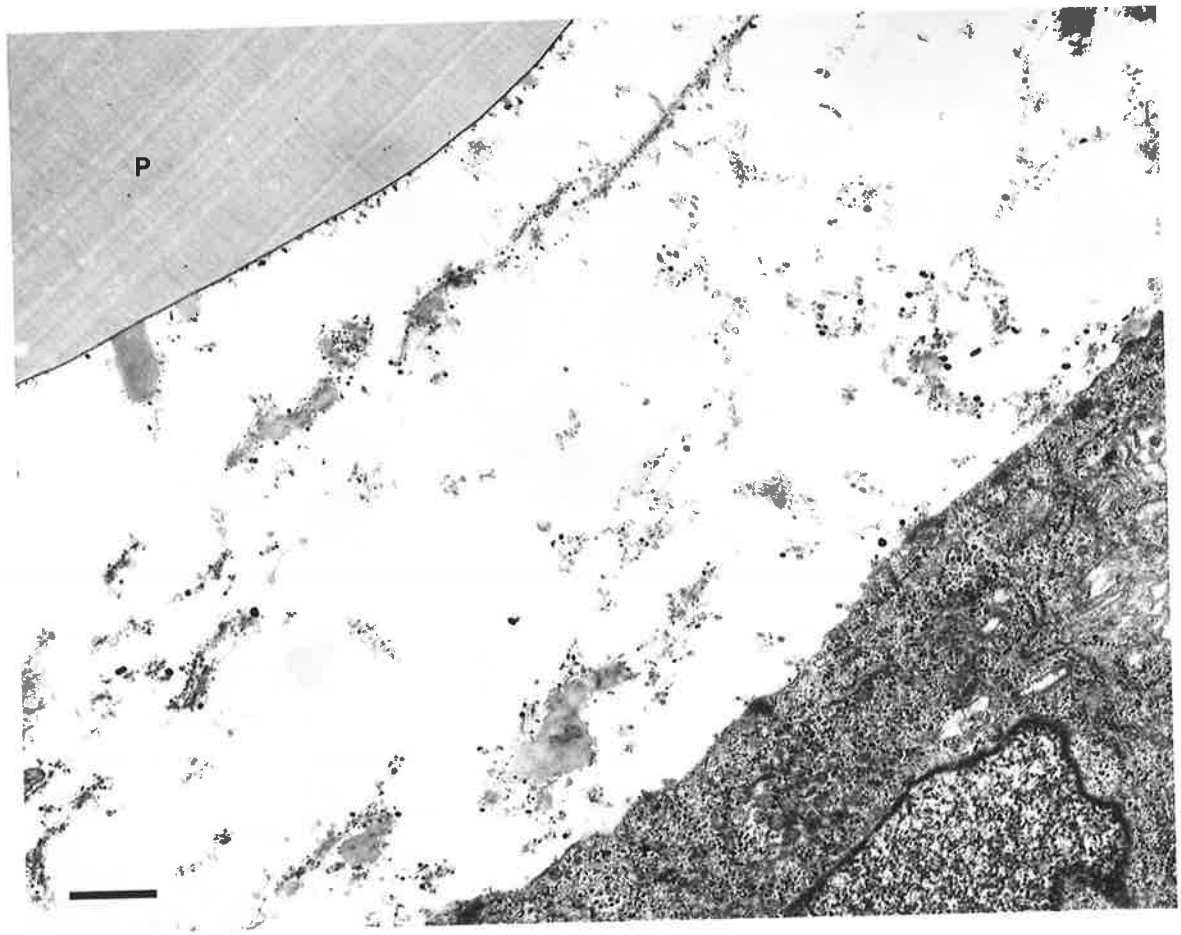

P

5.3.6 Effect of Ascorbate Supplementation on the Matrix Characteristics of Foetal Calf Auricular Chondrocyte Cultures

As described in section 5.2.6, some flasks of foetal calf auricular chondrocyte cultures were maintained after confluence in medium supplemented with ascorbate (50µg/ml). Cultures were fixed and processed for observation by electron microscopy at 28 days post-confluence.

a. Cellular morphology. The cell layer consisted of overlapping elongated cells of similar overall appearance to those observed in unsupplemented cultures (see section 5.3.5a). Cells were separated by a much more extensive intercellular matrix than in unsupplemented cultures, particularly in the basal layer of the culture (Figure 5.45). Necrotic cells were rarely observed, but lysosomes appeared frequently in the cytoplasm of viable cells. The organelle complement corresponded closely to that observed in unsupplemented cultures, with a prominent RER, golgi complex and numerous mitochondria. Fat droplets were occasionally observed, and cytoplasmic microfilaments appeared in small aggregations throughout the cells.

b. Matrix characteristics. The extracellular matrix was strikingly different to that observed in unsupplemented cultures, being dominated by large tracts of randomly oriented collagen fibrils (Figure 5.46) which demonstrated typical periodicity when seen in longitudinal sections.

Microfibrils were scattered throughout the matrix, occasionally forming small aggregates (Figure 5.47), but were much less conspicuous than in unsupplemented cultures. The ultrastructural characteristics of these microfibrils were identical to those described previously in unsupplemented cultures. Elastic fibres were found only in the deeper layers (Figure 5.48). Little microfibrillar material could be seen surrounding such elastic fibres, in contrast to those found in unsupplemented cultures.

Finely granular fibrillar material was often observed lying in close apposition to the cell membrane (Figure 5.49). This material did not have a discernible substructure, and was similar in appearance to that described for fibronectin-containing structures in cultures of human skin fibroblasts (Hedman et al., 1978) and myoblasts (Furcht et al.,

Figure 5.45. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes after supplementation with ascorbate. Low-power view showing general characteristics.

Culture fixed at 23 days after confluence. Note the extensive extracellular matrix in which collagen fibrils are the dominant component. Cell morphology is similar to that seen in unsupplemented cultures. X20,000. Scale = 1μm.

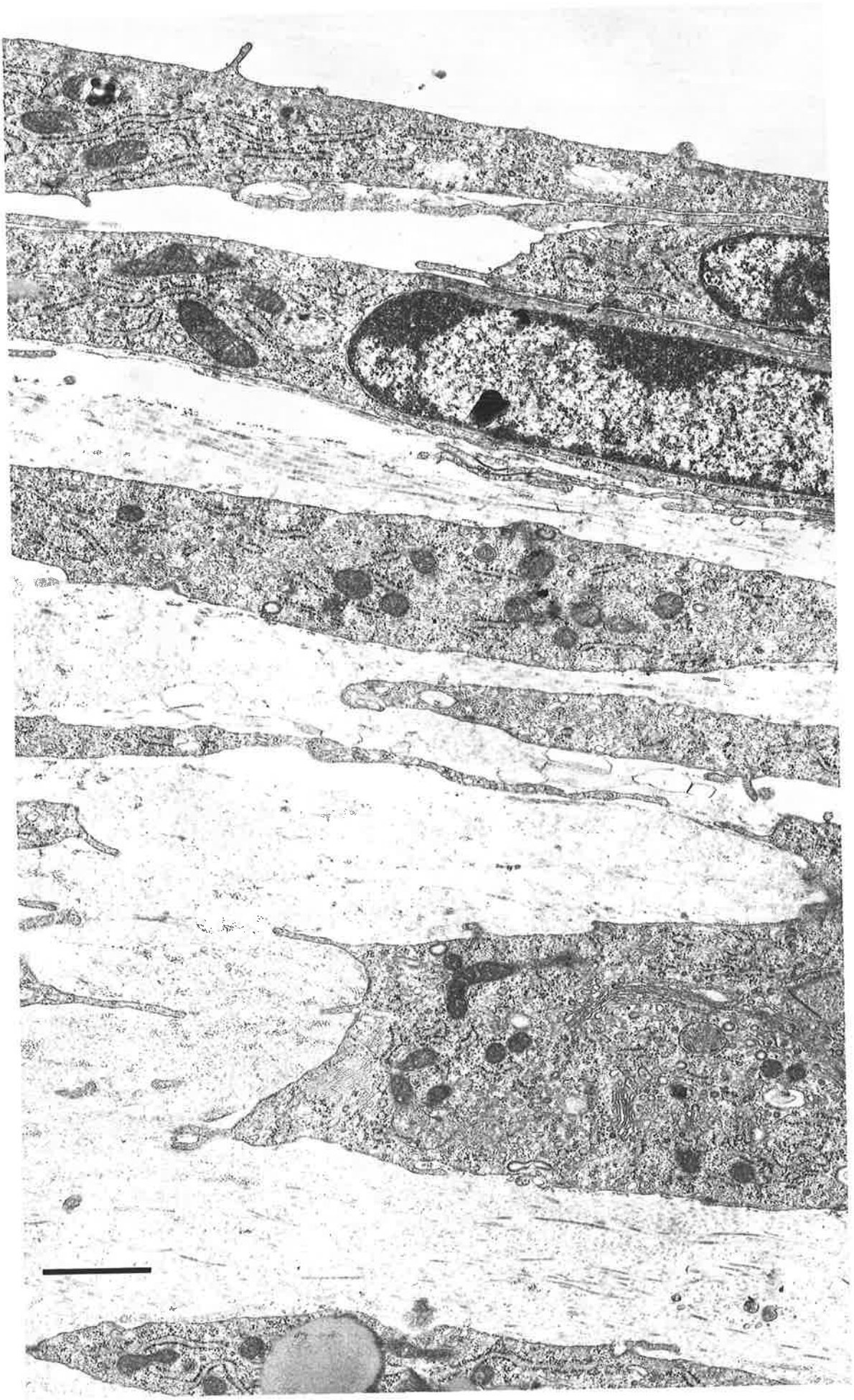
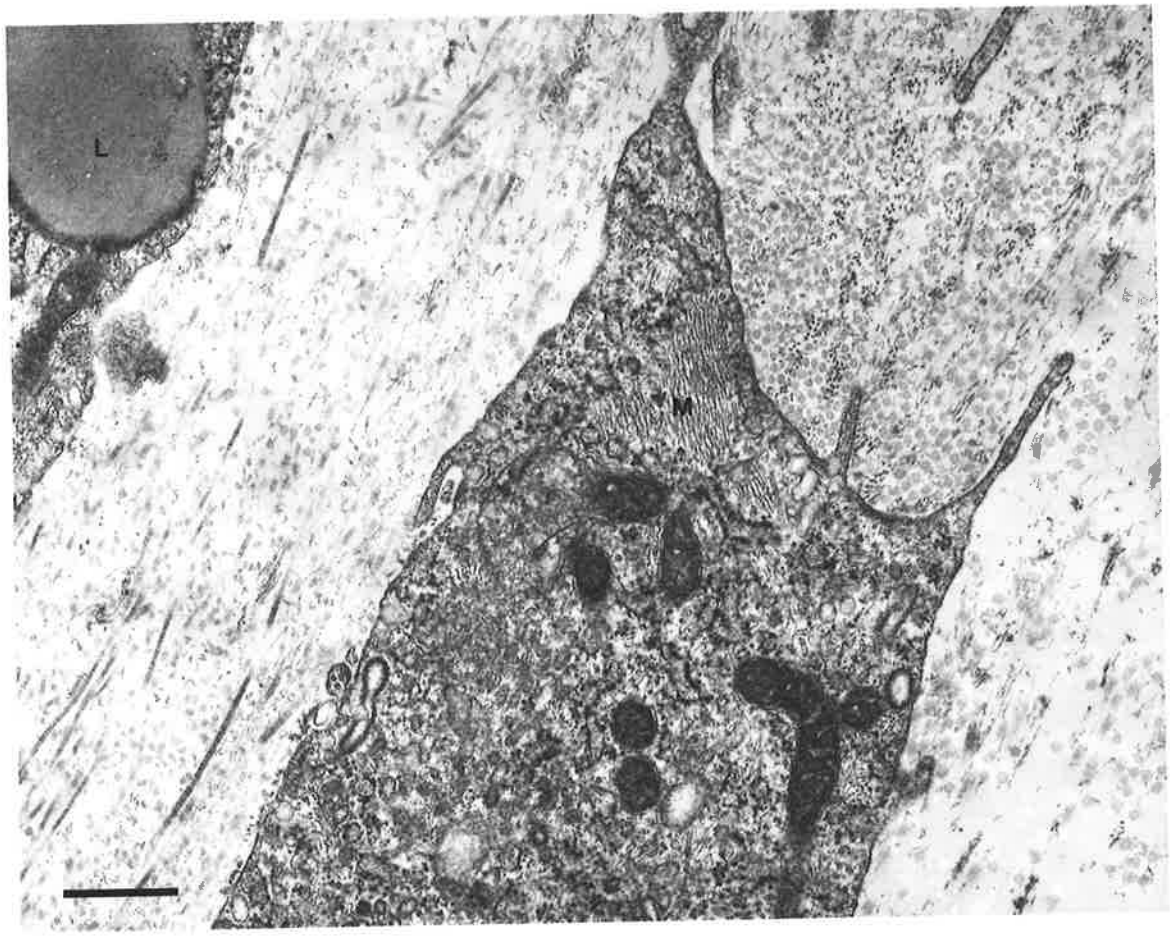


Figure 5.46. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes after supplementation with ascorbate

Higher power view of the same section as Figure 5.45. The cell on the right shows numerous mitochondria, rough endoplasmic reticulum and a small aggregate of cytoplasmic microfilaments (**M**). A lipid droplet (**L**) can be seen in an adjacent cell. Note the large numbers of collagen fibrils in the extracellular matrix. X30,000. Scale = 0.5μm.

Figure 5.47. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes after supplementation with ascorbate. High power view showing details of the matrix.

A small elastic fibre (**E**) can be seen, surrounded by collagen fibrils. Several small aggregates of microfibrils (↑) are present. The plastic culture surface (**P**) is at the bottom left of the micrograph. X80,000. Scale = 200nm.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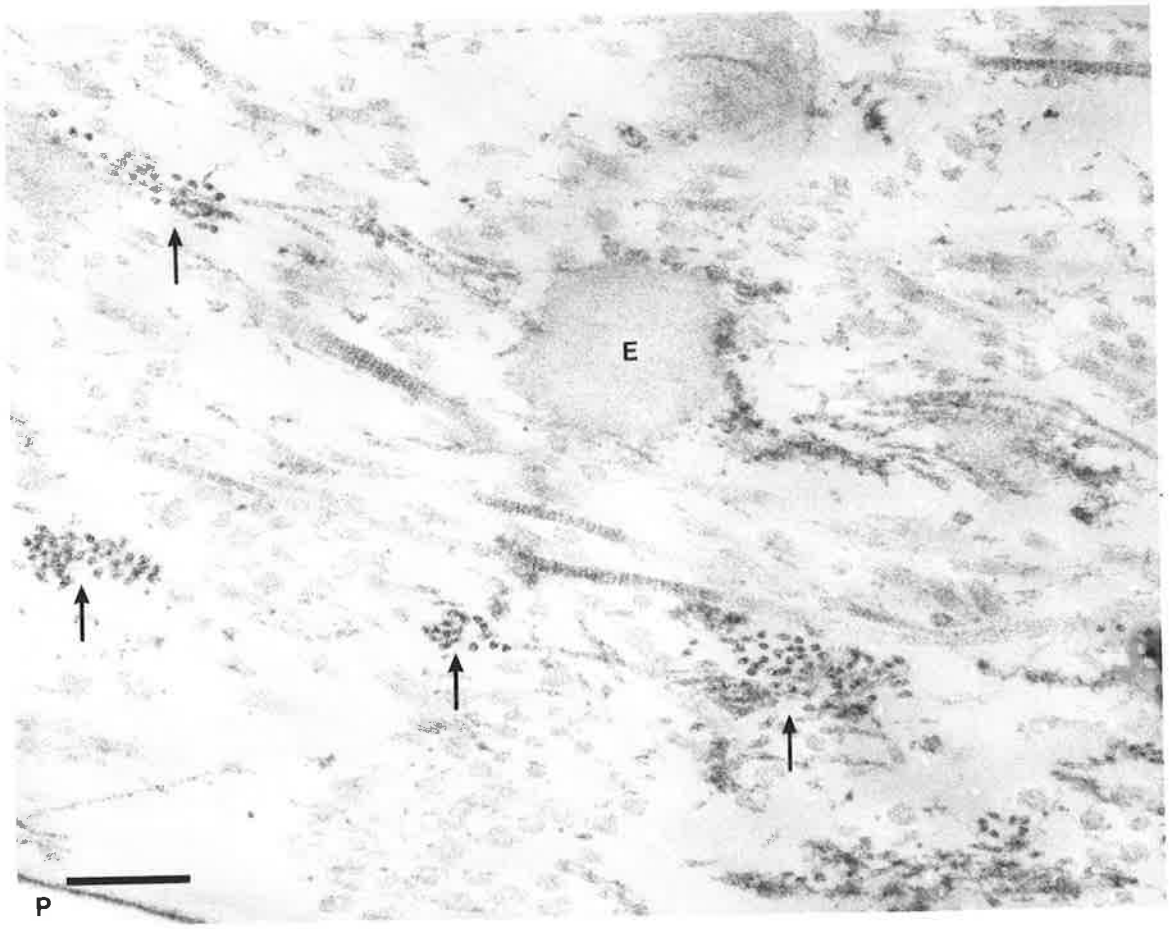
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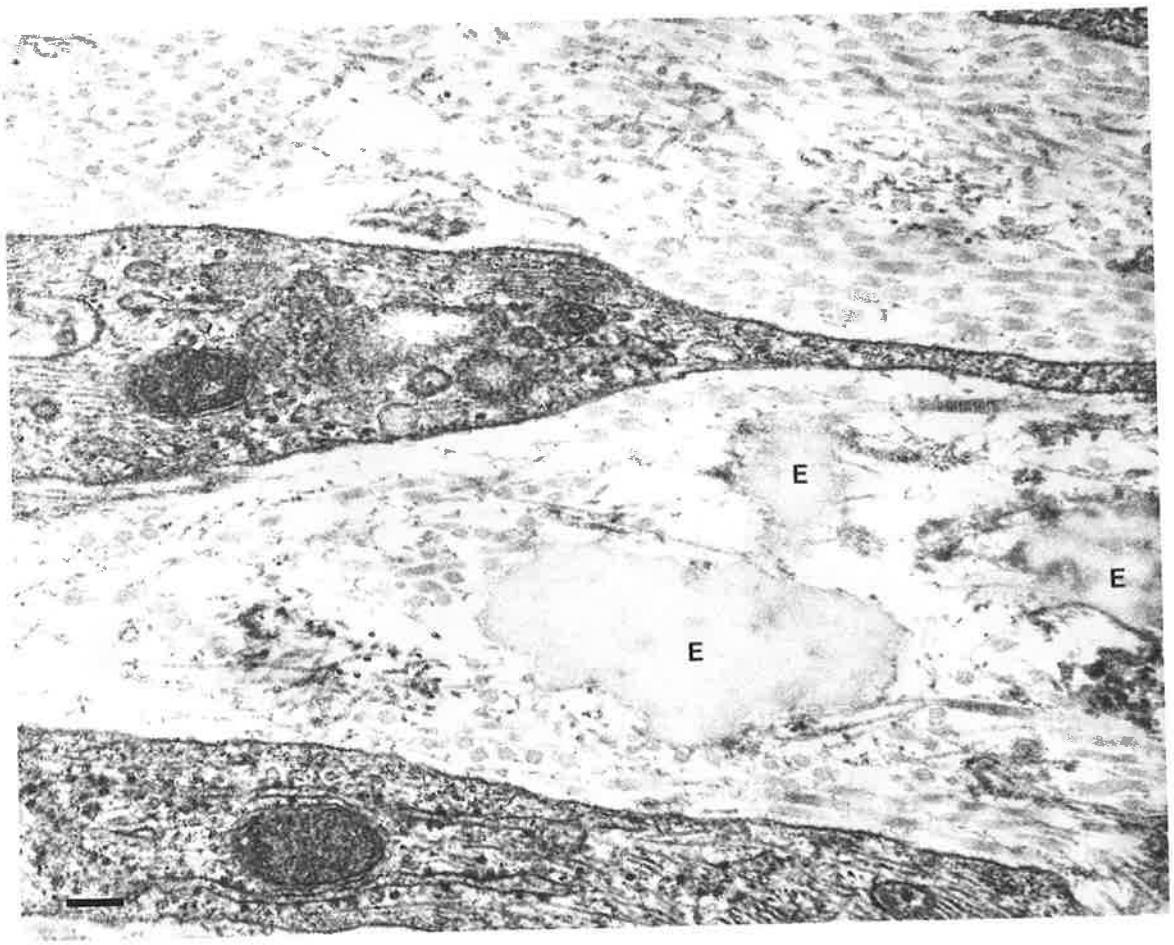
Figure 5.48. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes after supplementation with ascorbate. High-power view.
Several small elastic fibres (E) are shown in the deeper layer of the culture. Note the relative paucity of microfibrillar material in comparison to unsupplemented cultures. X38,000. Scale = 200nm.

Figure 5.49. Transmission electron micrograph of a culture of foetal calf auricular chondrocytes after supplementation with ascorbate. High-power view.
Note the strands of granular fibrillar material (↑) in close apposition to the cell membrane. Similar material is seen in association with adjacent collagen fibrils. X65,000. Scale = 200nm.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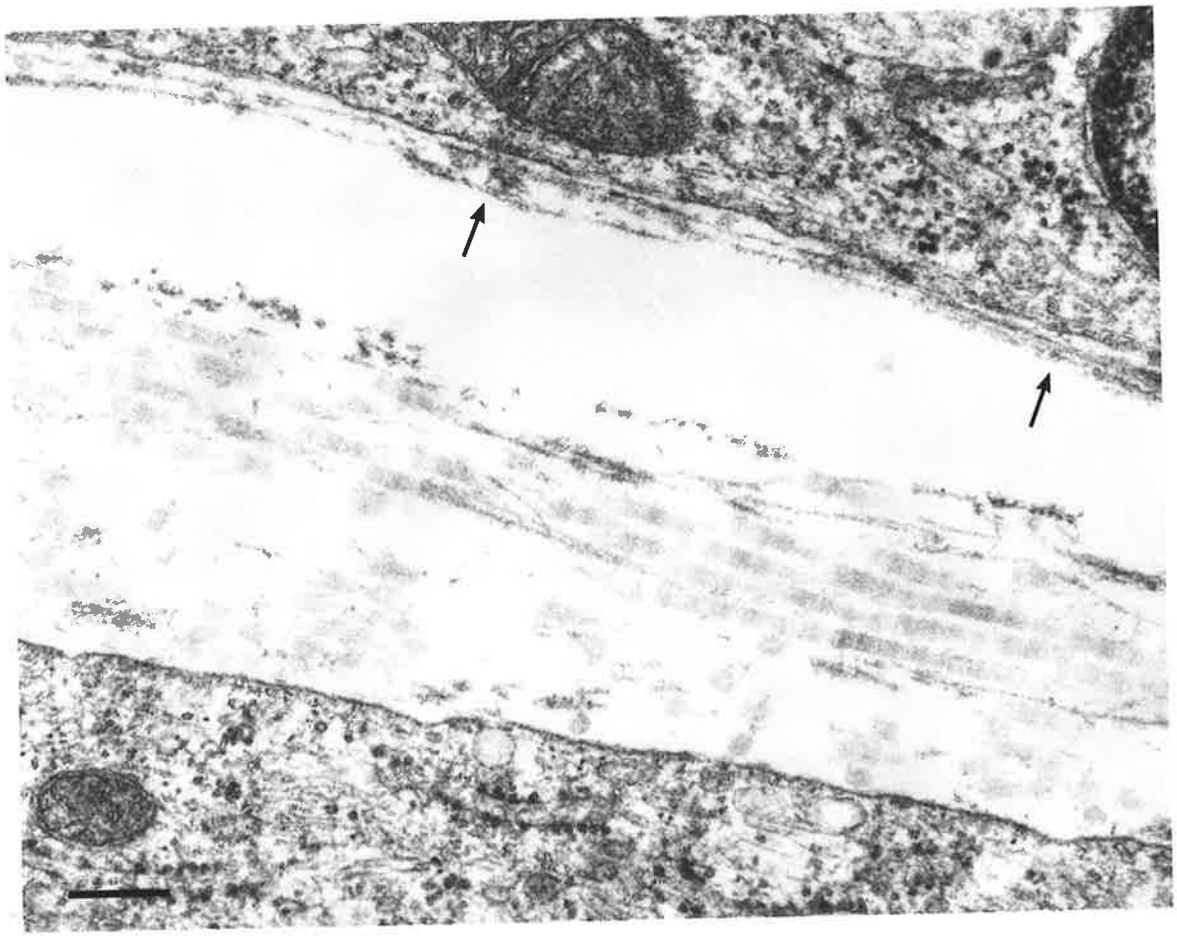


1978). Cultures of undifferentiated chondrocytes have also been demonstrated to produce fibronectin in vitro (Dessau et al., 1978). However further studies, perhaps by immunohistochemical labelling with anti-fibronectin antibodies, would be necessary to establish a relationship between the observed material and fibronectin.

No structures corresponding to the proteoglycan 'stellate reticulum' of intact foetal calf ear cartilage could be identified. This corresponded to the findings in unsupplemented cultures of both foetal calf (see section 5.3.5a) and neonatal rabbit (see section 5.3.5b) ear cartilage chondrocytes, but was in striking contrast to the huge amounts of proteoglycan matrix formed by young rabbit (section 5.3.5c) auricular chondrocyte cultures. These findings suggest that donor maturity is an important factor in the synthetic capabilities of cultured auricular chondrocytes.

In conclusion, it can be stated that the major effect of ascorbate supplementation on foetal calf auricular chondrocyte cultures was to promote the deposition of collagen fibrils, whilst relatively reducing the amount of microfibrillar material and elastic fibres.

5.3.7 Immunofluorescent Localization of Anti-MAg Antibodies in Foetal Calf Ear Cartilage

Immunofluorescent localization of anti-MAg antibodies in tissue sections and cell cultures of foetal calf ear cartilage was carried out as detailed in section 5.3.8. The results of these studies are described below.

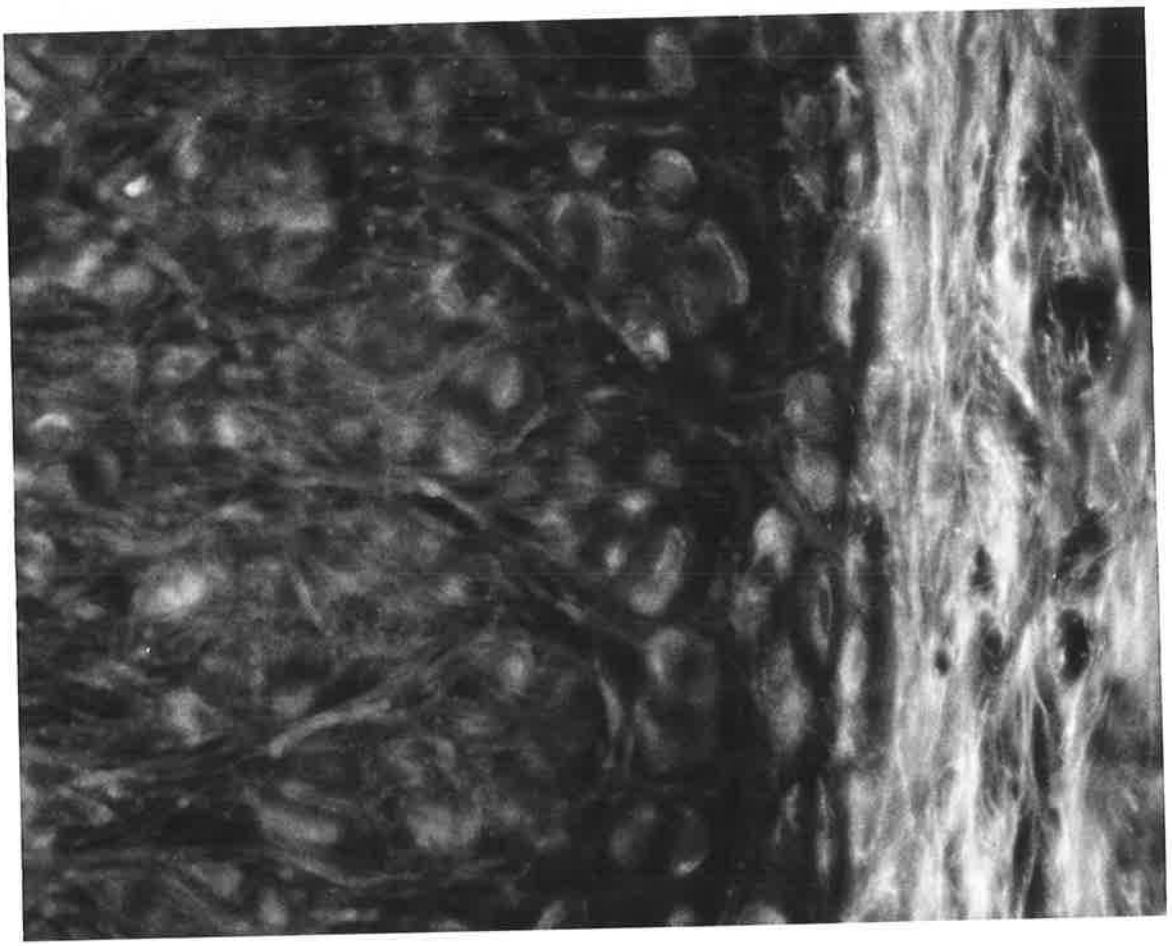
a. Tissue sections. Control sections of foetal calf ear cartilage showed very little autofluorescence of elastic fibres in the central and boundary zones of the tissue (Figure 5.50b). Sections treated with anti-MAg antibody were significantly brighter than control sections (Figure 5.50a) and individual fine, fluorescent fibres could be identified coursing through the intercellular matrix of the cartilage. These fibres formed a dense meshwork in the central zone, becoming smaller in the boundary zone before merging indistinctly into the perichondrium. Chondrocytes lying in lacunae showed a more homogenous dull fluorescence.

Figure 5.50. Immunofluorescent staining of native foetal calf ear cartilage with anti-MAg (ADS) IgG

Longitudinal sections of ear cartilage. The perichondrium is at the right of the micrograph. Magnification X250.

a. Section treated with anti-MAg (ADS) IgG. Note the dull fluorescence of elastic fibres in the cartilage matrix, which contrasts with the brilliant fluorescence of the perichondrium.

b. Control section treated with pre-immune rabbit IgG.



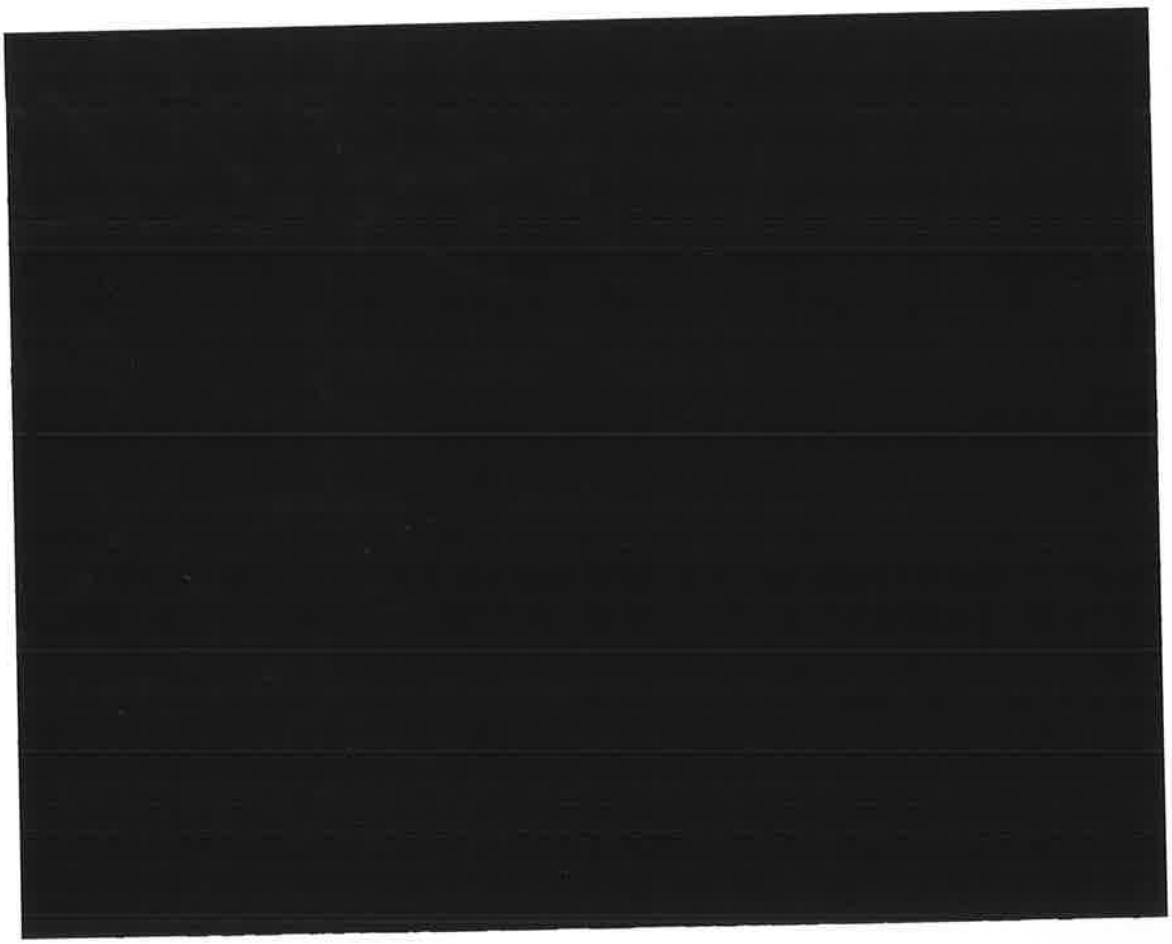
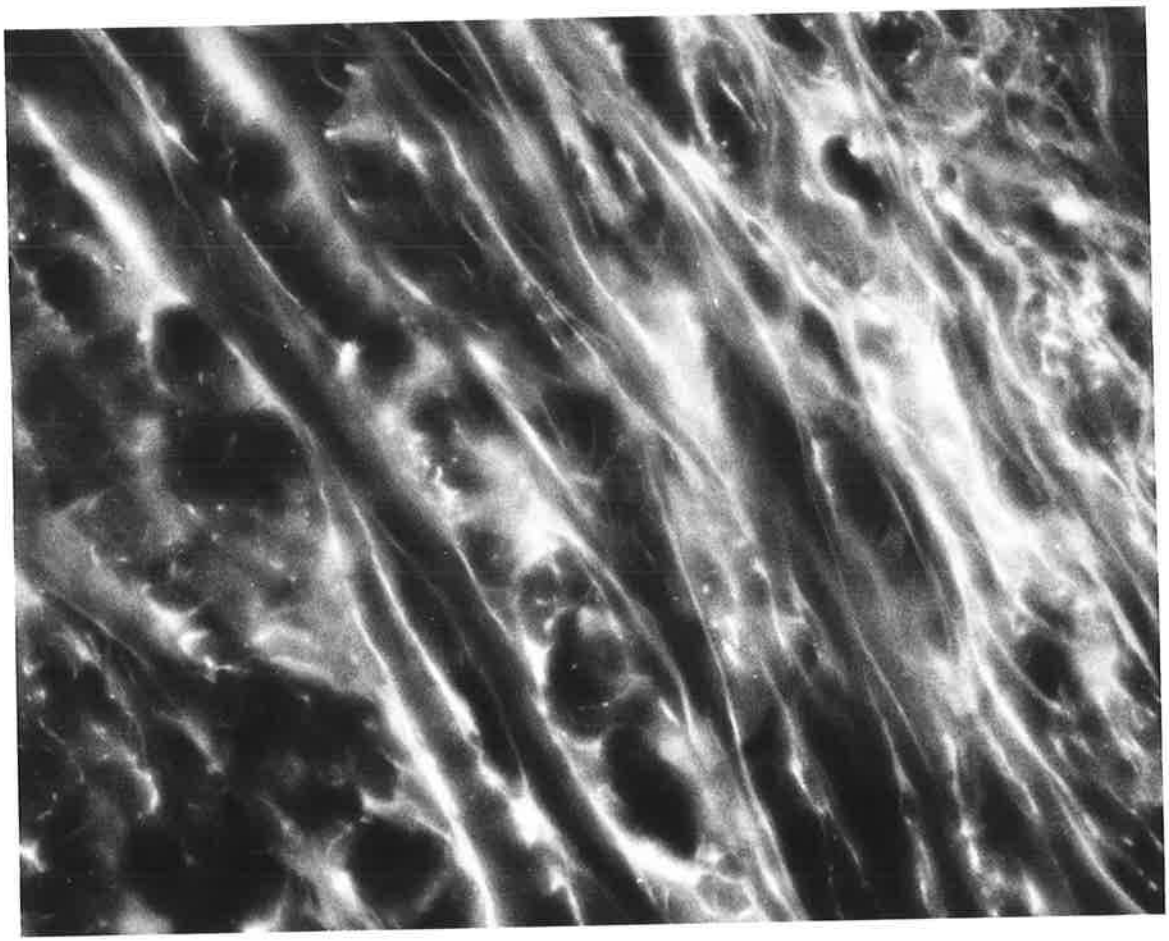


Figure 5.51. Immunofluorescent staining of the periochondrium of native foetal calf ear cartilage with anti-MAg (ADS) IgG

Magnification X400.

a. Section treated with anti-MAg (ADS) IgG. Note the brilliant fibrillary fluorescence. No cellular staining is evident.

b. Control section treated with pre-immune rabbit IgG.



The perichondrium showed a brilliant fibrillary fluorescence, being much brighter than the cartilage itself. Control sections, in contrast, showed only very small amounts of non-specific cell fluorescence, in quite a different pattern from that observed in anti-MAg treated sections (Figure 5.51). The pattern of staining in the perichondrium was clearly extracellular, with fine linear fluorescent fibres running parallel to the surface of the underlying cartilage, surrounding the fibroblasts.

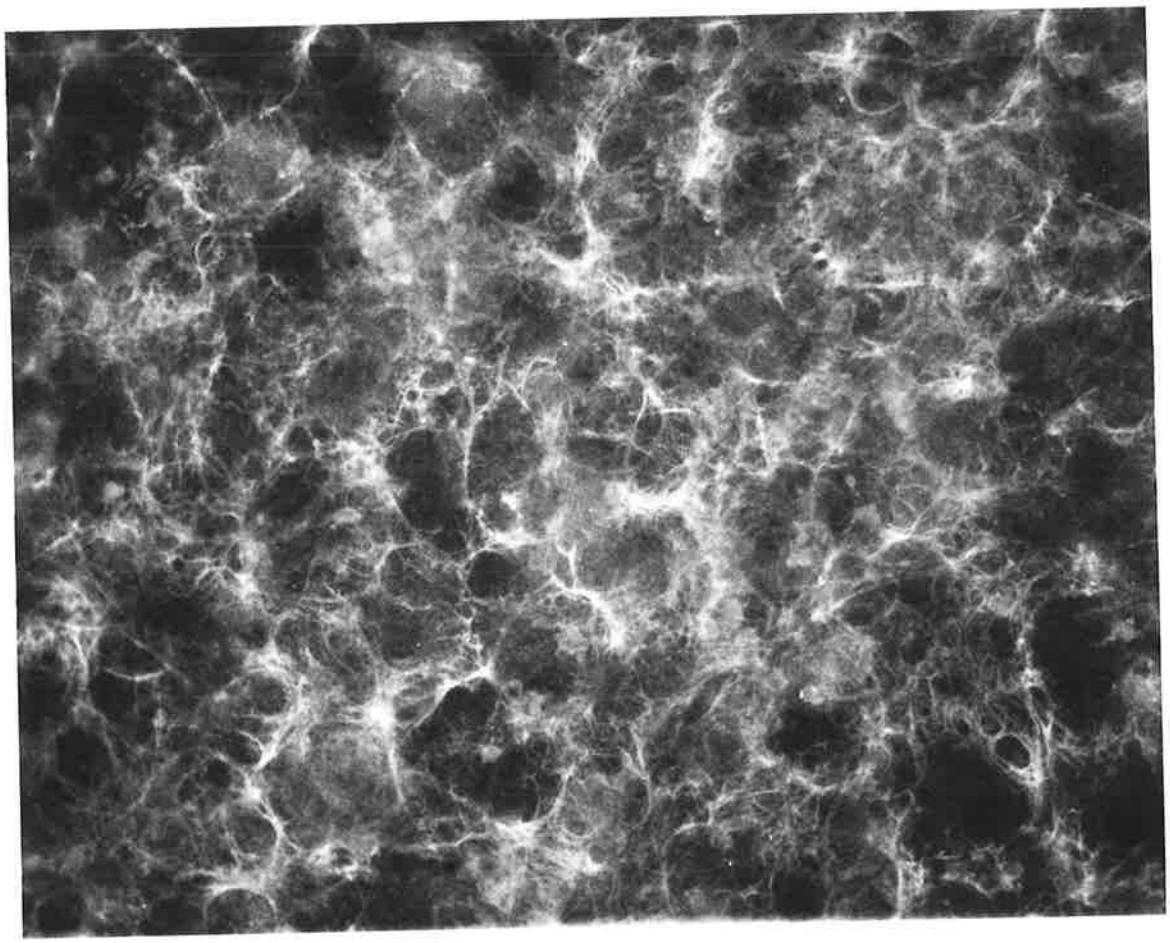
b. Cell culture. Isolated chondrocytes derived from foetal calf auricular cartilage were cultured in flasks containing glass coverslips. At 21 days post-confluence, the coverslips were removed, attached to glass slides and stained for fluorescence microscopy as described in section 5.2.8. Coverslips treated with anti-MAg IgG showed localization of antibody to fine fibres which formed a web-like network in the intercellular matrix, arching and ramifying around the cell spaces (Figures 5.52a and 5.53a). Control specimens showed minimal non-specific fluorescence only (Figures 5.52b and 5.53b). The fibrous meshwork so observed was much more dense than the delicate network of elastic fibres seen in similar cultures after staining with Miller's elastic stain (see section 5.3.4).

Figure 5.52. Immunofluorescent staining of a culture of foetal calf auricular chondrocytes at 21 days after confluence

Chondrocytes were grown on glass coverslips for use in immunofluorescence experiments as described in section 5.2.8. Magnification X250.

a. Section treated with anti-MAg (ADS) IgG. Note the extensive fibrous network in the extracellular matrix, surrounding cell spaces.

b. Control section treated with pre-immune rabbit IgG.



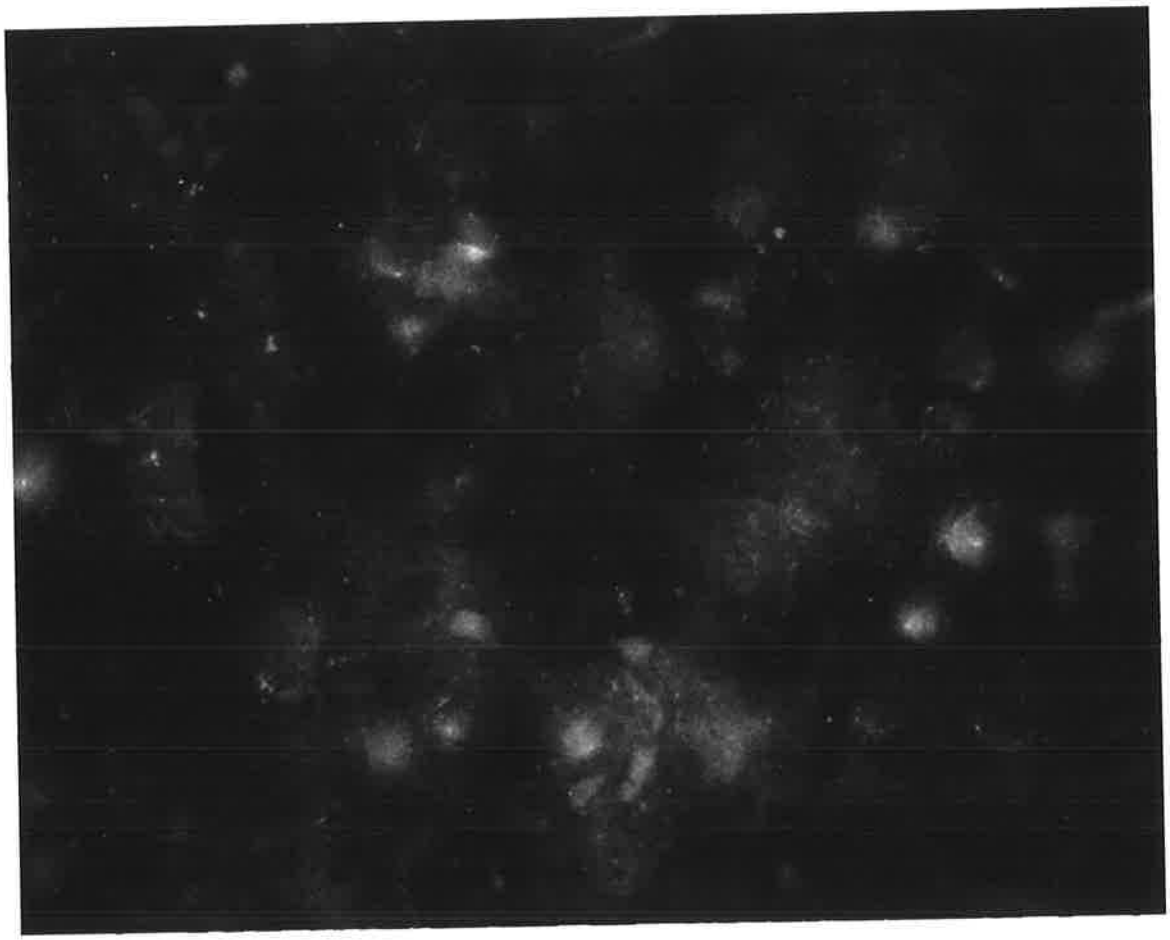
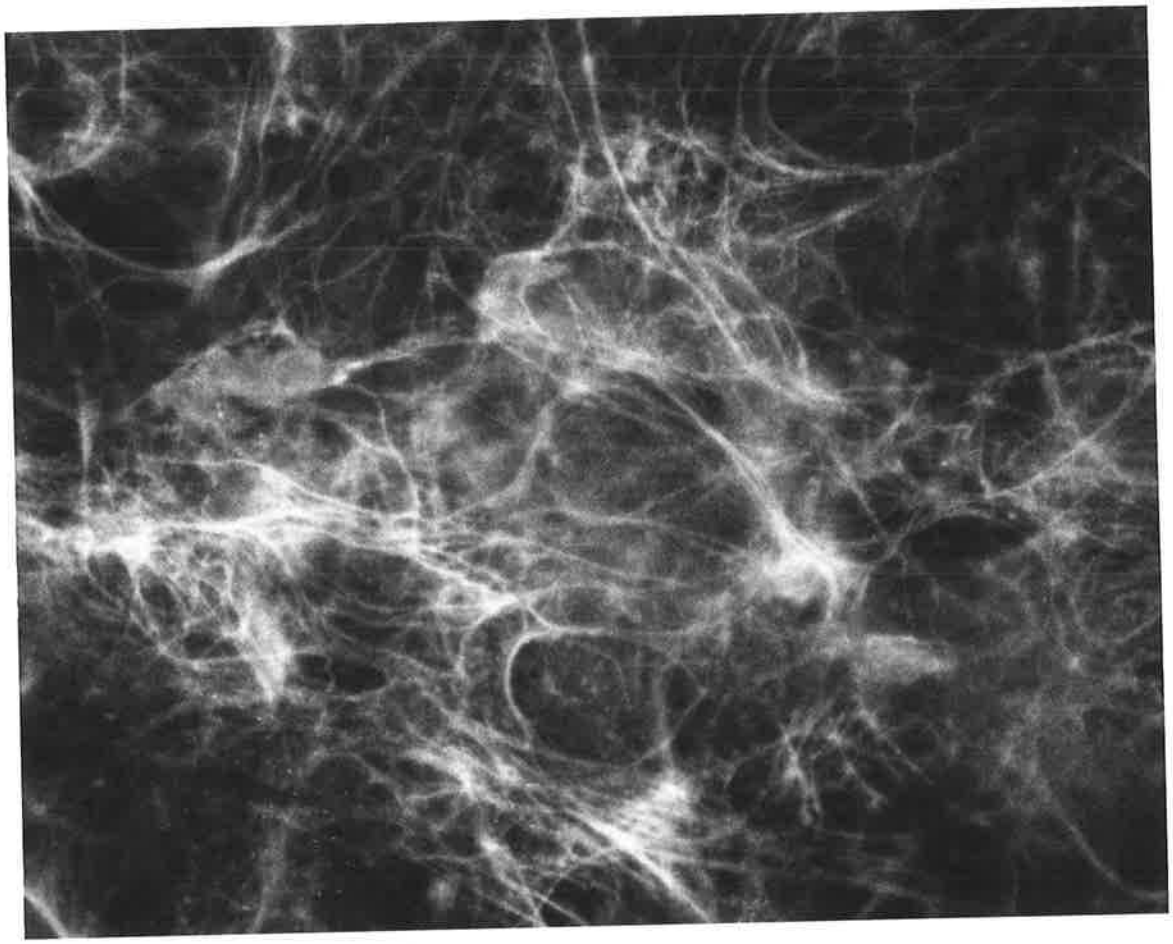


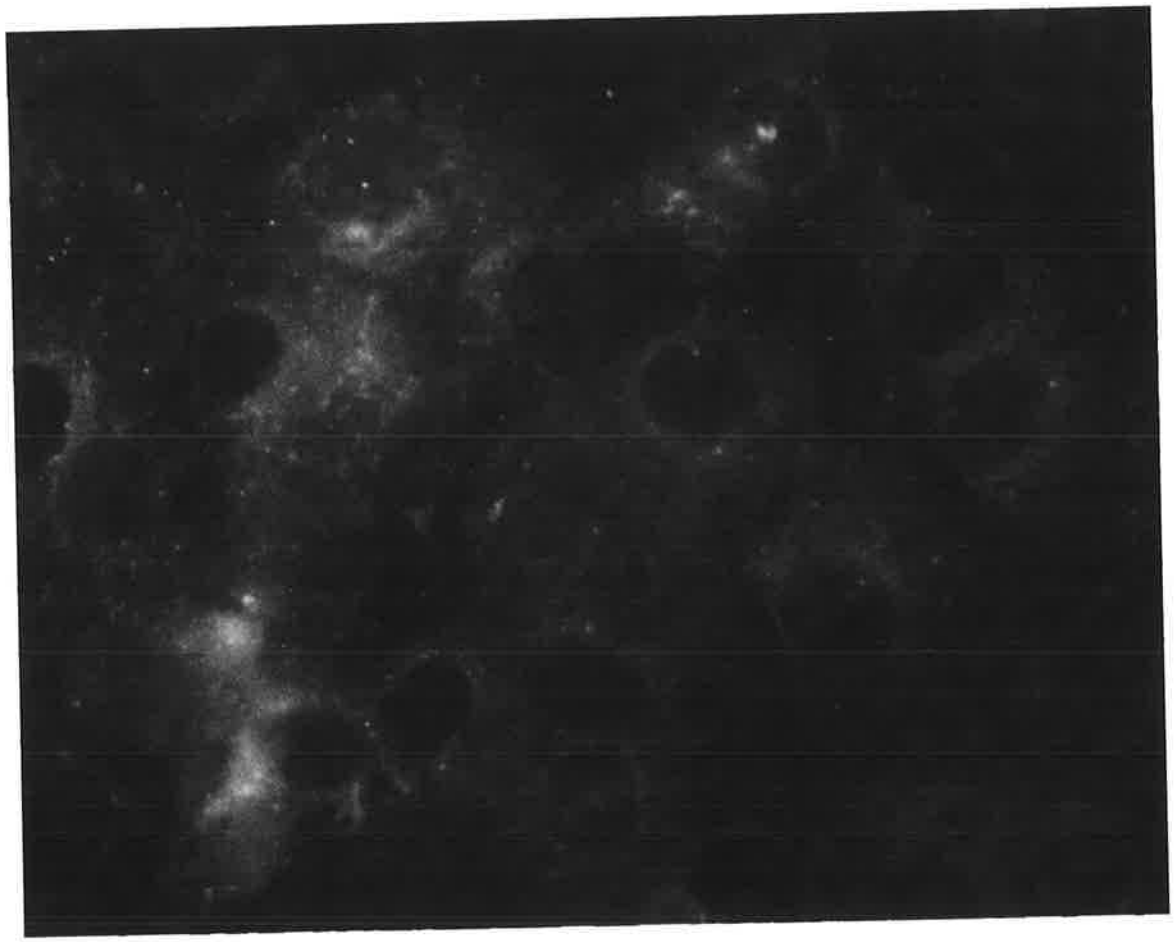
Figure 5.53. Immunofluorescent staining of a culture of foetal calf auricular chondrocytes at 21 days after confluence

Similar to Figure 5.52. Magnification X400.

a. Section treated with anti-MAg (ADS) IgG. High-power view emphasizes the fibrillary nature of the immunofluorescent staining.

b. Control section treated with pre-immune rabbit IgG.





5.4 DISCUSSION

5.4.1 Morphology of Native Ear Cartilages

The light and electron microscopic appearance of 220 day gestation foetal calf ear cartilage did not differ substantially from that previously described for developing ear cartilages of other species, including the rabbit (Sheldon and Robinson, 1958; Cox and Peacock, 1977; Quintarelli et al., 1979), mouse (Sanzone and Reith, 1976) and rat (Anderson, 1964; Serafini-Fracassini and Smith, 1974).

Foetal calf ear cartilage was found to be a morphologically more mature tissue than neonatal (2-day-old) rabbit ear cartilage. It contained larger cells, a better-developed intercellular matrix and more clearly defined cartilage zones. The ultrastructural characteristics of the matrix were qualitatively similar in both tissues, but foetal calf cartilage had a much more extensively developed proteoglycan 'stellate reticulum' and proportionately more collagen fibrils.

Young (16-day-old) rabbit ear cartilage was more advanced in development than either foetal calf or neonatal rabbit cartilages, corresponding more closely in appearance to 'adult' cartilage as described in section 1.7.1.

The certainty with which elastin-associated microfibrils could be identified by electron microscopic examination appeared to vary according to the relative proportion of proteoglycan present in the extracellular matrix of the tissues studied. Thus, although microfibrils were readily observed as a component of elastic fibres in neonatal rabbit ear cartilage (which contained little proteoglycan) they could not be demonstrated at all in young rabbit ear cartilage (which had an extensive proteoglycan matrix). In addition, elastic fibres in young rabbit ear cartilage appeared heavily encrusted with a surface coating of proteoglycan material. Microfibrillar structures were identified with some difficulty in foetal calf ear cartilage, which in matrix characteristics occupied an intermediate position between the neonatal and young rabbit cartilages.

A number of other investigators have noted a similar difficulty in identifying microfibrillar structures in more mature elastic cartilages, despite the apparent presence of such structures in younger tissues (Serafini-Fracassini and Smith, 1974; Sanzone and Reith, 1976; Cox and Peacock, 1977; Quintarelli et al., 1979). These findings suggest that a specific interaction may occur between matrix proteoglycans and the surface of elastic fibres. Such an interaction may involve microfibrils and thereby block sites on these structures which would usually bind heavy-metal stains, thus rendering them effectively invisible in the electron microscope (see section 1.7.3).

More recent work performed in this laboratory by Fanning and Cleary (1984) has provided experimental support for this view. These workers have investigated the effect of hyaluronidase digestion on the ultrastructural appearances of elastic fibres in auricular cartilage derived from foetal calves of 170 and 240 days gestation. Hyaluronidase digestion revealed typical elastin-associated microfibrils at the peripheries of elastic fibres, whereas in untreated specimens microfibrillar structures could not be readily distinguished. Further studies are in progress to determine which of the components of proteoglycans are responsible for this interaction with elastin-associated microfibrils. These findings suggest the possibility of specific interactions between elastic fibres and other components of the extracellular matrix, perhaps mediated by proteoglycans. This is discussed further in section 5.4.3.

Electron microscopic examination of the perichondrium of foetal calf ear cartilage showed it to be a dense, predominantly collagenous connective tissue containing a population of fibroblastic cells. Small developing elastic fibres were observed which had abundant elastin-associated microfibrils and which closely resembled those found in ligamentum nuchae. Little proteoglycan-like material was observed in this tissue. These appearances closely corresponded to the ultrastructural description of perichondrium in young rat auricular cartilage published by Serafini-Fracassini and Smith (1974). These authors found that although elastic fibres could not be demonstrated histochemically within this tissue, ultrastructural examination showed numerous bundles of microfibrils. Takagi et al. (1983) have also described small elastic fibres, composed predominantly of

microfibrils interspersed with small amounts of amorphous elastin, in the perichondrium of young rabbit ear cartilage.

It is probable that these microfibrillar aggregates are responsible for the oxytalan-staining fibres which have previously been described in the perichondrium of elastic cartilage from the bovine (Keith et al., 1977b) and rat (Bradamante et al., 1975; Kostovic-Knezevic et al., 1981). Such fibres have been shown to precede the appearance of mature elastic fibres in the developing auricular cartilage of the rat (Bradamante and Svajger, 1977).

5.4.2 Morphology of Auricular Chondrocyte Cultures

Primary cultures of enzymically-dissociated chondrocytes proved technically straightforward to establish and could be maintained for long periods without evidence of culture instability. As it has been shown that subculture of auricular chondrocytes may result in loss of biosynthetic capabilities, both for elastic fibres (Moskalewski et al., 1979) and for cartilage-typic proteoglycans (Madsen and Lohmander, 1979), primary cultures were utilized in all experiments.

When maintained under scorbutic conditions, the growth characteristics of cells derived from young tissues (foetal calf and neonatal rabbit) were markedly different from those derived from the (histologically) more mature 16-day-old rabbit ear cartilage. Foetal calf and neonatal rabbit auricular chondrocytes formed thin cell multilayers with a scanty extracellular matrix and little stainable elastin, whilst 16-day-old rabbit chondrocytes synthesized a thick cartilaginous membrane which contained very large amounts of proteoglycan and a meshwork of elastic fibres.

Ultrastructural observations of foetal calf and neonatal rabbit cultures confirmed the predominantly cellular nature of these cultures, little extracellular matrix being formed relative to the amount present in native cartilage. Elastic fibres were the dominant components of the matrix in both cases, collagen fibrils and proteoglycan material being virtually absent. In comparison, young rabbit chondrocytes synthesized a very extensive extracellular matrix which consisted mostly of a loose 'stellate

reticulum' of proteoglycan granules and microfilaments, and relatively few elastic and collagen fibrils. Single cells or small groups of cells were widely separated by large amounts of extracellular matrix.

Identical patterns of growth in culture were found in three separate experiments using cells derived from different animals of the same ages. These findings suggest that the observed differences in synthetic capabilities of isolated auricular chondrocytes in vitro may in part reflect those in vivo at a particular donor age.

Other histological studies of matrix synthesis by enzymically-isolated auricular chondrocytes in auto- and allo-geneic transplantation experiments are consistent with this hypothesis. Moskalewski and Rybicka (1977) have shown that transplanted auricular chondrocytes derived from one-day-old rabbits produced an extracellular matrix which contained many more elastic fibres than that elaborated by cells from animals 4 to 8 weeks old. Thyberg and Hinek (1977) have reported that transplanted cells from older animals (about 1kg in weight) reconstructed a matrix which was relatively depleted in elastic fibres when compared to native cartilage. These findings correspond with the earlier observations of Kawaik et al. (1970), who noted that cells isolated from adult rabbit ear cartilage reconstructed a matrix which contained very few elastic fibres.

Although relatively few studies concerning elastogenesis by monolayer or aggregated cultures of auricular chondrocytes have appeared in the literature, the information gathered so far also appears to favour an effect of donor age on in vivo synthetic capabilities. Moskalewski (1976; 1981) has demonstrated the production of morphologically-typical elastic fibres in aggregated cultures of enzymically-dissociated chondrocytes derived from 7- and 28-day-old rabbits, which were maintained under scorbutic conditions. He observed that in cultures of chondrocytes from older animals, the elastic fibre component of the matrix appeared less well developed.

Madsen et al. (1983) have recently published biochemical observations on the biosynthesis of matrix macromolecules by monolayer cultures of rabbit auricular chondrocytes, which are in good agreement with the morphological studies detailed above. These authors found that elastin synthesis (as measured by the valyl-proline

method of Hauschka and Gallop, 1979) was maximal in cells derived from young animals (1 to 2 weeks old), falling rapidly with increasing age. Collagen synthesis also was greatest in cells from 2-week-old animals, and declined with age. Proteoglycan production seemed less rigidly controlled than that of either elastin or collagen, a broad maximum occurring between donor ages of two weeks to 5 months.

It therefore appears likely that auricular chondrocytes in culture express different synthetic capabilities as a function of the degree of cellular differentiation of the donor tissue. Such phenotypic expression might be modulated by the extracellular matrix in a similar way to that demonstrated to occur in foetal bovine ligament fibroblasts by Mecham et al. (1984). Further experiments, combining morphological and biochemical analyses of the synthetic activity of auricular chondrocytes at a number of time points both pre- and post-natally, would allow comparison of these model systems.

5.4.3 Elastogenesis in Auricular Chondrocyte Cultures

a. Foetal calf and neonatal rabbit auricular chondrocytes. The studies reported in this chapter have shown that microfibrils of typical ultrastructural appearance were quite clearly visible in association with developing elastic fibres in scorbutic cultures of both foetal calf and neonatal rabbit auricular chondrocytes. Elastogenesis in culture appeared to proceed quite rapidly as elastic fibres were already present at 15 days post-confluence (the first time-point at which cultures were sampled). Microfibrillar aggregates, which were not associated with amorphous elastin, were also observed at this time. Examination of cultures at 35 days post-confluence revealed little qualitative difference in matrix characteristics.

These ultrastructural findings correspond closely to the observations of Moskalewski (1976; 1981). He showed that aggregated cultures of enzymically-dissociated auricular chondrocytes from 7-day-old rabbit ear cartilage would readily produce elastic fibres containing both elastin-associated microfibrils and amorphous elastin. Elastic fibres could be demonstrated by electron microscopic examination after 8 days in culture, and were morphologically similar to those found in vivo in developing ligamentum nuchae (Fahrenbach et al., 1966; Greenlee et al., 1966).

It can be concluded therefore that elastin-associated microfibrils are involved in elastogenesis in bovine and rabbit auricular cartilage both in vivo and in vitro. These studies suggest that elastic fibre formation proceeds in these tissues in a similar manner to that described in other non-cartilaginous tissues.

This interpretation is at variance with the assertion by Quintarelli et al. (1979) that microfibrils do not participate in elastic fibre formation in auricular cartilage. These workers based their conclusions on electron microscopic examination of native auricular cartilages from 2- and 16-day-old rabbits, in which they were unable to demonstrate elastin-associated microfibrils. However the published micrographs of this study are of insufficient resolution to allow independent verification of the absence of microfibrils. In addition, it has been demonstrated in the present study that microfibrils cannot be identified surrounding cartilage elastin in older animals, and conclusions based on observations of mature cartilage cannot be extrapolated to developing tissues (see also section 1.7.2). It should be noted that, although biochemical evidence of elastin synthesis by cultures of enzymically-dissociated chondrocytes derived from 2- and 16-day-old rabbit auricular cartilages was presented by Quintarelli et al. (1979), no ultrastructural examination of the cell layers was performed.

b. Young rabbit auricular chondrocytes. Elastic fibres produced by cultures of auricular chondrocytes derived from the ear cartilage of 16-day-old rabbits could not be shown to possess peripheral elastin-associated microfibrils on electron microscopic examination. Such fibres were heavily coated with proteoglycan material, closely resembling those observed in vivo in native ear cartilage. It should be noted that these young rabbit chondrocyte cultures synthesized large amounts of proteoglycan. In comparison proteoglycan was found to be a minor component of the extracellular matrix formed by cultures derived from foetal calf and neonatal rabbit ear cartilage.

Thyberg and Hinek (1977) have reported similar proteoglycan coating of newly-formed elastic fibres in the matrix of cultures of auricular chondrocytes derived from young rabbits of about 1kg in weight. They also observed that large amounts of proteoglycan were produced by these cultures.

The difficulties encountered in identifying elastin-associated microfibrils in young rabbit chondrocyte cultures contrasted markedly with the ease with which such structures could be found in cultures of foetal calf and neonatal rabbit chondrocytes. Thse observations reinforce the possibility of specific interactions between the surface of elastic fibres (or microfibrils) and proteoglycan molecules, which was discussed in section 5.4.1.

It has been shown in articular cartilage that proteoglycan molecules attach at regular points along collagen fibrils (Serafini-Fracassini et al., 1970; Myers et al., 1973; Scott and Orford, 1981; Poole et al., 1982). Proteoglycan classes in elastic cartilage have been found to resemble closely those in articular cartilage (Madsen and Lohmander, 1979; Madsen et al., 1983), and both tissues contain only type II collagen (Eyre and Muir, 1975). It is thus likely that auricular cartilage proteoglycan will interact with collagen fibrils in a similar fashion to that observed in articular cartilage. If a system of attachments were also present on elastic fibres (perhaps acting via the microfibrils), a mechanism would be provided for interlinking the collagenous and elastic networks in elastic cartilage in such a way as to enable differential movement between these networks during deformation of tissue. Such linkages might also facilitate resumption of the previous three-dimensional architecture on the release of tension. Proteoglycan molecules in articular cartilage are thought to occupy domains 3-5 times smaller than if they were fully extended (Hascall, 1977), allowing a degree of extensibility sufficient for this purpose.

It is of interest that proteoglycans with similar properties to those found in cartilage have been demonstrated in bovine aorta (Oegema et al., 1979; Kapoor et al., 1981; Schmidt et al., 1982) and human aorta (Salisbury and Wagner, 1981), tissues which also contain large amounts of elastin and collagen. Immunohistochemical studies have indicated that aortic proteoglycan is in part localized on the surface of elastic fibres as well as on collagen fibrils (Mangkornkanok-Mark et al., 1981; Bartholomew and Anderson, 1983). Therefore, although little information concerning interactions between elastin and proteoglycans is at present available (Velebny et al., 1981), the potential

importance of such interactions in elastic connective tissues suggests that further investigation would be of considerable value. Cultures of auricular chondrocytes which synthesize both elastin and proteoglycan (and collagen when supplemented with ascorbate) may prove to be a useful model system for such studies.

5.4.4 Effect of Ascorbate Supplementation on Foetal Calf Auricular Chondrocyte Cultures

Ascorbate supplementation of foetal calf auricular chondrocyte cultures resulted in a massive increase in collagen fibrillogenesis and in the amount of extracellular matrix produced, with a relative decrease in synthesis of elastic fibres and microfibrils (see section 5.3.6). The present study is the first which has compared the effects of ascorbate supplementation on the synthesis of matrix components by foetal calf auricular chondrocytes in culture. No other comparative studies of scorbutic and ascorbate-replete auricular chondrocyte cultures have appeared in the literature, although Thyberg and Hinek (1977) have reported ultrastructural observations on cultures of young rabbit auricular chondrocytes which were routinely supplemented with ascorbate. These cultures elaborated large amounts of extracellular matrix which was predominantly composed of collagen fibrils and proteoglycan, and in which relatively few elastic fibres were seen. The authors did not study cultures which had been maintained under scorbutic conditions. However it has been shown in cultures of other mesenchymal cells that ascorbate promotes collagen synthesis and fibrillogenesis, whilst suppressing the deposition of elastic fibres or biochemically-measurable amorphous elastin. The culture systems so far studied include foetal calf ligamentum nuchae fibroblasts (Jones et al., 1980) and smooth muscle cells derived from rat heart (Scott-Burden et al., 1979; De Clerck and Jones, 1980), and rabbit pulmonary artery (Dunn and Franzblau, 1982).

In all of these experiments, cultures were supplemented with a standard ascorbate concentration of 50μg/ml. More recently Faris et al. (1984) have demonstrated that very low levels of ascorbate may be sufficient to alter significantly the proportions of

collagen, elastin and lysyl oxidase synthesized by cultures of smooth muscle cells. These authors found that the amount of elastin deposited by rat aortic smooth muscle cells was considerably reduced by ascorbate supplementation of the medium at concentrations greater than 0.5μg/ml, whilst collagen synthesis was increased 4-fold in comparison to scorbutic cultures. They also found that lysyl oxidase activity decreased if cultures were supplemented with concentrations of more than 2μg/ml of ascorbate.

The mechanism by which ascorbate modulates the synthetic activities of cultured cells is not known. Dunn and Franzblau (1982) have suggested that the effects of ascorbate might be mediated by overhydroxylation of proline residues in secreted tropoelastin. This suggestion was based on the findings of Urry et al. (1979) who showed that hydroxylation of a synthetic elastin-like polypentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ (which coacervates at 37°C in a similar manner to tropoelastin) resulted in a higher temperature requirement for coacervation to proceed. As it has been postulated that coacervation of tropoelastin may be required for cross-linking to occur (Narayanan et al., 1977; 1978), overhydroxylation of proline residues in tropoelastin and consequent loss of the ability to coacervate at physiological temperatures might then result in decreased elastic fibre formation.

An alternative explanation has been put forward by Faris et al. (1984). They suggest that there may be multiple species of lysyl oxidase, and that ascorbate supplementation may selectively decrease activity of the enzyme specific for elastin cross-linking, leaving collagen fibrillogenesis unaffected.

Whatever the mechanism, these findings indicate the possibility of manipulating the synthetic capabilities of cultured cells by varying their extracellular environment. This approach might facilitate studies of the interactions of extracellular matrix components with each other, and the role of the extracellular milieu in directing cell functions. The preliminary study presented in this chapter suggests that auricular chondrocytes may be a promising model system in which such experiments could be undertaken. A more systematic biochemical and ultrastructural study of the effects of varying concentrations of ascorbate on the synthetic properties of these cells seems a logical first step toward this goal.

5.4.5 Immunofluorescent Localization of Anti-MAg Antibodies in Foetal Calf Ear Cartilage

a. Native ear cartilage. Although anti-MAg antibodies were shown to localize to elastic fibres in native foetal calf ear cartilage, the intensity of immunofluorescent staining obtained in this tissue was considerably less than that observed in ligamentum nuchae or aorta (see section 3.3.1). The relatively low fluorescent yield of elastic fibres in native ear cartilage following immunofluorescent staining with anti-MAg antibodies also contrasted markedly with the strongly positive localization demonstrated to occur in the perichondrium. This observation indicates that more antigenic binding sites are available to anti-MAg antibodies on perichondrial elastic fibres than on those fibres which lie within the cartilage plate itself.

In reference to these findings, it is of interest to note that 'masking' of antigenic sites by hyaluronidase- or protease-sensitive matrix components has been reported by a number of investigators who have used immunofluorescent techniques to stain interstitial collagens (Little and Chen, 1982; Bartholomew and Anderson, 1983; Evans et al., 1983) and matrix fibronectin (Holund and Clemmensen, 1982). It is therefore possible that the observed reduction in the number of antigenic sites available to anti-MAg antibodies on the elastic fibres of auricular cartilage may be due to an interaction of the elastin-associated microfibrils of these fibres with proteoglycan or other matrix protein components.

The electron microscopic appearances of perichondrial and cartilage elastic fibres are consistent with this possibility. Microfibrils of typical morphology were clearly seen in association with developing elastic fibres in the perichondrium, whereas microfibrils were much more difficult to resolve adequately as components of cartilage elastic fibres. These difficulties arose in part from the extensive coating of proteoglycan material which was observed on the surface of elastic fibres in the latter tissue. Investigation of the effect of hyaluronidase digestion on the fluorescent labelling pattern obtained with anti-MAg antibodies may therefore be of value to determine if the observed masking of microfibrillar antigenic determinants in cartilage is due to binding of proteoglycans.

b. Auricular chondrocyte cultures. Immunofluorescent staining of foetal calf auricular chondrocyte cultures with anti-MAg antibodies revealed a well-developed, web-like meshwork of fine fibrils located in the extracellular matrix. No cellular fluorescence was evident. Elastin-associated microfibrils of characteristic morphology have been shown by ultrastructural examination to be the dominant element of the matrix in these cultures (see section 5.3.5a). It is therefore likely that newly-synthesized microfibrils share antigenic determinants with those present in intact foetal tissues. This suggestion is consistent with the considerable tissue cross-reactivity exhibited by anti-MAg antibodies (see section 3.3.1). However, further ultrastructural studies are necessary to establish unequivocally that elastin-associated microfibrils are the only structures present in the culture matrix to which anti-MAg antibodies will bind.

It was noted in section 5.3.7b that the fibrillar network demonstrated in these cultures by immunofluorescent labelling with anti-MAg antibodies was much more extensive than the sparse elastic fibre matrix which was visualized after histochemical staining of the cell layer with elastic stains. This observation implies that microfibrils are capable of forming a fibrous network independently of the presence of insoluble elastin. Such a capability is consistent with the postulated role of microfibrils both in initiating elastogenesis and subsequently in directing the spatial orientation of elastic fibres, perhaps by acting as a scaffold for tropoelastin deposition (Sandberg, 1976; Ross et al., 1977). A specific interaction between microfibrils and monomeric tropoelastin may possibly provide a mechanism by which fibrous arrays of tropoelastin, deficient in cross-links, could be organized and stabilized. In order to investigate this possibility, studies are at present being undertaken in this laboratory to compare the immunohistochemical localization of anti-MAg and anti-tropoelastin antibodies in cultures of foetal calf chondrocytes.

5.4.6 Conclusions

Comparative ultrastructural studies of foetal calf, neonatal (2-day-old) rabbit and young (16-day-old) rabbit ear cartilages have shown that foetal calf ear cartilage from

animals of 220 days gestation has a morphological maturity which lies intermediate between the two rabbit cartilages. Elastin-associated microfibrils were visible at the periphery of elastic fibres in the matrix of foetal calf and neonatal rabbit ear cartilage, but appeared to be masked by surface condensation of proteoglycan-like material in young rabbit ear cartilage. It was noted that the certainty with which elastin-associated microfibrils could be identified appeared to be inversely related to the histological maturity of the tissue.

Cultures of enzymically dissociated foetal calf and neonatal rabbit auricular chondrocytes, when maintained under scorbutic conditions, produced a scanty extracellular matrix. Elastic fibres, with typical elastin-associated microfibrils, were the dominant components of this matrix and very little proteoglycan or collagen was seen. In contrast, young rabbit auricular chondrocytes accumulated an extensive, predominantly proteoglycan matrix which contained relatively few elastic fibres. As in native ear cartilage, elastin-associated microfibrils could not be identified due to proteoglycan encrustation of the surface of these elastic fibres.

In common with other mesenchymal cell cultures, ascorbate supplementation of foetal calf auricular chondrocytes resulted in a massive increase in collagen fibrillogenesis with apparent suppression of elastic fibre formation.

Immunofluorescent labelling of native foetal calf ear cartilage revealed a low amount of antibody binding to the elastic fibre network within the cartilage itself when compared with the brilliant fluorescence of the adjacent perichondrium. Labelling of auricular chondrocyte cultures derived from this tissue demonstrated a fibrillar meshwork in the extracellular matrix, implying that microfibrils formed in vitro are antigenically similar to those found in intact tissues.

These studies have firmly established the association of microfibrils with developing elastic fibres in elastic cartilage, both in vivo and in vitro. In addition, it is suggested that elastic fibres in auricular cartilage may undergo a surface interaction with matrix proteoglycan, which may mask the staining of microfibrils so that they cannot be recognized electron microscopically.

It is concluded that foetal calf auricular chondrocyte cultures are a potentially useful model system for the study of elastogenesis in vitro. In particular, their ability to synthesize both components of the elastic fibre suits them ideally to immuno-histochemical and biochemical experiments designed to isolate microfibrillar components.

CHAPTER SIX

CONCLUDING SUMMARY AND DIRECTIONS FOR FURTHER STUDY

6.1 IMMUNOLOGICAL STUDIES

The currently accepted view of elastin fibrillogenesis, which ascribes a central role to elastin-associated microfibrils, is based largely on high resolution electron micrographic studies. However, little is known of the macromolecular structure of these microfibrils. The studies of Ross and Bornstein (1969; 1970), succeeded by those of Kewley et al. (1977a,b) and Sear et al. (1978; 1981a,b), have proved particularly influential in establishing methodology for the study of elastin-associated microfibrils, but there is reason to question some of the findings and conclusions of these studies concerning the composition of 'microfibrillar proteins' (see section 1.3).

Experiments were therefore designed to allow critical re-examination of this data. A 'microfibrillar antigen' (MAg) extract was isolated from foetal calf ligamentum nuchae by a procedure based on that of Kewley et al. (1977a), incorporating several modifications in an effort to reduce contamination of the resultant extract. Thus a more rigorous pre-treatment protocol was introduced, collagenase was purified to eliminate non-specific activity and extensive use was made of protease inhibitors. Despite these precautions, analysis of MAg (Prosser et al., 1984) has revealed it to be a heterogeneous mixture of proteins, which were extremely insoluble under non-dissociative conditions. Similar findings may be inferred from other studies of Ross and Bornstein type 'microfibrillar protein' extracts (Muir et al., 1976; Sear et al., 1977b; 1978; Bach and Bentley, 1980; Serafini-Fracassini et al., 1981a,b). The heterogeneous nature of these extracts strongly implies that (in the absence of a specific 'marker' amino acid or peptide) amino acid analysis is unlikely to be useful in identifying their macromolecular components.

Electron microscopic monitoring of the extraction residues confirmed that elastin-associated microfibrils were removed from elastic fibres by reductive GuHCl extraction. However, in contrast to the findings of Ross and Bornstein (1969), it was established that microfibrils were significantly solubilized by GuHCl solutions throughout the extraction procedure, in the absence of reducing agents. These observed differences in the extractability of microfibrils may relate to the more finely homogenized state of tissues extracted in the present study.

An antiserum to MAg, although producing only a single precipitin band on immunodiffusion against the original extract, was shown by a sensitive ELISA technique to be polyspecific. Contaminant activity was removed by affinity chromatography to produce a highly-specific antibody preparation, referred to as anti-MAg (ADS) IgG. It was found that the preliminary non-reductive GuHCl extracts, SG and SGCG, contained antigenic material which cross-reacted with anti-MAg antibodies. The presence of such antigenically active material is consistent with the progressive solubilization of microfibrils noted during the extraction procedure.

Light and electron microscopic tissue localization experiments confirmed that anti-MAg (ADS) IgG antibodies bound specifically to elastin-associated microfibrils. CL glycoprotein was isolated in this laboratory near the end of these studies (Gibson and Cleary, 1982), and it was recognized at this time that anti-MAg (ADS) IgG contained contaminant low-level activity against CL glycoprotein. This observation can be correlated with the minor additional labelling of thin (3-5nm) matrix filaments observed in experiments with anti-MAg (ADS) IgG antibodies, which was similar to the tissue distribution observed with labelled anti-(CL glycoprotein) antibodies. Subsequent work (M A Gibson and J C Fanning, unpublished observations) has shown that adsorption of contaminant anti-(CL glycoprotein) activity from anti-MAg (ADS) IgG abolished the labelling of these thin filaments, whilst specific antibody binding to elastin-associated microfibrils was unaffected.

Although the tissue localization of anti-MAg antibodies has some features in common with that of the Kewley anti-'microfibrillar protein' antibody preparation (Kewley, 1977a,b), the considerably wider distribution of the latter antiserum points to it being quite polyspecific. The presence of such contaminant activity is probably responsible for the immunoprecipitation of the claimed microfibrillar glycoprotein MFP I with the Kewley antiserum (Sear et al., 1978; 1981a,b). Recent studies suggest that MFP I and CL glycoprotein are related species, and that both may be forms of 'type VI' collagen (Gibson and Cleary, 1983; Heller-Harrison and Carter, 1984; Knight et al., 1984). As CL glycoprotein is not related to elastic fibres (Gibson and Cleary, 1983), it is most unlikely that MFP I is derived from elastin-associated microfibrils.

In conclusion, a major achievement of the present study has been to provide a highly-specific, well-characterized antibody probe with which to investigate further the macromolecular composition of elastin-associated microfibrils. Subsequent work performed in this laboratory (M A Gibson and E G Cleary, unpublished observations) has confirmed the usefulness of this antibody preparation. In these experiments, anti-MAg antibodies have been used to monitor the presence of antigenic components in fractions of GuHCl extracts prepared from foetal calf ligamentum nuchae. The crude extract was first separated into fractions of different molecular size by gel filtration chromatography. It was found that material present in fractions less than 200,000 daltons in size was able to absorb out the anti-microfibrillar activity from anti-MAg (ADS) IgG (demonstrated by fluorescent antibody localization in tissue slices).

The material from this fraction was further subfractionated on a Sephadex G200 column under dissociative conditions (4M GuHCl). Three subfractions were obtained and were used to raise antibodies in rabbits. All three antibody preparations reacted against the original MAg extract. However, immunofluorescence studies showed that only antibodies developed against the small molecular weight fractions (less than 50,000 daltons) gave precise tissue localization to elastic fibres without any contaminant interfibrillar localization. As this antibody does not exhibit any cross-reactivity with tropoelastin or α-elastin it is highly probable that its activity is directed toward microfibrillar components. This relationship is at present being established by immunoelectron microscopic techniques.

Should this be a specific 'anti-microfibrillar' antibody, it could be used to screen the low molecular weight antigenic mixture after resolution of its components by polyacrylamide gel electrophoresis. Antigenically-active species could then be identified by an 'immuno-blotting' technique (such as the 'filter affinity transfer' procedure described by Erlich et al., 1979), and macromolecules of interest isolated by preparative gel electrophoresis.

The way is thus open for the positive identification of the first truly 'microfibrillar' species. It is hoped that the use of the recently-described low-temperature embedding

techniques with Lowicryl media and improved electron microscopic markers such as immuno-gold (Roth, 1983) will facilitate more thorough description of the tissue distribution and cellular origin of such macromolecules.

6.2 FOETAL CALF LIGAMENTUM NUCHAE FIBROBLAST CULTURES

As the extreme insolubility of MAg extract precluded the use of an anti-MAg antiserum in immunoprecipitation experiments, attention was given to tissue culture as a potential source of soluble microfibrillar components. Studies were made of foetal calf ligamentum nuchae fibroblasts, based on the approach of Sear et al. (1978; 1981a,b).

Ultrastructural studies of the cell layer of cultures maintained under scorbutic conditions demonstrated the accumulation of morphologically-typical microfibrils. However, amorphous elastin was not detected. These observations are consistent with the biochemical studies of Mecham et al. (1981; 1984), who found that cultures maintained under similar conditions would produce tropoelastin, but not cross-linked insoluble elastin.

Although ligament fibroblast cultures were shown (by the use of radio-labelled precursors) to secrete newly-synthesized macromolecules into the culture medium, exhaustive attempts to immunoprecipitate antigenically cross-reacting material with anti-MAg antibodies were unsuccessful. These findings suggest that microfibrillar components may be rapidly insolubilized in the matrix. It is possible that simple reductive GuHCl extraction of the cell-free culture matrix may solubilize these microfibrils, thus providing a soluble starting material for analysis as outlined in the previous section.

Comparison of the results of this experiment with those reported by Sear et al. (1978; 1981a,b) further indicates that the antiserum used in their studies (prepared by Kewley et al., 1977a) is directed toward quite different antigenic determinants from anti-MAg antiserum. Studies are being undertaken in this laboratory to determine whether MFP I can be immunoprecipitated from ligament fibroblast culture medium with anti-(CL glycoprotein) antibodies.

6.3 STUDIES OF AURICULAR CARTILAGE

During the course of these studies, evidence was published which appeared to question the invariable association of microfibrils with elastogenesis (Quintarelli et al., 1979). If,

as was claimed, microfibrils did not participate in elastin fibrillogenesis in ear cartilage, the basic hypothesis concerning the role of microfibrils would require major re-assessment. For this reason, a comparative ultrastructural study of elastic fibres in foetal calf ear cartilages, both in vivo and in vitro, was undertaken.

These experiments (Chapter Five) have established clearly the association of microfibrils with developing elastic fibres both in native ear cartilages from foetal calf and neonatal (2-day-old) rabbit, and in primary cultures of enzymically-dissociated chondrocytes derived from these tissues. Electron microscopic observations were confirmed by immunofluorescent experiments with anti-MAg (ADS) IgG which showed the presence of material related antigenically to microfibrils in association with elastic fibres in native foetal calf ear cartilage. Similar studies on cultures of foetal calf auricular chondrocytes demonstrated a network of fine matrix fibrils, which appeared far more extensive than expected from histochemical staining of the cell layer for elastic fibres.

These findings indicate that, contrary to the assertions of Quintarelli et al. (1979), elastogenesis in ear cartilage proceeds in a manner apparently similar to that described in other developing elastic tissues. Moreover, the observation that microfibrils are able to form fibrillar arrays in the absence of stainable elastin indicates their possible function in the morphogenesis of elastic fibres.

Comparative studies of more mature ear cartilage from 16-day-old rabbit have suggested the possibility of a specific interaction between elastic fibres and proteoglycan, which 'masks' the microfibrillar component. Subsequent experiments based on this observation (Fanning and Cleary, 1984) have shown that hyaluronidase digestion of sections of foetal calf ear cartilage revealed microfibrils at the periphery of elastic fibres, which were obscured in untreated sections by surface condensation of proteoglycans. The possibility of a structural relationship between proteoglycan and elastic fibres is also relevant to other elastic tissues, such as aorta, in which significant quantities of proteoglycan are present.

Although these studies are of a preliminary nature, they indicate that donor age and ascorbate supplementation may have major effects on the synthetic capabilities of cultured auricular chondrocytes. Thus it may be possible to modulate their synthetic activities so as to produce a matrix which contains specified proportions of microfibrils, elastic fibres, collagen and proteoglycans. More systematic examination of the synthetic capabilities of cells derived from both pre- and post-natal tissues would be of value in defining this possibility. Such a study could be extended to investigate some of the factors which influence cellular differentiation, along the lines of the experiments performed by Mecham et al. (1981; 1984) on foetal calf ligamentum nuchae fibroblasts.

In regard to these proposals, it should be noted that maturation of rabbit auricular cartilage has been shown to proceed sequentially, beginning at the base of the pinna and gradually extending to the tip. Thus at any time point during the development of the cartilage, there are marked regional differences in the cell and matrix characteristics. This has also been shown to be true in foetal calf ear cartilage (E G Cleary and J C Fanning, unpublished observations). From a practical viewpoint, it is therefore necessary to specify precisely the part of the pinna from which cultures are derived. More importantly, these observations indicate the presence of a mechanism by which the state of differentiation of cells in different areas of the same organ may be varied. As cartilage has no blood supply, it is possible that such a control system may be mediated via cell-matrix interactions.

It has already been noted that whilst foetal calf ligamentum nuchae fibroblasts synthesize large amounts of microfibrils in culture, they do not produce amorphous elastin. However, auricular chondrocytes, derived from the same animal, and maintained under identical conditions, will readily produce both components of the elastic fibre. This is a significant observation, as microfibrillar bundles (of identical morphology to elastin-associated microfibrils) have been shown to persist throughout adult life in certain tissues, including the ocular zonules (Streeten et al., 1983) and the skin (Cotta-Periera et al., 1976; 1977; Kobayasi, 1968; 1977) and do not appear to undergo elastin deposition. Investigation of the apparent discrepancy in synthetic activities

between these two culture systems may therefore enable understanding of the factors which regulate production of the two components of the elastic fibre.

In conclusion, these preliminary studies on foetal calf auricular cartilage have indicated that it is a promising model system in which to examine aspects of elastogenesis. In addition, auricular chondrocyte cultures offer the possibility of insights into cell-matrix interactions and the control of cellular differentiation, particularly with respect to elastin biosynthesis. These studies have stimulated subsequent work in this laboratory to define the histological and ultrastructural development of this tissue (E G Cleary and J C Fanning, work in progress). Studies of the structural interactions between elastic fibres and proteoglycan, and the possible role of proteoglycans in elastogenesis are planned.

6.4 CONCLUDING REMARKS

It seems appropriate at this point to offer some personal speculation as to the nature of elastin-associated microfibrils. It is my view that the 'hollow' core of microfibrils seen in the electron microscope may be composed of tropoelastin chains, coacervated or loosely cross-linked into a fibrous form. This tiny elastic fibril may be 'packaged' in a sheath of spiral glycoprotein chains, perhaps 6-8 per microfibril, which have the ability to bind (or may even incorporate) lysyl oxidase. This sheath may protect the tropoelastin fibril from enzyme attack during transport to the site of elastic fibre formation.

Although the association of lysyl oxidase with elastin-associated microfibrils has been postulated by a number of authors (reviewed by Cleary and Gibson, 1983), until recently it has not been possible to prepare an antibody to lysyl oxidase of sufficient purity and reactivity to allow meaningful tissue localization studies. However, Kuivaniemi et al. (1984) have now described the preparation of an antibody which appears highly specific for lysyl oxidase. Immunolabelling experiments with this antibody may at last prove if lysyl oxidase is, in fact, related to microfibrils.

The composite model proposed is consistent with most of the observed properties of microfibrils. Thus, as elastin biosynthesis begins in developing tissues, microfibrils

would appear and aggregate (by an unknown mechanism) into arrays. Subsequent cross-linking of tropoelastin fibrils into amorphous elastin may be associated with degradation of the glycoprotein coat and loss of its cationic staining properties, explaining the increased electron-lucency of mature elastin.

However, stabilization of the glycoprotein coat might allow persistence of microfibrils into adult life in special situations such as ocular zonules and skin. These 'mature' microfibrils may have a core of more highly-cross linked elastin, conferring on them both elastomeric properties and tensile strength.

In summary, it appears that the study of elastin-associated microfibrils is entering an exciting phase. It is anticipated that within the next few years the biochemical composition of microfibrils will become clear, allowing a more precise understanding of their true function.

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