CELLULAR AND MOLECULAR CHANGES IN CALCIFYING TENDINITIS.

BY

SUSAN RACHEL HANN.

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Doctorate of Philosophy in the Faculty of Science,
University College and Middlesex Medical School,
University of London.

Department of Experimental Pathology,
Institute of Orthopædics,
Royal National Orthopædic Hospital,
Stanmore, Middlesex.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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To my family, with love.

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ABSTRACT

Calcifying tendinitis can be a debilitating disease and occurs when mineral becomes deposited in tendon. The tendons situated in the rotator cuff of the human shoulder are particularly vulnerable. The pathogenetic mechanisms of this disease are unknown, but chondrogenic modulation has been proposed by Uhthoff (1975 et seq.) and this particular theory has been investigated.

Cellular, molecular and enzymic features of pathological tendon have been compared and contrasted with normal tendon and articular cartilage, in addition to ossifying muscle.

Fresh tissue samples were taken in the operating theatre, sub-divided, then processed by several different methods.

Pathological specimens showed a variety of morphological changes. Ultrastructural observations in the regions around the deposited mineral enabled a detailed description of the pathological tissue and the associated calcific deposits.

Alkaline phosphatase is frequently associated with calcification but in mineralising tendon, enzyme activity was not evident.

Immunocytochemistry used to investigate the possible presence of collagen types II, VI, hyaluronic acid binding region protein and chondroitin- 4 and 6- sulphates, molecules that may be associated with a pathological cellular modulation in the tendon. No collagen type II was observed in the calcifying tendon. Thus, we suggest that a chondrogenic modulation is an unlikely causal mechanism of the pathology.

Microanalytical studies compared the calcium to phosphorous ratios of mineral found in individual patients. Statistically significant ratios were obtained from hydroxyapatite crystals found in ossified tendon, compared with the less electron dense crystals of calcospherites located in the same tissue section. The ratios were all closer to the expected value for hydroxyapatite rather than pyrophosphate.

Although it is not yet possible to provide a specific pathological mechanism for calcifying tendinitis, it appears not to be akin to endochondral ossification or primary bone formation, but may have features unique to tendon mineralisation.

CONTENTS TABLES.

CHAPTER ONE: MORPHOLOGY AND ULTRASTRUCTURE.

CHAPTER TWO: ALKALINE PHOSPHATASE IN CALCIFYING TENDINITIS.

CHAPTER THREE: IMMUNOLOCALISATION OF MATRIX MACROMOLECULES.

CHAPTER FOUR: MICROANALYSIS OF CALCIFIC DEPOSITS.

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INTRODUCTION.

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Calcifying Tendinitis.

The human shoulder joint has been the evolutionary result of adaptations giving intricate forelimb manoeuvrability. The advanced and complex shoulder mechanism of the human 'front leg' facilitates numerous extensions and rotations not possible for quadrupeds. Possibly because of its complexity, versatility having been achieved at the price of robustness, the shoulder is prone to a large number of different defects. One of these, calcifying tendinitis and its pathological mechanism, is the subject of this thesis.

Calcifying tendinitis is among the commonest causes of musculo-skeletal disorders involving the shoulder region and is important because of its painful incapacitating nature. The disease has been the subject of very few scientific studies and there are limited epidemiological data available. As amorphous deposits occur in apparently previously normal tissue with no detectable abnormality in calcium and phosphorous serum levels, the disease is currently classed in the pathogenetic group of idiopathic soft tissue calcification disorders (Connor, 1983).

The possibility of minor trauma in the tendon some years prior to the onset of mineralisation cannot be ruled out as significant in the aetiology, although few patients recall such injuries. Presumably such lesions are small and occur a considerable length of time before the feeling of pain associated with the disease.

Other names given to the condition include calcific periarthritis, which emphasises the extra-articular site of the deposit and peri-articular apatite deposition which defines the type of deposit.

Apatite is actually calcium hydroxyapatite a compound with the formula $Ca_{10}(PO_4)_6(OH)_2$. Although apatite deposition may affect almost any tendon and is indeed a part of the normal progression of tissue changes from tendon to fibrocartilage, being present just at the attachment to bone, the formation of deposits in the main body of the tendon is a pathological process often occurring in the rotator cuff region of the shoulder joint.

Clinical History and Tissue Pathology.

Clinical features of the condition vary. According to Bosworth (1941) about 43% of calcifying tendinitis patients show no real pain whereas others have acute pain and inflammation. While degrees of pain are notoriously difficult to evaluate, Bland et al. (1977) state that if a calcific mass reaches 1.0-1.5 cm. in diameter it always becomes symptomatic. Clinically an inflammatory response is associated with acute shoulder pain.

Bosworth also reported that of the shoulders of 138 patients with demonstrable calcific deposits, 64 (46%) had bilateral deposition and of those with unilateral deposits, the right shoulder was involved twice as often as the left. Bland's statistics (1977) give the occurrence of multiple or bilateral deposits to be as high as 78.5% in calcifying tendinitis patients. No study has been made to directly correlate right handedness with the predilection of the right shoulder to deposit formation, but increased occurrence on the right lends favour to the theory of trivial injury initiating the disease process.

Diagnosis is by radiography, which can clearly demonstrate the presence of calcific nodules (fig:1). Biochemical and haematological tests are not useful and will only show evidence of non-specific inflammation (Faure and Daculsi, 1983). Calcifying tendinitis is rarely associated with metabolic abnormalities of calcium and phosphorous. Infection has never been associated with the formation of calcific deposits, (Wilson, 1939; and agreed by other authors). The complaint often resolves spontaneously. However, persistant cases may require more aggressive treatment including surgery, which is normally successful. Some deposits may be washed out with a large bore needle, but this is harder and often unsuccessful.

Calcific deposits in the rotator cuff are most frequently located in the supraspinatus tendon but also appear in the infraspinatus, less often the teres minor and rarely in the subscapularis tendons (Bosworth, 1941). The deltoid and teres major muscles along with those adjoining the aforementioned rotator cuff tendons are the

Figure 1.

X-ray demonstrating a calcific deposit in the supraspinatus tendon (Patient C).

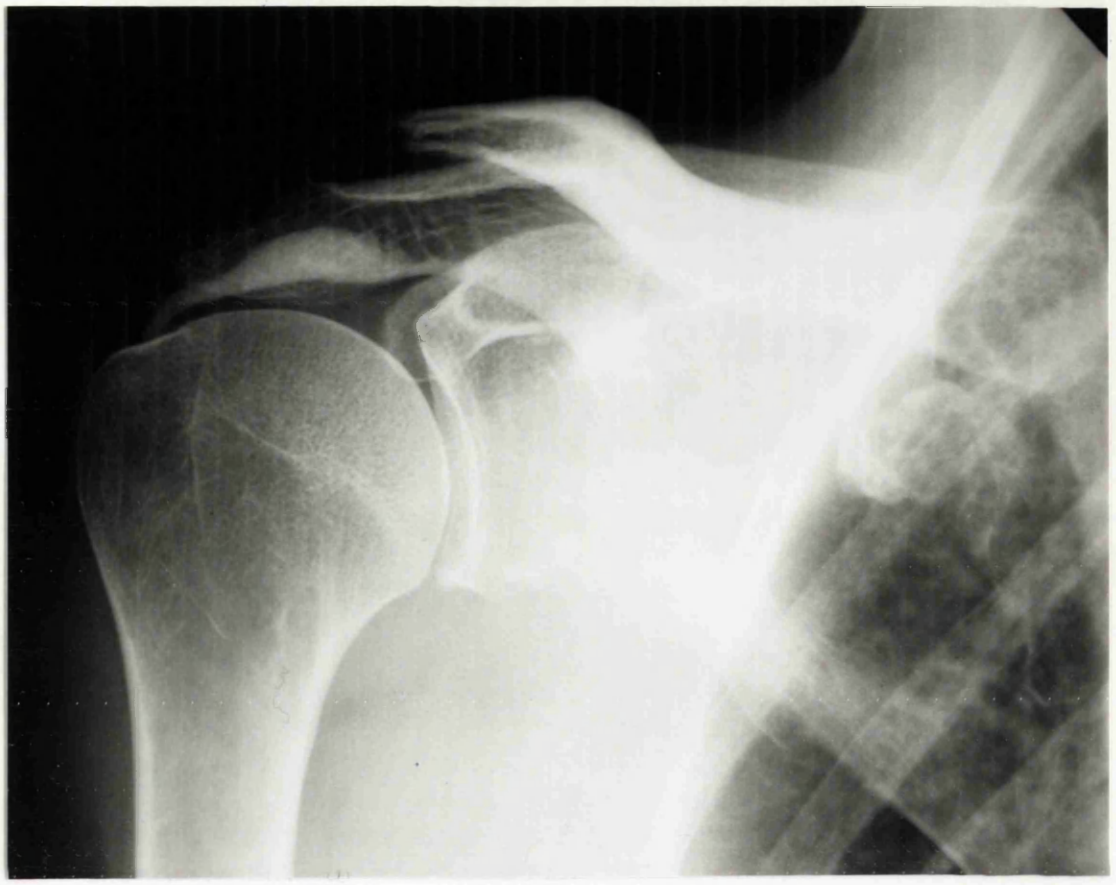
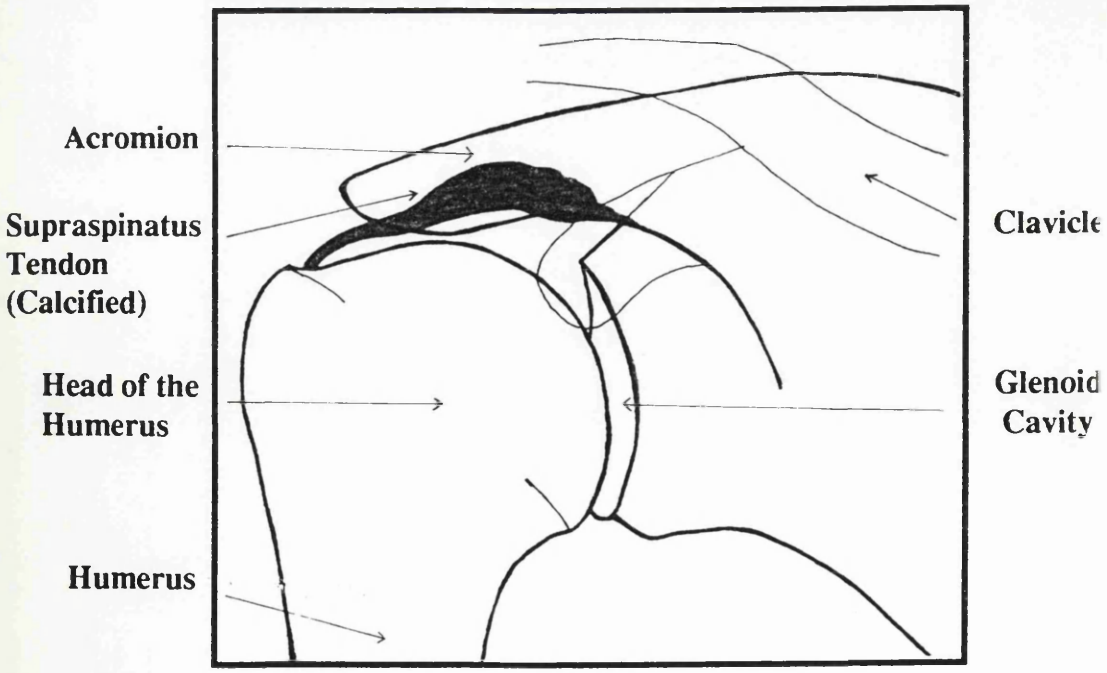


Diagram showing important features of the X-ray above.



scapulohumeral group, that pass from the scapula to the humerus. Both the supraspinatus and infraspinatus tendons arise from and cover the dorsal surface of the scapula. The supraspinatus stabilizes the head of the humerus as the deltoid abducts the limb. It is important to note that the supraspinatus tendon passes under the acromion in the region where the tendon bends to mould with the curve of the shoulder. This is before it inserts into the greater tuberosity of the humerus and hence is in close apposition to both the acromion and the head of the humerus. Due to reduced vascular perfusion, this part is frequently referred to as a "critical zone", where tissue hypoxia could occur (Rothman and Rubin. 1967). It is in this region of the tendon that the calcific deposits are often located. The subscapularis is the only rotator cuff tendon that inserts into the lesser tuberosity rather than the greater tuberosity. The rotator cuff tendons are broad, flat and about two and a half centimeters in length. As the capsule of the glenohumeral joint is formed by a merger of the tendons it is impossible to dissect the tendons from the capsule. Rotator cuff tendons are located directly under, and firmly attached to, the sub-acromial bursa which coats part of the greater tuberosity and continues onto the tendon upper surface. The function of bursae is to provide easy gliding movement, without any real articular contact or redistribution of load, where two connective tissues must move on one another. In this case the sub-acromial bursa allows smooth motion between the acromion and adjoining ligamentous arch (coracoacromial arch) above, and the rotator cuff tendons below. In calcifying tendinitis patients the bursa may become secondarily inflamed as the result of calcific deposits in the tendon causing rupture and movement of mineral into the bursal sac. Thus, calcific bursitis can be clinically coexistent with calcifying tendinitis. For a detailed description of the shoulder joint and its mechanics see Bland et al., 1977.

The late ninteenth and early twentieth centuries saw the first serious investigations of the anatomy of the shoulder joint. Calcified deposits around the shoulder were first identified by Baer in 1907 who described the deposit as 'dense cheesy material'. In 1934, Codman published a book giving a detailed account of his lifetime's study of 'The Shoulder'. This text is still widely quoted and has been an important foundation

for subsequent studies. Apart from describing the normal shoulder Codman made a study of pathological conditions and his book includes a chapter on 'Calcified deposits in the supraspinatus tendon'. From this it became generally accepted that in calcifying tendinitis, degeneration of tendon fibres precedes calcification. Codman describes the pathological lesions and outlines some of the contemporary theories as to the causes and mechanisms of deposit formation. His own hypothesis was that tissue degeneration occurred long after acute or chronic injury. The histology of a pathological lesion being due mainly to the faulty repair of an injury in tissue that had little vascularity and was slow to restore itself. One contemporary Meyer (see Codman, 1934) having made an anatomical study, suggested that the supraspinatus may be attenuated as a result of friction in overuse, but Codman only accepts this possibility if inflammation with resulting attenuation and atrophy are assumed as intermediate pathological states. Another colleague Moschcowitz (1915), considered the primary process to be tendinitis. Subsequently, the classical hypothesis for the mechanism of mineralisation favoured a local and initial necrosis of the tendon leading to the deposition of calcific material, although some calcifications were thought to occur in the absence of any necrotic phenomenon.

It was another forty years before new ideas emerged and real progress was made in the study of pathological human tendon undergoing calcification. Up to this time, both tendon rupture and calcifying tendinitis were considered to be degenerative diseases of the rotator cuff tendon of the shoulder (Steinbrocker 1972). Then Uhthoff (1975) proposed calcifying tendinitis to be an active cell-mediated calcification by postulating that a fibro-chondrogenic transformation (henceforth referred to as modulation) takes place within the tendon and thus chondrogenic cells are responsible for the deposition of mineral. Uhthoff et al. (1976) and then Sarkar and Uhthoff (1978) decried suggestions of an underlying degenerative process and this was further confirmed by Jozsa et al, (1980) who concluded that a hypoxic alteration of the tendon occurs without necrosis or inflammation. Rathbun and Macnab (1970) noted that the zone of relative avascularity of the supraspinatus tendon corresponds to the most

common site of breakdown changes in the rotator cuff. It is in this critical zone that rotator cuff tendinitis, calcification and spontaneous ruptures most often occur.

Myskiw et al., (1978) have investigated the role of devascularisation and foreign body implantation in promoting tendon calcifcation in rabbits and suggest a model for chronic tendinitis. Devascularization resulted in dehydration. The subsequent death of tendon cells, gave a release of lytic enzymes causing in vivo calcification in tendon. Further, they found that when tendon was implanted with rabbit fur, an intact blood supply permitted hyperhydration giving rise to an inflammatory response. Cell death was believed to be the result of toxin accumulation rather than the osmotic imbalance caused in the devascularised tendon. The release of lytic enzymes occurred, as with the devascularised tendon, but with moderate calcification resulting, insufficient to cause the same degree of maceration and damage to the collagenous matrix. These authors concluded that crystalization of calcium and phosphate ions results from death of cells, regardless of the cause. In the supraspinatus tendon the "critical zone" is avascular (Rathbun and Macnab, 1970), so the devascularised area in the rabbit model is interesting as it enables examination of tendon that is without blood vessels. Myskiw et al., (1978) believe that the water in the tendinous matrix is replaced by inorganic matter during calcification quoting Deakins work (1942) for support and do not consider the possibility of cellular modulation occurring prior to deposition of calcium.

Faure and Daculsi (1983) in reviewing the topic of calcifying tendinitis note that the intracellular or extracellular site of the first deposit is still unclear. However, one of the interesting features of calcifying tendinitis is that the deposits can spontaneously resorb, a phenomenon reported to be coincident with severe pain when, during resorption, hyperaemia prevails (Uhthoff and Sarkar, 1978). In 1975, this group reported the presence of macrophages phagocytosing calcium and noted the similarity to the 'foreign body giant cells' reported in experimental extraosseous tissue calcification (Seifert 1970). This may explain some of the confusion as to whether the deposit occurs intra- or extra-cellularly.

Tissue anatomy and morphology.

The normal but variable structures of the different tissues that might have some involvement in the pathological processes are outlined, including descriptions of the characteristic cell types and the components of the surrounding cell matrices. The mature tissues implicated in the disease are tendon, which is the host tissue, cartilage, which is possibly an intermediary of the pathological calcification, and bone, which is necessarily rich in minerals of similar type, if not identical, to the pathological deposits. While the matrices of tendon, cartilage and bone are histologically separable, these connective tissues have the same basic structure, consisting of cells of mesodermal origin suspended in varying amounts of viscous gel or ground substance (Gardner and O'Rahilly, 1968). Strength and stability are obtained by the presence of collagen fibrils, the types and quantities being tissue specific. Bone also contains minerals that give it rigidity.

Tendon.

Tendon is the strong fibrous connection between a muscle and a bone. The tough regular arrangement of collagen fibrils laid in large uniaxial bundles gives the tendon a high tensile strength and low elasticity. It is the specific spatial pattern of fibril bundles and their zig-zag crimped arrangement that gives tendon its characteristic histological appearance. The fibril bundles together with the tendon fibroblasts are, in most tendons organised in fascicles (Elliot, 1965). Fascicles possess a crimp structure and within neighbouring fascicles there is a strong tendency for crimping alignment (Kastelic et al., 1978). The fascicles are enclosed within the paratenon, a sheath built of loose and dense connective tissue. Larger tendons are tightly packed bundles of fascicles, each bundle wrapped in cylinders of loose connective tissue, called the endotenon (Davison, 1982).

Human tendon has not been well characterised but many scientific data available on animal tissues have proved to be also directly applicable to humans (Gardner and O'Rahilly, 1968). Birk and Trelstad (1985; 1986) investigated the synthesis and

* For a review see: Uitto J and Perejda AJ (Eds) 1987. Chapter 12. Posttranslational Modifications of Collagen and Their Alterations in Heritable Diseases. Connective Tissue Disease. Molecular Pathology of the Extracellular Matrix. Volume 12. The Biochemistry of Disease. Marcel Dekker, inc. New York and Basel. 263-292

deposition of collagen in chick embryo tendon and related the intra-and extra-cellular compartmentation to collagen fibrillogenesis, fibril bundle formation and tissue morphogenesis. Where it is necessary to minimise friction against adjacent surfaces tendons are often enclosed within slippery, collagenous tendon sheaths. In an area where it may slide over hardened surfaces tendon may also form fibrocartilage (Gillard et al, 1977).

The cells and surrounding matrix components of interest in this thesis are those in 'normal' healthy tendon, those found in associated non-pathological tendon once the condition has become apparent, those that may have mediated during the development of the disease, and those present as a result of the pathological condition. Information may be derived about the condition by assessing the presence or absence of specific tissue components. Cell types are important in identification of ongoing processes within the tissue and knowledge of the intra-cellular components allows assumptions to be made about the synthetic activity levels, mitotic status, and phagocytotic resorption. This information is vital for the characterisation of cell types and cell status.

Cells. In young and active tenocytes the cytoplasm is abundant and basophilic because of the high concentration of rough endoplasmic reticulum. In mature cells the cytoplasm is less dense with a few fine granules and occasional fat droplets, and they have nuclei that are relatively large, with prominent nucleoli. Their shape is flattened and irregular in outline, with branching processes. In longitudinal section they appear spindle shaped whereas a cross-section gives a triangular or rounded appearance. In old and inactive fibroblasts the cytoplasm is sparse, the endoplasmic reticulum scanty and the nucleus flattened and heterochromatic. For details of age-related morphologial changes in rabbit tendon, see Ippolito et al., (1980).

Collagen. The collagen molecule is synthesized by the tenocyte initially within the cell as a larger precursor (procollagen) then secreted and cleaved extracellularly to become collagen (Branwood, 1963; Porter, 1964),. The components of 'normal' tendon may include at least three of the known collagen types (currently thirteen collagen types have been localised but this figure needs regular revision (Burgeson, 1988)).

Type I collagen the most widely found, is abundant in tendon and is known to coexist with type III collagen (Keene et al., 1987). Unlike the collagen type II of cartilage, the type I collagen in tendon associates into thick parallel bundles.

Type III collagen is frequently associated with the processes of development (Epstein and Munderloh, 1975) and repair (Williams et al, 1984), although the ratio of types I and III collagens varies considerably between tissues. According to Amiel and Kleiner (1988) there is no detectable collagen type III in tendon but it makes up about 10% of the collagen in periarticular ligaments.

Type VI collagen forms microfibrils in the extracellular matrix of adult rat tail and embryonic chick tendon (Bruns et al.1984,1986) and may have a role to play in restricting the diameter of type I collagen fibrils. Banded aggregates of collagen with ~100nm periodicity, located in connective tissues, are assumed to be collagen type VI (Bruns et al., 1986; Keene et al., 1988). Collagen with this periodicity has been implicated by association with various diseased tissues, particularly tumours (Buckwalter et al., 1979).

Type XII collagen is expressed in embryonic chick tendons (Dublet and van der Rest, 1987) and has demonstrated similarities with collagen type IX found in cartilage.

Other minor components of tendon include, proteoglycans (Scott,1981 and 1984; Vogel et al.,1984), structural glycoproteins, plasma proteins and a variety of small molecules. Fibronectin is secreted by the synovial cells (Banes et al,1988) in an avian model, and in equine tendon it is reported that the concentrations synthesized increased in healing tendon, disappearing once repair is complete (Williams et al, 1984).

Regional variation in tendon structure. Investigations by Daniel and Mills (1988) into cell types and matrix components of non-pathological rabbit flexor tendons have shown two distinct biomechanical regions. Their experiments showed that adult tendon cell cultures continued to express region-specific phenotypes and thus yielded two distinct populations of cells. From the compressed regions chondrocyte-like cells and abundant matrix were obtained. In vitro, these synthesized high molecular weight chondroitin sulphate proteoglycan. Immunohistologically, chondroitin sulphate and

keratan sulphate could be located in this region only. Tensional regions gave rise to typical tendon morphology with elongated cells, sparse matrix and parallel bundles of collagen fibres. In vitro there was synthesis of a dermatan sulphate rich, low molecular weight proteoglycan. This confirms the findings by Vogel et al., (1986) that the glycosaminoglycan content in compressed distal regions of bovine flexor tendon are up to 50% higher than in the proximal region.

Confirmation of keratan sulphate rich areas has been made in studies on adult bovine flexor tendon (Vogel and Thonar, 1988) and in chick embryo tendon (Craig et al.,1987). Quantitative immunoassay has shown keratan sulphate to be present in at least 500-fold higher amounts in the fibrocartilaginous region of tendon that is subjected to compressive forces in vivo than in more proximal regions subjected only to tensional forces (Vogel and Thonar, 1988).

Tendons have their own blood vessels and can heal if the tissue is stitched together after injury, but little is known about the mechanism of tendon repair. The loose areolar tissue (paratenon) that surrounds the tendon specializes to form a tendon sheath where the tendon bends. The inner part of the sheath closely wraps the tendon and the outer part may be fixed locally to adjacent bone. Between the two wrappings a thin lubricant solution which includes hyaluronic acid reduces frictional resistance to the sliding of the tendon (Davison, 1982).

Riederer-Henderson et al. (1983) studied the extracellular matrix differences between 17-day chick embryo tendon and synovial fibroblastic cells. On the day of isolation, in primary culture, the tenocytes were found to produce 30% of their total protein as type I collagen whereas the synovial fibroblasts synthesized 10% of their total protein as a mixture of types I (~70%) and III (~30%) collagen. The tenocytes were also found to produce two to three times more sulphated glycosaminoglycans than the synovial cells. Kain et al. (1988) investigated regional differences in matrix formation in the healing flexor tendon of chickens and found that the sheath integrity (excision or repair) had no effect on the net matrix formation of the healing tendon or nutrient uptake (Peterson et al.,1985). Therefore, from these observations, differences that exist

between synovial fibroblasts, sheath fibroblasts, and tenocytes are assumed not to be of significance in the tendon repair process. However, in a chapter on the repair of tendons (Peacock, 1984) establishes that the spindle-shaped cells of mature tendons are relatively inactive fibrocytes and that few of the cells are capable of synthesizing fibrous protein. He concludes that healing after surgery occurs through the formation of a dense connective tissue scar dependent upon the migration of cells from other areas which have the ability to synthesize fibrous protein, mucopolysaccharides, etc.

The binding of many tendons to bone occurs through a fibrocartilaginous transition zone, the length of which according to Hirsch and Morgan (1939) ranges between one and two millimeters in infants and in adults, at insertions from a few micra to one millimeter. In the fibrocartilage, the cells are more chondrocytic in appearance with increasing proximity to the bone. The matrix becomes decreasingly collagenous and the proteoglycan rich, cartilaginous ground substance is apparent. "Sharpey's spicules or fibres", first described in 1849, were at one time believed to be mineralised cartilage or bone interdigitating with collagen fibrils close to the bone. They are now considered to be collagen fibres between columns of rounded cells (Cooper and Misol, 1970). The fibres consist mostly of type I collagen molecules (Eyre and Muir, 1975). It is unclear whether the chondrocyte-like cells of fibrocartilage produce any of the type II collagen characteristic of hyaline cartilage (Davison, 1982), but a mixture of collagen types I and II have been observed around the hypertrophic cells in epiphyseal growth plate (von der Mark and von der Mark, 1977) and could occur here.

At the musculo-tendinear junction the collagen fibrils of tendon attach to the sarcolemma of the fibrillar proteins of muscle. Other tendon fibrils appear to penetrate and perhaps fuse with the collagen of the perimysium and epimysium (Davison, 1982).

Cartilage.

Cartilage, which occurs in three forms, hyaline, yellow elastic and white fibrous, has a high matrix to cell volume ratio. Because white fibrocartilage is composed, in the main, of type I collagen bundles, interspersed with chondrocytes it has many physical and

histological properties of tendon. Fibrocartilage is a strong connective tissue found at the insertion of the supraspinatus and other rotator cuff tendons into the bone of the humeral head.

Elastic and hyaline cartilage contain distinctive large round chondrocytes, however, when clustered together the adjacent cell surfaces are flat. The large spherical nucleus is surrounded by dense rough endoplasmic reticulum. Characteristically the cells have many short processes and are enclosed in lacunae, set in a more solid matrix. Chondrocytes may appear in clusters that are assumed to be derived from a single cell by mitosis. Chondroblasts are young or dividing chondrocytes. Elastic cartilage contains elastin and collagen type II which is the primary collagen component in hyaline and elastic cartilage matrix (Linsenmayer and Hendrix, 1980). Both types of cartilage are avascular, have no lymphatic vessels, and are not innervated. Hyaline cartilage is found in the foetal skeleton, epiphyseal growth plate, articular cartilage and other sites. Elastic cartilage is found in regions of the ear, epiglottis and the pharyngotympanic tube (Craigmayle, 1986).

Collagen. Collagen type II has a fibrillar triple helical structure the fibrils of which are generally smaller in diameter than those of collagen type I. However, it is glycosylated to a much greater degree than the latter (Kuhn, 1987). Collagen type II provides a strong meshwork that supports and strengthens the tissue. Other minor collagens reported in cartilage are:

Type V collagen was first reported to be present in cartilage by Rhodes and Millar (1978) but this has since been questioned by Ayad et al., (1984) who suggest cross-reaction occurred between the earlier type V polyclonal antibodies with type VI collagen later localised in cartilage.

Type VI collagen, in articular cartilage, is located in chondrocyte lacunae. The lacuna is a basket-like capsule termed a chondron, believed to be rich in minor collagens (Poole et al., 1984), particularly collagen type VI (Ayad et al., 1984), which is also found in the inter-territorial matrix.

Type IX collagen is also a proteoglycan by virtue of a glycosaminoglycan side chain.

It is thought to interact between collagen fibrils (Muller-Glauser et al., 1986) as evidence from immunogold localisation demonstrates that type IX collagen is co-distributed throughout the matrix with type II collagen fibrils, and a collagen network stabilising role is proposed.

Type X is a major biosynthetic product of chick hypertrophic cells (Schmid, 1985a, b) being present in all cartilages which participate in endochondral bone formation, but not present in those that remain cartilaginous (Linsenmayer et al. 1988). The collagen type X molecule is about half the size of a typical interstitial collagen (types I and II). Two possible roles for this collagen type have been postulated (Linsenmayer et al., 1988). The molecule may be incorperated into the matrix vesicle membrane by virtue of a COOH- insertion sequence that is its terminal globular hydrophobic domain. However, more recent evidence shows that there is no direct association between matrix vesicles and collagen type X. Rather the molecule is associated with collagen type II (Poole and Pidoux, 1989). An alternative theory is that type X collagen is involved in matrix degradation, being more susceptible to breakdown by vertebrate collagenase than collagen type II, thus participating in the formation of the advancing marrow cavity.

Type XI collagen was originally isolated from growing human cartilages (Burgeson and Hollister, 1979) and is thought to be co-distributed with type II collagen throughout the cartilaginous matrix (Eyre et al. 1987).

Cartilage; Non-Collagenous Matrix. The ground substance of connective tissues is the non-fibrous viscous gel containing a high proportion of water in which the cells and other components are embedded. Cartilage has a very high extracellular water content which may help in promoting molecular movement compensating in part for the lack of vascularity. The glycosaminoglycans which form part of the cartilage matrix are keratan sulphate, chondroitin-6-sulphate, chondroitin-4-sulphate, dermatan sulphate and hyaluronan (Hardingham, 1986). The glycosaminoglycans help regulate the diffusion and flow of other molecules through the cartilage and give the tissue its resistance to compression, together with durability, resilience, and ability to organise and maintain a high concentration of water. Proteoglycans vary considerably in size, electrolytic

charge and composition, and in cartilage are known to have two levels of organisation. Monomers consist of central core protein filaments with attached glycosaminoglycan chains whereas aggregates consist of central hyaluronan filaments with multiple attached monomers. Most aggregated monomers have a thin segment which attaches to the hyaluronate filament and this probably represents the keratan sulphate rich region of the protein core. There is also a peripheral thick segment that usually represents the chondroitin sulphate rich region, but in some monomers this can also be part of the keratan sulphate rich region. For a recent review of the electron microscopy of cartilage proteoglycans, see Buckwalter and Rosenburg (1988). Equilibrium density centrifugation studies on intact proteoglycans extracted from cartilage have demonstrated that proteoglycans have the ability to aggregate (see Carney and Muir, 1988). Whilst in cartilage the proteoglycans have the ability to form macromolecular aggregates in the presence of hyaluronan (Hardingham and Muir, 1973), the ability of tendon proteoglycans to form similar aggregates has yet to be demonstrated. Region associated variations in size of proteoglycan populations in tendon have been shown (Vogel et al., 1986: Vogel and Thonar, 1988). In tendon, aggregation could be specific to fibrocartilaginous regions. There is also a variety of minor glycoproteins found in human cartilage which include fibronectin (Weiss and Reddi, 1981), chondronectin (Hewitt et al., 1982), and anchorin CII (see Fernandez et al.,1988).

Bone.

Bone is a rigid form of connective tissue, normally confined to the skeleton, which has both mechanical and metabolic functions. It is initially laid down in in a thin layer (membrane) as in the bones of the face, the skull vault and the clavicles, or via cartilage (endochondral ossification) as in all other major bones. Macroscopically, two types of bone, spongy (cancellous) and compact (dense) may be distinguished. Spongy bone consists of slender irregular trabeculae which branch and unite forming a network, the spaces being filled with bone marrow. Compact bone appears solid, except for microscopic spaces. The difference between the two types of osseous tissue depend

only on the amounts of solid matter and the size and number of spaces. Almost all bones contain both spongy and compact types, however, the amount and distribution of each varies. Except over their articular surfaces, bones are coated with specialized connective tissue called the periosteum. The endosteum is a similar, but less developed, connective tissue layer that lines the marrow cavity and marrow spaces. The production of bone has only rarely been reported in human tendon (Uhthoff 1975), the common mineral deposits found in calcifying tendinitis resembling neither compact nor spongy bone.

Cells. Three cell types peculiar to bone are recognised, for a more detailed description of these cell types see Vaughan (1981a).

Osteoblasts lay down bone matrix found at the surface of bone, referred to as osteoid tissue, where osseous matrix is being deposited. Osteoblasts differ in appearance in young and adult bone formation. In actively growing bone the cells are columnar in shape, each with several long processes, an eccentric nucleus, a prominent Golgi body, many mitochondria and basophilic cytoplasm. In mature bone osteoblasts are more elongate and are often referred to as lining cells. Mineralisation follows matrix production but the role of the osteoblast in this process is not well understood. However, osteoblasts do produce the enzyme alkaline phosphatase, which is strongly implicated in mineralisation.

Osteocytes are osteoblasts trapped within the bone matrix. They are smaller and less basophilic than osteoblasts and they appear not to demonstrate alkaline phosphatase activity. Osteocytes have numerous interconnecting cytoplasmic processes which extend along canaliculi. Each cell retains some rough endoplasmic reticulum and Golgi apparatus, and the cytoplasm also contains fat droplets and some glycogen.

Osteoclasts are multinuclated giant cells which vary greatly in size and the number of nuclei they possess. They are found in close association with the surface of bone, where they are involved in resorptive activities.

Collagen and mineral. Collagen forms about 19.5% by weight of the composition of fresh bovine cortical bone (Vaughan, 1981a). Type I collagen makes up about 97% of

this total and type V collagen about 3%. Data from other tissues suggests that these may exist as copolymers in the same heterotypic fibrils. (Niyibizi and Eyre, 1988). The collagen molecule is considered to have intrafibrillar spaces to accomodate most of the apatitic mineral. However, the collagenous fibrils themselves contribute greatly to the strength and resilience of bone. The mineral of bone accounts for approximately two thirds of its total weight. Inorganic salts that are responsible for bone hardness and rigidity include calcium phosphate (about 85%), calcium carbonate (about 10%) and small amounts of the fluorides of calcium and magnesium (Leeson and Leeson, 1976). Calcium phosphate appears in different forms according to the calcium to phosphorous ratio. Hydroxyapatite the main mineral in bone was thought to be the substance deposited in calcifying tendinitis (Moseley, 1969). However, the calcifications from shoulder tendon sheaths have been shown to consist of carbonated apatites and are thought to be a mixture of several phases of different nature and crystallinity (Faure et al., 1982).

Development of tissue types.

Information about the differentiation of cell types could provide clues as to the reason for mineral deposition in calcifying tendinitis. Cartilage, muscle, tendon, and other connective tissues that comprise the extremities are derived from homogeneous-appearing population of mesenchymal cells of the embryonic limb bud (Gardner and O'Rahilly, 1968). Although these mesenchymal cells appear identical, they consist of distinct precursor cell populations that can differentiate into specific tissue types (Dienstman et al.,1974 and others). Some undifferentiated cells are believed to remain in the tissues, providing a postnatal source of new cellular elements. Hence the fibroblasts involved in tendon repair are thought to be mesenchymal cells that were latent in the tissue prior to damage.

Evidence of differentiation into specific cell lineages comes with the production of tissue specific proteins (eg. collagen type II by chondrocytes). This must be preceded by molecular events at the genetic level which account for the commitment of

mesenchymal cells in the limb bud to form specific tissue types. Chondrogenesis from single limb mesenchyme cells has been demonstrated. This has been defined by the appearance of a pericellular alcian blue staining of the matrix, and intracellular type II collagen as demonstrated by indirect immunofluorescence with monoclonal antibodies, and the presence of clonable cartilage cells (Solursh et al. 1982). The differentiation of cartilage cells from single mesenchyme cells in vitro and in vivo occurs only when the cells are rounded, so cell shape changes have been proposed as one factor that could mediate effects of cell-cell interaction in the process of differentiation (Archer et al. 1982). There is also general agreement that osteoblasts are derived from the mesenchyme (Bassett 1962).

There are two distinct types of osteogenic precursor cell:

The determined osteogenic precursor cells are found in the marrow stroma and thought not to migrate in the blood (Friedenstein 1973,1976). These cells have the characteristics of stem cells since they are capable of self-replication and of generating differentiated cells. It is unlikely that these cells would be located away from their natural sites, such as the deep layers of the periosteum and the marrow stroma.

The inducible osteogenic precursor cell is present in the connective tissue framework of many tissues other than bone marrow and may circulate in the blood (Ashton et al. 1980). Unlike the determined type, these cells will not form bone in the absence of stimulation and any bone production continues only whilst the inducing agent is present (Friedenstein 1968, 1973; Owen 1970). The stimulus needed to activate the inducible osteogenic precursor cells could be trauma, contact with certain cell types or with bone matrix proteins or a combination of these factors. Therefore, another possible mechanism for the pathological mineral deposition in human tendon which should be given consideration is the stimulation of inducible osteogenic precursor cells latent in the connective tissue, to produce non-ossifying mineral deposits.

Strong evidence indicates that osteoclasts, are derived from different precursors to the osteoblasts, osteoclasts being derived from the haemopoietic stem cells of the marrow, probably via monocytic cells (Buring 1975, Jotereau and Le Douarin 1978,

Vaughan, 1981) . Osteoblasts and osteoclasts cannot transform from one cell type to the other. Therefore, the multinucleate nature of the osteoclast is seen as the incidental result of macrophages attempting to ingest bone in close apposition to other macrophages, then fusing to become multinucleate cells (Chambers, 1980).

Cellular differentiation in skeletal tissues was extensively reviewed by Hall (1970) in an article that concentrated on inter-transformations between bone and cartilage. Morphological form as the main criterion of differentiation was questioned due to evidence that stem cells in vitro can show more than one line of differentiation, depending on the microenvironment (Fell and Mellanby, 1952). A study of cell proliferation and specialisation during endochondral osteogenesis in young rats (Young, 1962), demonstrated that cells could revert to an undifferentiated state and then reform as a different cell type (Cottrill et al., 1990). Although the stimulus is unknown, germinal cells become cartilage under the influence of intermittent pressure and tension acting on membrane bones at points of articulation with other elements of the skeleto-muscular system (Hall, 1968). This is perhaps more relevent to the mechanism involved in ectopic bone formation in cases such as myositis ossificans, where endochondral ossification is evident. In calcifying tendinitis specimens, individual cells are described as chondrocyte-like but the tissue surrounding these cells does not appear cartilaginous. However, intermittent pressure and tension is a pattern that fits with normal use of an arm and hence can be envisaged as a stimulation for the initiation of mineralisation in the shoulder . The supraspinatus tendon is located directly below the acromion and is effectively between this and the head of the humerus in a site that would be subjected to intermittent tensional and compressive forces.

Embryonic chick and foetal rat sequentially express osteoblast markers during the development of the extracellular matrix. Type I collagen and alkaline phosphatase are synthesized throughout proliferation and matrix mineralisation, whereas osteocalcin and osteopontin are expressed after the proliferative period. In order to meet one criterion set for osteoblasts in tissue culture, type III to type I collagens must be expressed in a ratio of less than 1 to 9 (Spelsburg et al. 1988). The presence of osteoblast markers in

calcifying human tendon would demonstrate an osseous stage in the pathological process. Collagen types I and III are both components of normal tendon matrix and were therefore, not useful in this investigation. Alkaline phosphatase is a precursor of mineralisation whether via endochondral ossification or membranous bone formation . The production of osteocalcin or osteopontin would indicate the abnormal presence of bone specific cell-types.

Endochondral ossification: Bone growth occurs both in length and girth. The increase in girth comes through deposition of periosteal bone. Length increase is a result of epiphyseal growth of long bones and vertebrae. Growth plates are present at both ends of all the major long bones, however, growth at one end always exceeds that at the other. The process of mineralisation in the growth plate has been studied in detail but is still not fully understood. However, endrochondral ossification is used as a model to explain those concepts that are understood about chondrocytes causing calcification.

Remodelling of bone: This occurs throughout life but is extensive during childhood. In the adult about 3.5% of the total skeleton is being remodelled at any given time (Rasmussen 1968). Remodelling is achieved by the combined efforts of osteoclasts and osteoblasts (Horowitz et al., 1988). The phagocytic activity is confined to the osteoclast whilst the osteoblast is believed to facilitate the process by the production of stimulating factors and specific enzymes that result in bone resorption (see Chambers, 1980). The osteoblasts lay down the replacement matrix, but this matrix may be at another location.

Fracture repair: Apart from bone growth and remodelling, osteogenesis is also required for fracture repair. Direct osteogenesis occurs from the periosteum. In addition a mass of hard tissue forms in the marrow cavity, which first forms cartilage, and then bone via endochondral ossification. The mechanical conditions under which a fracture is healing influences the number and distribution of cells which differentiate into chondrocytes (Ashhurst, 1986).

Calcified tendon: Here a dynamic cycle of mineral deposition and resorption is believed to be occurring simultaneously within the tissue (Uhthoff et al., 1975), so that at any point the observation of phagocytic-type cells is expected. Macrophages have much in common with osteoclasts and are probably derived from the same stem cells (Owen 1980). Both have the ability to merge and form giant cell complexes (foreign body giant cells). Thus, macrophages demonstrated to be phagocytosing mineral in tendon are carrying out the equivalant task of osteoclasts on bone.

Calcification.

Much of the work on the initiation of calcification has been on the epiphyseal plate, a growth unit composed of hyaline cartilage found at the ends of developing long bones. This model of mineralisation has been important as the temporal and spatial sequence of events can be demonstrated whilst growth is in progress. The epiphyseal plate is divided into several histologically distinct zones. From the closest joint towards the growing bone, these zones are termed the reserve cell zone, the proliferating cell zone, the hypertrophic cell zone and the mineralised zone. Calcification begins only in the upper hypertrophic cell zone, with the deposition of hydroxyapatite onto the longitudinal septa of extracellular matrix between the longitudinally oriented columns of enlarged chondrocytes. Although the transverse septa contain the same proteoglycan and collagen types they do not mineralise. Thus, the search for nucleating factors has been primarily centred on the cellular and extracellular constituents of the mature cell region and the hypertrophic cell zone.

Calcification processes are assumed to be similar whether occurring normally, pathologically, or idiopathically. Most forms of calcification are now believed to be initiated by membranous organelles whether extracellular calcifying "matrix vesicles" or intracellular mitochondria. Matrix vesicles are submicroscopic, membrane-invested, extracellular particles (about 200nm in diameter) which promote calcification through calcium binding phospholipids and phosphatase activity. Electron microscopic examination of the epiphyseal plate reveals membrane bound vesicles in the

extracellular matrix which, although present throughout the epiphyseal plate, are primarily found in the mineralising region. Their distribution in the cartilage matrix almost exactly corresponds to that of subsequent calcification. Initial needles of hydroxyapatite are exclusively associated with those matrix vesicles located in the longitudinal septa of the hypertrophic cell zone (Anderson, 1967; Bonnuci 1967; Bernard and Pease 1969). Further evidence included a demonstration that the first identifiable crystals of apatite arise within matrix vesicles, often in close apposition to the inner leaflet of the matrix vesicle membrane (Anderson, 1976). The suspension of an isolated proteolipid in a mineralising solution resulted in the spontaneous formation of vesicles, and the subsequent accumulation of calcium phosphate salts on the vesicular membrane (Ennever et al. 1976) showed that the mechanism was independent of surrounding tissue components. These discoveries complemented the investigation and analysis of the enzymes found in hypertrophic cell zone matrix vesicles. This revealed the presence of enzymes localized in the vesicle membrane that were activated just before the onset of apatite deposition (Matsuzawa and Anderson, 1971). Ali and collegues (1970) reported that, of a number of enzymes assayed in isolated matrix vesicles, the highest relative specific activities were seen for alkaline phosphatase, pyrophosphatase, ATPase and 5'-AMPase. These enzymes could increase the local concentrations of phosphate in the microenvironment of the vesicular membrane, or even perhaps within the vesicle. The levels of activity of these enzymes in chondrocyte membrane fragments were considerably lower.

Other workers have found evidence that these activities may be manifestations of a single phosphatase in the vesicle membrane perhaps acting as a phosphotransferase. (Majeska and Wuthier 1975 ; Cyboron et al.1981). Ali also demonstrated that matrix vesicles, isolated from the hypertrophic cell zone, bound Ca^{45} in vitro with the subsequent formation of hydroxyapatite (Ali and Evans 1973). Experimental work confirms that calcium probably accumulates in matrix vesicles as they approach the calcification front (Ali 1976). Wuthier (1977) demonstrated that the concentration of calcium in matrix vesicles is 25-50 times greater than that found within adjacent

chondrocytes. Hence, a mechanism for concentrating calcium within matrix vesicles must exist, suggesting that components of the membrane such as phosphotidyleserine, may be involved in calcium binding and accumulation. The weight of evidence has led to matrix vesicles being accepted as initiators of mineralisation in the epiphyseal growth plate (for a review see Ali, 1983).

In calcifying tendon, Jozsa et al. (1980) found abundant morphological evidence that calcification occurs within extracellular vesicles in the extracellular spaces, and the aggregation of these calcifying vesicles to form larger deposits results in complete destruction of their vesicular structure. This same mechanism was reported by Kim et al. (1976) in the human aortic valve and aortic media.

One animal model used to investigate tendon mineralisation is the rat. The achillies tendon, when cut, mineralises on repair via endochondral ossification (Rooney et al., 1988). Studies on the calcification and ossification of fibrocartilage in the attachment of the patella ligament in the rat (Badi, 1972) showed that chondrification occurred prior to mineralisation.

However, other factors are involved in mineral formation and matrix vesicles should not be considered in isolation. Mitochondria use a forceful, inwardly directed calcium and phosphate transport mechanism and are believed to mineralise when the normal cellular calcium homeostasis malfunctions, resulting in cell death. There has been speculation on mitochondral involvement in the growth plate mineralisation and Brighton and Hunt, 1974 correlated reduced internal calcium concentration in mitochondria with their relative progression though the growth plate and hence increased extracellular calcium levels. Intracellular pathological calcification is usually initiated by mitochondria (Anderson, 1986) but calcifying tendinitis is not believed to be initiated intracellularly although Faure and Daculsi (1987) feel that further studies are needed for this to be clarified.

A dominant feature of several theories of the local mechanism of biological calcification has been that an organic (non-vesicular) matrix functions as a nucleating substrate to induce the formation of a mineral phase from a metastable soluble phase

(Glimcher 1959; Solomons and Neuman 1960). The suggested mechanism involves a reduction of the surface energy and/or the number of ions present in a critical nucleus required for phase change and thereby a reduction in the energy barrier for hydroxyapatite formation.

The sub-structure of collagen type I, the main structural protein of both bone and tendon has internal space that becomes filled with hydroxyapatite in mineralising tissues (see Glimcher, 1976) and could be the site of initiation of calcification. The physical biochemistry of calcification was extensively reviewed by Wadkins et al. in 1974.

Regardless of the initial mechanism of crystal formation, after mineral initiation the proliferation of mineral crystals is dependant on regulatory factors, such as extracellular calcium and phosphorous and other mineralisation inhibitors and promoters. However, the introduction of just a few nucleating crystals of hydroxyapatite into a homeostable body fluid will lead to the rapid accumulation of apatite because nucleating crystals serve as templates for new crystal formation. There are a variety of animal models for tendon mineralisation. The best characterised is the domestic turkey leg tendons which mineralise as part of the natural ageing process and hence give a natural, spatial and temporal model. Landis (1986) reports the presence of 'small vesicular structures' in the initial stages, located between collagen fibrils which contain mineral. Then, as calcification progresses, crystals are predominantly associated with collagen. The collagen alone is believed to be directly responsible for the progressive mineral deposition within the tissue as a whole. In avian tendon, Landis concludes that vesicles are necessary for calcification of other organic matrix components such as the adjacent collagen fibrils.

Other factors thought to have a role in mineralisation include lipids (Boskey et al., 1978), non-structural proteins and inhibitory substances, for instance proteoglycans (Di Salvo and Schubert. 1967; Chen and Boskey, 1986).

Higher organisms possess mechanisms that control crystal growth. Given its exponential nature, uncontrolled crystal growth is very injurious to the organism.

Various workers have postulated and demonstrated the presence of inhibitors of calcification or crystal growth as a contolling mechanism (Howells and Pita, 1976; Campion and Dieppe in press). There is no reason to suppose that all calcifying vertebrate tissues do so by the same mechanisms.

The involvement of matrix vesicles, mitochondria and matrix nucleation catalysts in the complex process of calcification is probable and evidence for all can be sought by histological and ultrasructural examination of specimens from calcifying tendinitis patients.

The data presented in this thesis relate to an examination of the apparent changes associated with calcification in the rotator cuff tendons. By first characterising the nature of these changes into cause or effect groups based on histological observations, then studying some tissue samples in more detail, information has been obtained about the probable mechanisms causing calcium deposition and about the cellular activities involved in controlling the pathological condition.

Appended to this thesis is a table showing the clinical details of each patient from whom tissue samples were examined and a case report from one patient.

CHAPTER ONE.

MORPHOLOGY AND ULTRASTRUCTURE.

MORPHOLOGY AND ULTRASTRUCTURE

1.1 INTRODUCTION

The exact mechanism of calcification in tendon remains uncertain. Observations by Refior et al., (1987) confirmed previous reports (Uhthoff 1975; Uhthoff et al. 1976; Uhthoff and Sarkar 1978; Sarkar and Uhthoff 1978; Uhthoff and Sarkar 1989) that there was fibrocartilage within the tendon, but Refior et al. (1987) believed this was a result of degenerative change. Such inherent degeneration is also believed to be responsible for rotator cuff tears. This challenges Uhthoff's theory (1975) that the cells resulting from chondrogenic modulation subsequently mediate calcium deposition, a mechanism entirely different from the degeneration responsible for rotator cuff tears. The suggested mechanisms causing deposition of mineral in tendon, to date, arise from morphological and ultrastructural observations made on examination of tissue from the area of deposit formation, combined with relevant knowledge of the patient's medical history.

Uhthoff and Sarkar (1978) discuss four phases in calcifying tendinitis which are: Pre-calcific, formative, resorptive, and restitutive. They suggest that these are cyclic in occurrence so all stages could be going on at any one time. Theoretically, if this were the case there should be evidence for all of these stages in pathological tissue from each patient. Because of this, it was deemed necessary to spread the investigative techniques between as much pathological tissue as possible. This work has been carried out on human tissue taken from tendons that would eventually be repaired, but which have been assisted by the surgical removal of calcific deposits. However, only very small samples were available for this study. This severely limits the number of different investigations that were possible on a given specimen and choices were made in order to maximise the amount of information about the disease process in each patient. In order to facilitate observation of basic morphology and ultrastructure (see diagram: 1.1), it was necessary to select the larger portion of each tissue sample for histological and electron microscopical examinations.

Throughout this thesis, reference to pathological specimens is based on their relative location compared to the bulk of mineral as determined by the surgeon. Hence, the roof and floor specimens were pieces of tissue taken from just above and just below the deposit respectively, the walls were taken from the edge of the deposit. Often these samples would have mineral attached.

In this chapter there is a summary of observations of many previously described characteristics of calcified tissue and surrounding areas, together with a detailed account of the tissues implicated in the pathological mechanism. Subsequent chapters describe the results obtained from applying specialised techniques to gain more information about individual components involved in the pathological condition.

Obtaining whole tendons from post-mortem cases with no known history of shoulder pain permitted a study of the gross morphology of the supraspinatus tendon and enabled comparative estimation of the sites and sizes of the calcific nodules (fig 1:2). Longitudinal section of the whole tendon demonstrates the structural architecture, and allows observation of differences in the tissue types adjacent to, or integrated with, the tendon.

It must be remembered that the calcifying tendinitis patients, apart from demonstrating natural variation between biological samples, have been injected with pharmacological substances in the course of treatment, as well as having physiotherapy sometimes with ultrasonication, all deliberately intended to alter the course of the disease and affect the pathology. Details of the patients records with respect to their age, the length of time that they have suffered prior to surgery and the pre-operative treatments are appended to the back of this thesis along with a typical case report for one patient.

Specimens from three patients with myositis ossificans were also collected and are used for comparison of the pathological mechanisms observed in this disease with those seen in the calcified tendons.

1.2 GENERAL METHODS

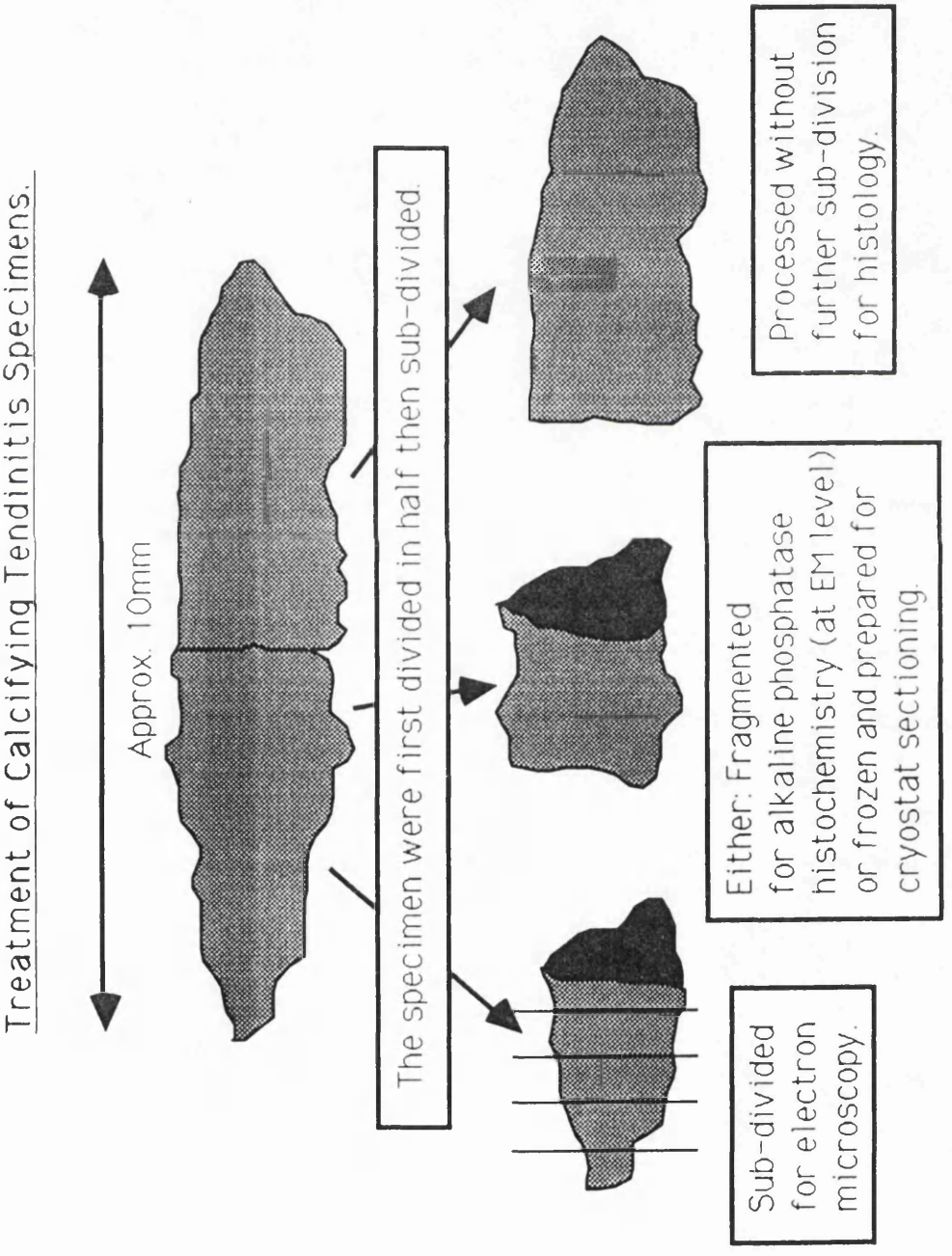
This general methods section describes in detail the tissue processing schedules that were used for studies reported in this chapter. In subsequent chapters reference to these general methods is made where appropriate.

To summarise the table containing patients details (appendix I) specimens were taken from a total of 11 patients, seven females. One patient had previously had a calcific deposit removed from her contralateral shoulder, another had pain in his other shoulder but no deposit was radiographically demonstrated. All patients were aged between 30 and 55 years, and the pre-operative onset of pain varied from nine months to four years. Nine right shoulders contained deposits and two left. Nine of the patients had deposits in the supraspinatus tendon, one was in the infraspinatus and one in the subscapularis tendon.

Treatment of calcifying tendon specimens. Samples were immediately placed in appropriate fixative or 'snap' frozen in liquid nitrogen in the operating theatre to reduce any degenerative changes that would occur with time (diag:1.1). Each tissue sample from the calcifying tendinitis patients (table 1.1) was divided and parts wax embedded for histology and resin embedded for electron microscopy (EM). A number were also frozen for histochemical and immunological studies (see chapters two and three). Selected specimens were left undecalcified before histological processing and all were undecalcified for EM processing.

Treatment of post-mortem tissue. At post-mortem the supraspinatus tendons from both shoulders (of two cases, aged 55 and 84), were removed. The first was sliced transversly into three portions and a small piece of tendon cut out from the centre at the point nearest the insertion of the tendon into bone and from the two interfaces of sub-division. These were placed in fixative for electron microscopy. The large segments were put into sterile saline for transportation. As soon as was practical (about two hours after

Diagram 1.1


Treatment of Calcifying Tendinitis Specimens.
Approx. 10mm
The specimen were first divided in half then sub-divided.
Processed without further sub-division for histology.
Either: Fragmented for alkaline phosphatase histochemistry (at EM level) or frozen and prepared for cryostat sectioning.
Sub-divided for electron microscopy.

Specimens taken from patients:	A	B	C	D	E	F	G	H	I	J	K
Tendon junction											
Supra-infraspinatus	X	-	X	X	-	-	-	-	-	-	-
Subscapularis											
Without deposit	-	-	-	-	-	-	-	-	-	-	X
With deposit	-	-	-	-	-	-	-	-	-	-	X
Infraspinatus											
Without deposit	-	X	-	-	X	X	X	X	-	X	-
With deposit	X	-	-	-	-	-	-	-	-	-	-
Supraspinatus											
Without deposit	-	-	X	-	-	-	-	X	X	X	X
Adjacent to deposit	-	X	-	-	-	-	X	X	X	-	-
Roof of the deposit	-	-	-	-	X	-	-	-	X	X	-
Wall of the deposit	-	X	X	X	-	R	X	X	X	X	-
Floor of the deposit	-	-	-	-	X	-	X	X	-	X	-
Mature deposit	-	-	X	X	X	-	X	X	-	X	-
Acute phase deposit	-	-	-	X	-	-	-	-	-	X	-
Bursa											
With deposit	-	-	-	-	-	X	-	-	-	-	-

Table 1;1 Samples Taken From Patients A to K

KEY	
Specimen	X
No Specimen	-
Resorbed Specimen	R

* Formol saline consists of 10% formalin (this is 40% formaldehyde w/v) containing 0.9% (w/v) NaCl and .005% w/v bromcresol purple as a pH indicator (yellow at pH 5.2 to purple at pH 6.8 and above).

* Specimens from patients A and B were processed undecalcified, all subsequent samples were decalcified for histological processing when mineral was demonstrated in the tissue by x-ray.

removal) the three portions were orientated in cryomountant on cork and frozen by immersion in liquid nitrogen before being stored frozen (-70°C) for cryostat sectioning.

The second tendon from each patient was divided longitudinally into two, and placed in formol saline. After a minimum of 48 h. fixation specimens were dehydrated through a graded series of ethanol solutions, cleared in chloroform, infiltrated at 60°C under vacuum with wax and embedded. One half of the tendon was cut in transverse section, the other longitudinally. Wax blocks were sectioned at 6 μm on a sledge microtome.

Histological processing (Calcifying tendinitis and myositis ossificans specimens). Specimens were fixed in formol saline for a minimum of 48 hours before being X-rayed using a Faxitron X-ray machine. Those specimens which contained an observable amount of mineral were generally decalcified, using neutral (14.3%) EDTA (Hillman and Lee, 1953). Specimens were then dehydrated through a graded ethanol series, cleared and wax infiltrated in a tissue processing machine before embedding in wax.

Histological sections were cut on a sledge microtome and serial 7μm sections were stained with Ehrlich's haematoxylin and eosin (Lillie, 1954), azur A for metachromasia (Hughesdon, 1949), alizarin red (McGee-Russell, 1958) or Masson's trichrome (Masson, 1928).

The histological processing and staining of pathological specimens were carried out in the Department of Morbid Anatomy, Institute of Orthopaedics, R.N.O.H. Sections were photographed on a Zeiss photomicroscope III or an Olympus BH-2 photomicroscope.

Transmission electron microscopical (T.E.M.) preparative techniques. (All specimens). **Processing:** Tissue blocks about 1 mm^3 or less were fixed for 2 h. in 2.5% glutaraldehyde (Agar scientific) in 0.085M sodium cacodylate buffer (pH 7.4) on ice. Specimens were then washed in three changes of 0.085M sodium cacodylate buffer containing 0.2M sucrose (wash buffer pH 7.4) prior to secondary fixation with 1% osmium

tetroxide in 0.085M sodium cacodylate for 60 min. at room temperature (RT). Specimens were washed in sodium cacodylate buffer before dehydration in the following ethanol series (BDH):

50%	2 x 5 min.
70%	2 x 5 min.
90%	2 x 5 min.
100%	3 x 10 min.
propylene oxide (Agar Scientific)	2 x 15 min.
araldite (neat) CY212*	2 x 1 h.

Specimens were transferred into fresh araldite CY212 (Agar Scientific) and mixed at room temperature. * CY212 was mixed according to manufacturers instructions but excluding the addition of methyl phthalate (plasticiser).

Embedding.

Specimens were transferred into fresh araldite CY212, labelled and polymerised at 65°C for 48 h., then stored at room temperature.

Sectioning.

Thick and ultra-thin sections were cut using an LKB mkII ultramicrotome. Sections 1.0 µm thick were dried onto glass slides and stained with toluidine blue (Richardson et al. 1960) to orientate the tissue. Ultrathin sections (70-100nm) were mounted on copper grids. Where necessary these were precoated with 0.45% pioloform (Agar Scientific) dissolved in chloroform (BDH).

Staining of ultra-thin sections: Sections were stained by flotation, on drops of saturated uranyl acetate (UA) solution (Watson 1958) for 30 min at room temperature and on drops of lead citrate (LC), (Reynolds 1963) for 5 min at room temperature in the presence of sodium hydroxide pellets.

Polysaccharides. The PA-TCH-SP is a PAS-type staining method for polysaccharides in electron microscopy (Wattel 1974). Ultrathin sections of osmicated araldite embedded specimens were picked up onto gold grids. Grids were immersed in 1% aqueous (aq.) periodic acid (stored at 4°C before use) then washed for 3 x 5 min. in distilled

water. Grids were immersed in 0.2% Thiocarbohydrazide (TCH) in 20% acetic acid (A.A.) for 18 h. Sections were brought back to water by immersion in the following solutions: 2 x 1 min. in 10% A.A., 20 min. in 5% A.A., 20 min. in 2.5% A.A., 20 min. in 1% A.A.,then 2 x 15 min. in distilled water. Sections were immersed for 30 min. in 1% aq. silver proteinate in the dark at RT. Sections were washed for 3 x 5 min in distilled water, and further stained with Reynolds lead citrate (LC) for added contrast. Either the periodic acid or the TCH step onwards were omitted as controls.

Ultra-thin sections were viewed and photographed on a Philips C.M.12 transmission electron microscope.

1.3 RESULTS

The descriptions given are of normal or pathological supraspinatus tendon and the surrounding tissues. Pathological samples have also been obtained from the infraspinatus and subscapularis tendons (patients A and K: table 1.1). The deposition of mineral in these is assumed to be the same process as that which has occurred in the supraspinatus tendons.

Figure 1.1 shows a full length 'normal' supraspinatus tendon not containing a calcific deposit, removed at post-mortem from a 55 year old lady. The complete tendon is about 5cm in length and a pale cream colour, however, there is some overlap with the supraspinatus muscle which is red. For comparative purposes a calcific deposit (removed from patient J) is shown. It can be seen that the deposits can occupy a considerable volume of the entire tendon. The deposit has been cut open to expose its chalky cream contents. In typical deposits the area of calcification is less well defined and not enclosed within a single fibrous nodule.

Figure 1.3a is a longitudinal section through the 'normal' supraspinatus tendon stained with haemotoxylin and eosin (H and E). The zone of fibrocartilage in front of the region of insertion can be observed (fig: 1.3b) and an adjacent section has been stained with alizarin red for calcium salts, to show the mineral at the point of insertion where the tendon attaches to bone (fig:1.3c). Within the tendon the fascicular

compartments are seen to be divided by loose connective tissue. The other connective tissues to be observed are muscle fibres from the connecting supraspinatus muscle, the epitenon and the sub-acromial bursa that are inseparable and the synovial lining cells found inside the bursa. Blood vessels are sparse but in the 'critical zone' this tendon is perfused by a number of small capillaries and at higher magnification evidence of mucoid degeneration can be seen. Within small blood vessels, plasma and red blood cells can be identified.

Fibroblasts are connective tissue cells, but more specific is the term tenocyte that is used to describe the cells that are within a normal tendinous matrix (fig: 1.3d and 1.5a). Other fibroblasts found in the vicinity of the pathological changes, include those cells of the loose connective tissue surrounding the tendon, those of the bursa and synovium and the muscle cells.

The area of mineral deposition could only be stained with alizarin red on undecalcified sections (fig: 1.4a) whereas in decalcified tissue it was apparent where the mineral had been situated by the amorphous acellular non-tendinous tissue that remained (figs: 1.10a and b). Prior to decalcification tissue was X-rayed to demonstrate the presence of mineral. The mineral frequently coincided with areas of metachromasia as shown by azur A staining. The tendon in areas of calcification is disorganised (fig: 1.4b and c) and does not demonstrate the normal alignment of collagen fibrils and crimp structure found in non-pathological tendons.

Between regions sampled from different patients there were features of the pathology common to many of the observed tissue sections. The morphological descriptions of each sample has not been included to avoid repetition but the main features are described.

By low magnification electron microscopy, deposits appear rounded and focal in nature (fig:1.8d). The size of deposits, however, was extremely variable and often these were close to more organised peripheral tissue (fig: 1.4e).

In regions of calcification and the surrounding tissue, capillaries and blood vessels were sparse and there were no apparent associations between these and the calcific

deposits. Often red blood cells were seen within loosely packed areas of sections not associated with vessels and these were assumed to be either haemorrhagic as a result of surgery or ill-defined sinuses (fig: 1.4c).

Lipid content was not routinely investigated, as the chemicals used prior to wax embedding strip the tissue of its lipids. However, by electron microscopy large lipocytes were observed in some pathological tendon and also in the relatively 'normal' specimens from the same patients, and in post-mortem tendon. The lipid did not appear to be associated with the calcific deposit.

The floor of the deposit from patient C gave a good demonstration of metachromatic tissue (fig: 1.4c and d) although similar observations were made from the roof of the same nodule. This heterogeneous form of tissue was generally apparent in all the boundary areas of the deposit in samples that did not appear to have signs of resorption. Here the chondrocyte-like cells believed to mediate calcium deposition (Uhthoff, 1975) can be seen (figs: 1.4e and f) and ultrastructurally this cell type does have a more rounded appearance with short cell processes and a pericellular lacuna which are very unlike normal tenocytes (figs: 1.5b and 1.6b).

* The specific PAS stain for polysaccharides showed that the concentration of glycogen within the chondrocyte-like cells did not appear to be substantially more than in tenocytes (fig: 1.6a and b).

Ultrastructurally, a range of cell types and metabolic activity was observed apart from the chondrocyte-like cells already described and these were found to be associated with fibrocartilage. A selection of cells have been shown (fig:1.7 a-d) to demonstrate the variety seen in one patient (B). Fibroblastic cells with numerous cell processes located peripheral to the edge of mineral islands could be seen and also cells with few processes within a well defined lacunae (fig: 1.7b). The holes observed in this and other cells are assumed to be fixation artefacts, due either to osmotic imbalance, where swollen mitochondria have burst within the cell or the remaining space from vacuoles that previously held mineral. Mineral has dropped out of the section from the island of deposit close to the cell and presumably it is missing from within these intracellular

* See page 46.

holes whether it was within a mitochondrion or a vacuole.

Figures 1.7, c and d, show scavenger cells that contain mineral. Both are located within a heavily mineralised region of tissue. The former is more fibroblastic in shape and it could be speculated that this cell was located within the tissue prior to calcium deposition (tissue macrophage). The larger calcospherites located within the other phagocytotic cell are similar in shape and size to the spherical deposit making up the body of large masses of mineral (fig: 1.8e). It is possible that accumulation of mineral into the larger deposits is partially due to congregation of these scavenger cells that then become necrotic through taking in excessive amounts of calcific deposit. This cell is similar to the blood bourne macrophages having the more typical 'kidney shaped' nucleus.

There were also signs of necrosis with cell remnants occasionally being found in the area of calcification, where other cells had swollen or burst mitochondria. The swollen and burst mitochondria were in cells local to the calcific deposit, those cells distant from the calcific deposit in the same section had 'normal' mitochondria.

Particular attention was paid to vesicular structures within the matrix so as to ascertain whether these were the initial foci of mineral deposition. Matrix vesicles were found in pathological tendon and around the foci of calcification but similar vesicular structures were seen in 'normal' tendon. Mineralised nodules of matrix vesicle size were also observed in regions of calcification (fig 1.8a). Packing of crystals among collagen fibres was observed in association with both the calcific deposit ultrastucture (fig: 1.8b), and in the area peripheral to the bone in patient D (fig: 1.8c).

Figure 1.8d shows that the size of deposit was not uniform and these calcospherites were often within the substructure of larger deposits (fig:1.8d). Fig: 1.8f shows the 'needle-like' crystals that form the deposits.

Two types of deposit removed from patient D were described by the surgeon. Firstly a mature deposit, and it was in this specimen that bone was found (fig: 1.9 a to d). In the same region as the bone, nodules of calcific deposit similar to those found in the other patients were also evident (fig: 1.9c). Secondly, acute phase deposits were

less structured with the consistancy of 'tooth paste' and consisted of loose 'needle-like' crystals of hydroxyapatite.

Throughout the pathological specimens taken from patient (H), and also observed in localised areas of sections from other patients, there was an influx of inflammatory cells inter-mixed with nodules of mineral (fig: 1.10 a). The general appearance in these sections was that the nodules were confined in a highly cellular tissue and of a smaller size possibly due to their containment and resorption. Foreign body giant cells were seen to be associated with areas of resorption (fig: 10.b).

For comparative purposes specimens removed from myositis ossificans patients, and treated by the same protocols, demonstrate the nature of both endochondral and membrane bone formation in pathological circumstances. Fig: 1.11a clearly shows chondrocytes in a cartilaginous matrix quite unlike that observed in the calcifying tendinitis specimens even in the region peripheral to the bone found in patient D (fig: 1.9 a-d). The fibrocartilaginous transition zone seen in figure 1:11b has similarities with the areas seen in patient D (fig: 1.9a).

* The PAS stain for polysaccharides was applied to clearly demonstrate the similarity in glycogen storage patterns between the cells in figure 1.6. The structure marked x is not associated with glycogen storage as has beeen previously suggested (Uhthoff and Sarker 1978).

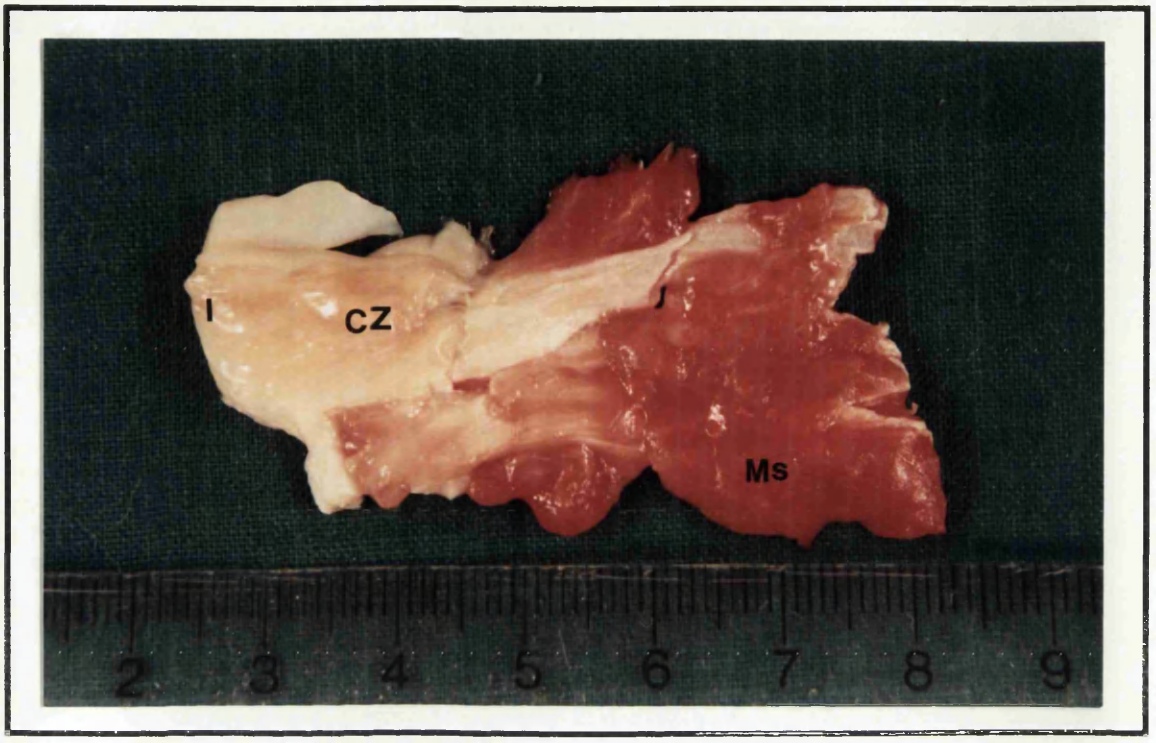
FIGURES.

Figure Legends

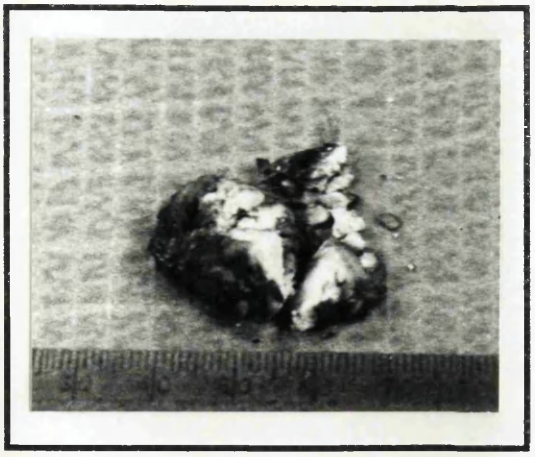
Fig: 1.1 A whole supraspinatus tendon removed at post-mortem from a 55 year old lady is shown. The region of insertion of tendon into bone is labelled (I) and the overlapping supraspinatus muscle (Ms). The usual location of the calcific deposits in the supraspinatus tendon is at the 'critical zone', indicated here as CZ.

Fig: 1.2 (a and b) A calcific nodule was removed intact from patient J and cut open to expose the cream coloured contents (M). This deposit is very compact with a chalky textured centre. The area of deposition is usually less well defined and not enclosed within a single fibrous nodule.

1.1 a



1.2 a and b.



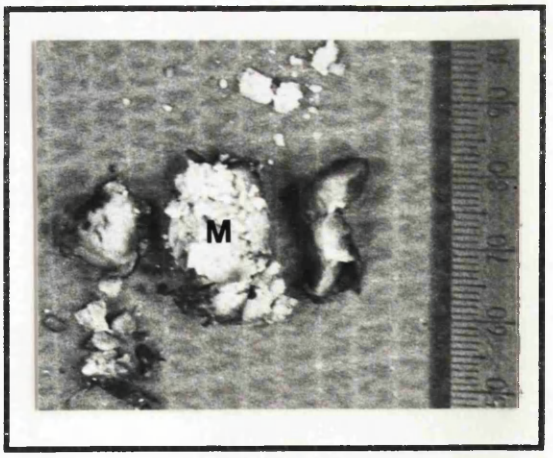


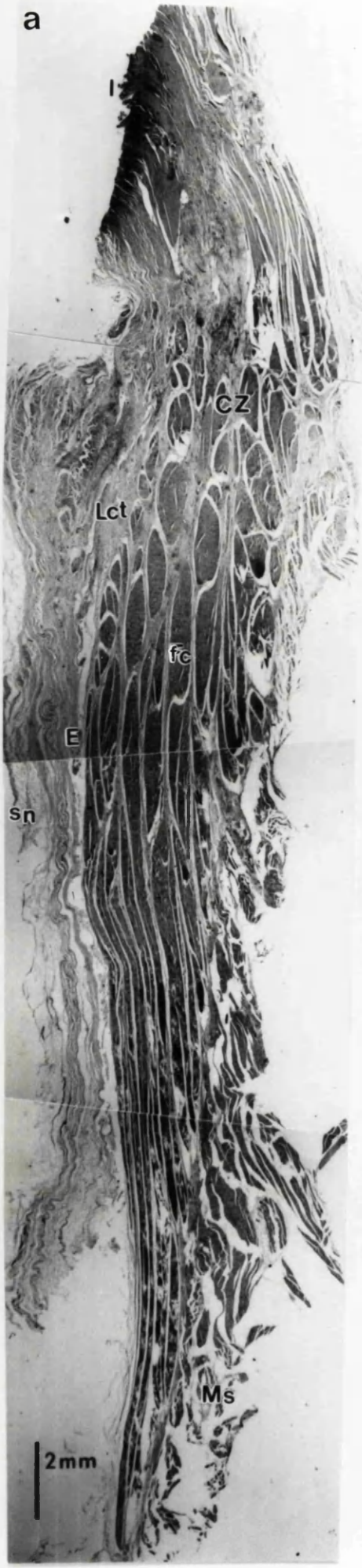
Fig: 1.3a Longitudinal section through the 'normal' supraspinatus tendon stained with H and E. The region of insertion is indicted (I). The tendon fascicular compartments (fc) are seen to be divided by loose connective (Lct) tissue within the tendon. The other connective tissues to be observed are muscle fibres (Ms) from the connecting supraspinatus muscle, the epitenon (E) and the sub-acromial bursa that are inseparable and the synovial lining cells (sn) found inside the bursa. The 'critical zone' is indicated (CZ).

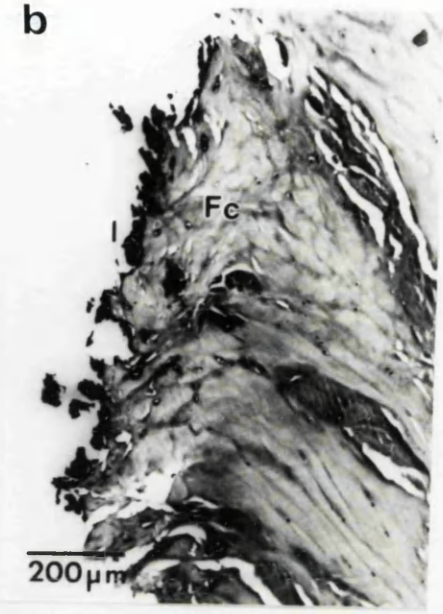
Fig: 1.3b In this enlargement, the zone of fibrocartilage (Fc) adjacent to the region of insertion (I) can be clearly seen in longitudinal section by H and E staining.

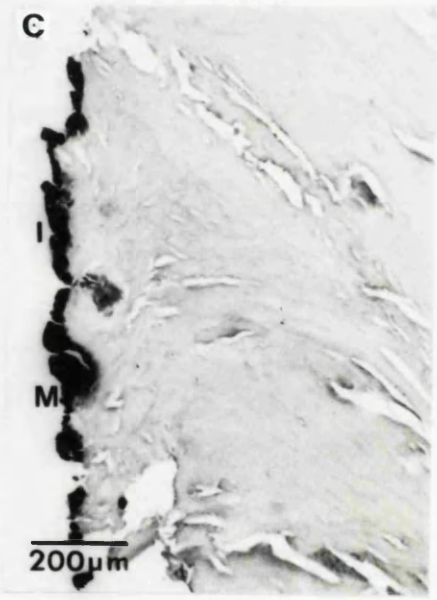
Fig:1.3c An adjacent section to that described above has been stained by Alizarin red to show calcium salts (M) at the point of insertion (I), where the tendon attaches to bone. Mineral was not found in the post-mortem tendon away from this zone.

Fig:1.3d Higher power micrograph of a fascicle, shows the alignment in longitudinal section, of tenocytes within the collagenous matrix in 'normal' tendon.

1.3







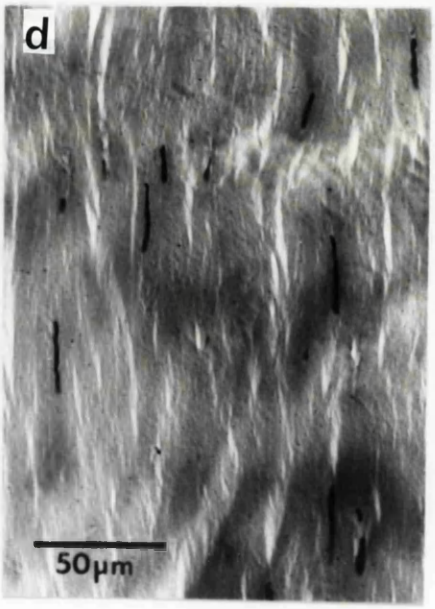


Fig: 1.4a Alizarin red stain for calcium salts demonstrates the enormous amount of mineral (M) in pathological tendon removed from the wall of the deposit in Patient B.

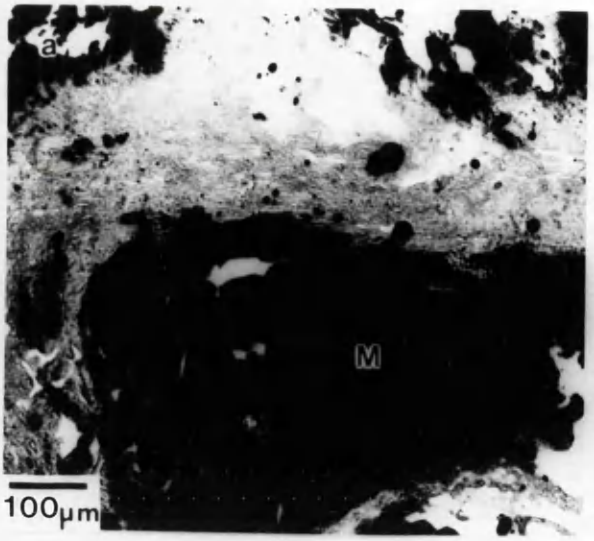
Fig:1.4b H and E stain of adjacent section to 1.4a demonstrates the extent that the tendon structure is disrupted by the mineral deposition (M). The tendon in all areas of calcification was disorganised and the normal alignment of collagen fibrils and crimp structure (seen by polarised light microscopy) found in 'normal' tendons was not evident.

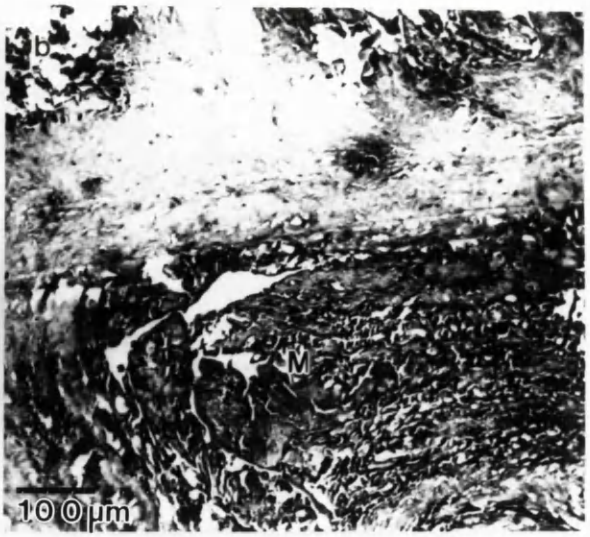
Fig: 1.4c H and E stained section of the floor of the deposit from patient C. The decalcified tissue retains indications of the areas that were mineralised (M). There is also infiltration of red blood cells (rbc) into this region. The arrow points to the cluster of cells stained by azur A in the next micrograph (1.4d).

Fig: 1.4d Azur A stained section of the same area shown in 1.4c demonstrates that the tendon is metachromatic. The pericellular matrix of the rounded chondrocyte-like cells was metachromatic. The arrow points to the cluster of cells stained by H and E in the last micrograph (1.4c).

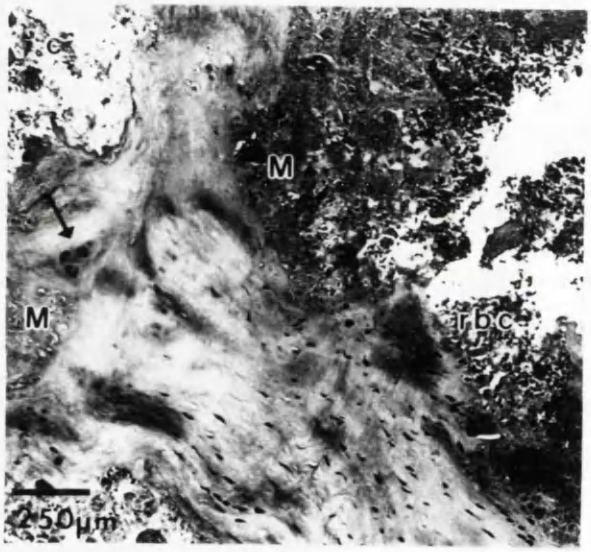
Fig: 1.4e H and E stained section from the roof of the deposit from patient E. There were areas of 'normal-looking' tendinous fibres (tf) within the same sections as the fibrocartilage (fc) and often close to mineralised nodules (M). Note the rounded chondrocyte-like cells (arrow) which are enlarged in figure 1:4f.

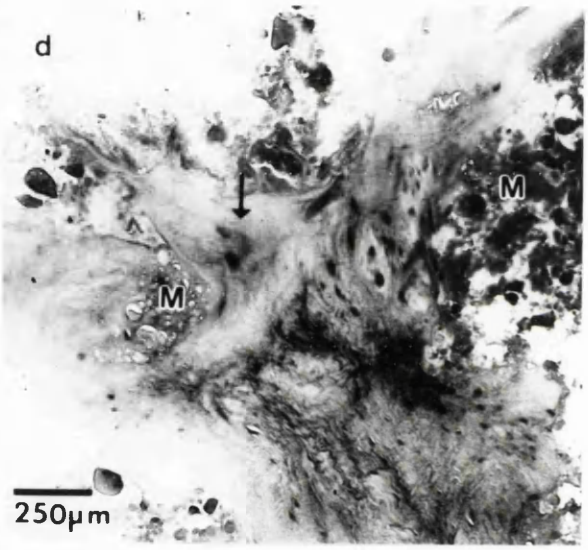
Fig: 1.4f Enlargement of the chondrocyte-like cells (arrow) found in the roof of the deposit from patient E, stained by H and E (as indicated in figure 1.4e). Compare the abnormal round or polygonal cells with the usual long, spindle shaped, tenocytes (t).

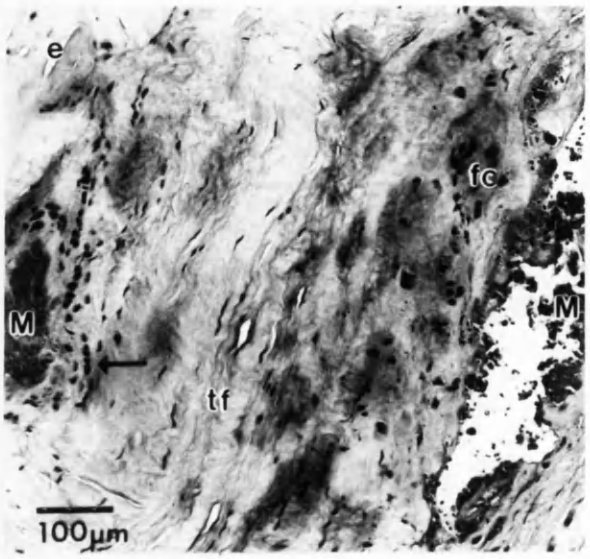

a
M
100µm


b
M
100µm

1.4


c
M
M
rbc
250µm


d
M
M
250µm


e
fc
M
M
tf
100µm

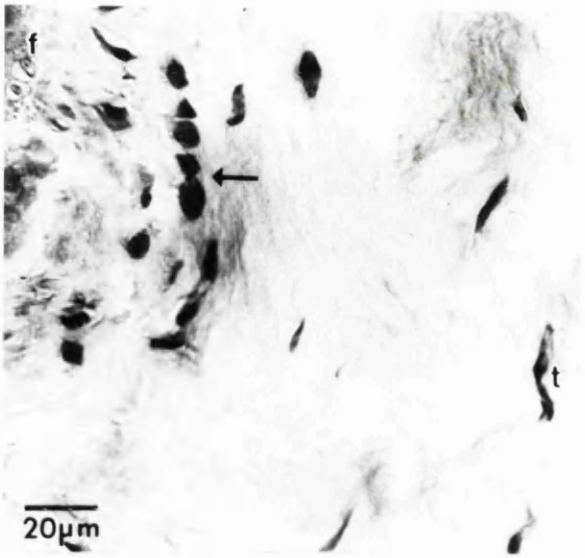
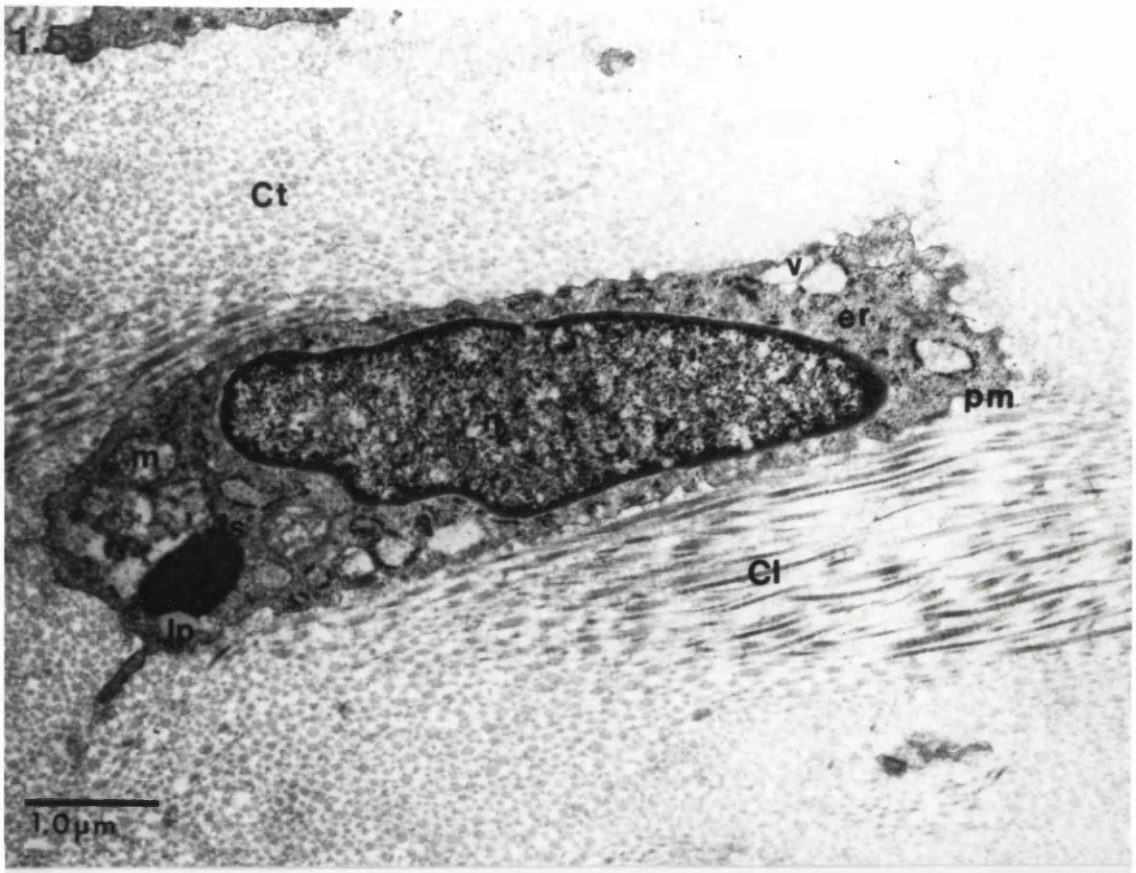
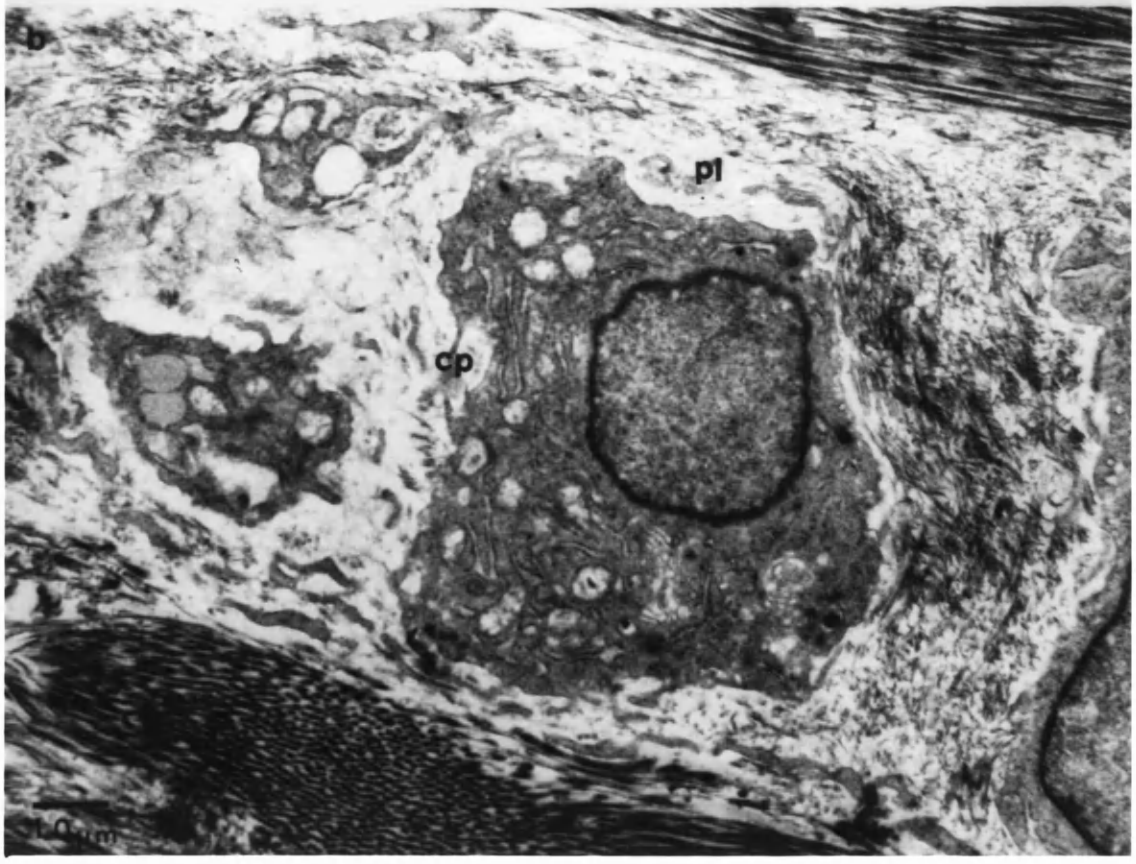

f
t
20µm

Fig: 1.5a Electron micrograph of a tenocyte located in a region of supraspinatus tendon from an area distant to the calcific deposit. Organelles can be observed within the cytoplasm and have been labelled accordingly; the nucleus (n), mitochondrion (m), endoplasmic reticulum (er), vacuole (v), lysosome (ls), lipid droplet (lp) and the cell is bounded by the plasma membrane (pm). The extracellular matrix contains collagen fibrils mainly in transverse section (Ct) although just below the cell the fibres are seen in longitudinal section (Cl).

Fig: 1.5b Electron micrograph of a chondrocyte-like cell type that has a more rounded appearance. They were generally larger than the tenocytes and also appeared to have a larger cytoplasm to nucleus volume ratio. This cell has numerous short cell processes (cp) and a distinct pericellular lacuna (pl), which contains fine fibrils as compared to those seen further away from the cell.


1.5
Ct
v
er
pm
n
m
lp
Cl
1.0µm


b
pl
cp
1.0µm

A periodic acid schiff (PAS) stain has enabled a comparison between the glycogen storage pattern in the normal tenocytes compared to the chondrocyte-like cells found in pathological tendon.

Fig: 1.6a PAS stained section through a typical tenocyte, demonstrates the presence of intracellular glycogen visualised here by the deposition of silver granules where there is a concentration of polysaccharides within the cell (arrow).

Fig: 1.6b and insert. PAS stained chodrocyte-like cell, demonstrating that the large vacoules located within the cytoplasm are not glycogen rich (x). The enlarged area shows the polysaccharide-silver complex is mainly deposited within the cell cytoplasm (arrow).

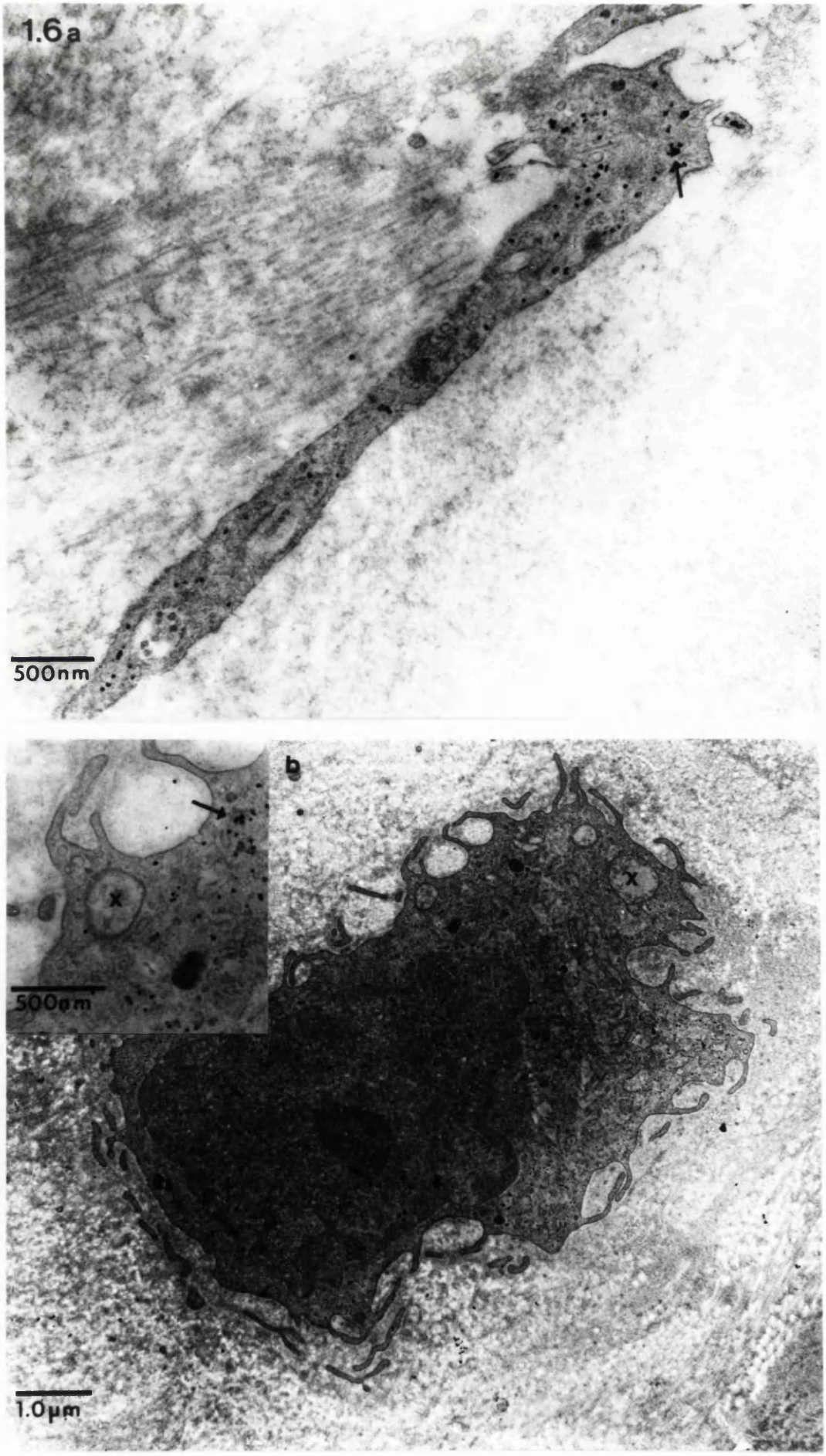

1.6a
500nm
b
X
500nm
X
1.0μm

Fig: 1.7a Fibroblastic cell with numerous short cell processes, close to the mineral M). Patient B, non-osmicated tissue but stained with uranyl acetate (UA) and lead citrate (LC).

Fig: 1.7b Fibroblastic cell surrounded by a pericellular lacunae (pl) in close proximity to the mineral (M). There are signs of necrosis as the cell has a dark, shrunken nucleus and the mitochondria appear to have swollen and burst (m). This appearance frequently occurred where cells were close to the mineral. Patient B, osmicated, UA and LC stained.

Fig:1.7c Other cells appeared to show evidence of phagocytosed mineral, presumably demonstrating the mechanism of resorption. Patient B, non-osmicated and stained with UA and LC.

Fig:1.7d Micrograph showing large scavenging macrophage phagocytosing calcospherites from the extracellular matrix. Note the kidney-shaped nucleus (n). The tissue is non-osmicated and stained with UA and LC.

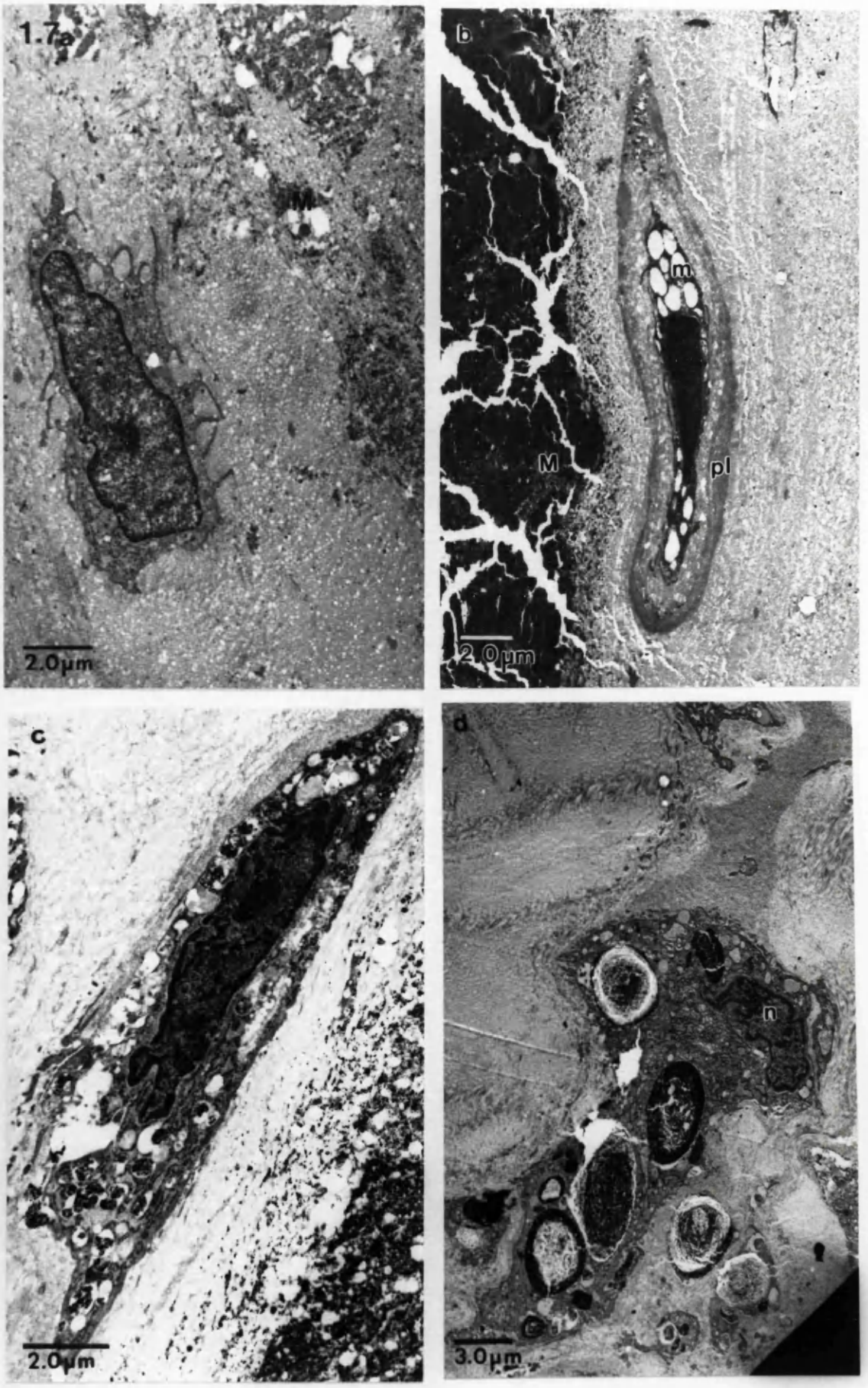

1.7a
2.0µm
b
m
M
pl
2.0µm
c
2.0µm
d
n
3.0µm

Fig: 1.8a Micrograph showing vesicular-sized structures which are found in the matrix of normal tissue and around the foci of calcification. These struc tures located in pathological tissue were rich in mineral but no binding membrane was apparent. The section has been stained with UA and LC but not been osmicated.

Fig: 1.8b Micrograph showing that the mineral (M) often appeared to accumulate between collagen fibres (C). This tissue is from patient B and has been osmicated, UA and LC stained.

Fig: 1.8c Micrograph showing a packing of crystals among collagen fibres and can be observed in association with both the calcific deposit ultrastucture and here in the area peripheral to the bone from patient D. The tissue was not osmicated but stained with UA and LC.

Fig: 1.8d Larger deposits are referred to as calcospherites (Cs). These were often found in the less cartilaginous regions of pathological tendon and do not appear to be orientated with regard to collagen fibres. This tissue is from patient C and has not been osmicated or stained.

Fig: 1.8e 'Pebbled' sub-structure observed within a calcospherite.
Calcific deposits vary considerably in size from the large nodule removed complete from patient J (Fig: 1.1b and c) to the very fine 'needle-like' crystals that form the larger deposits causing circular whirled sub-structures. Patient C unstained.

Fig: 1.8f Crystals within calcospherites appear as fine 'needles'when slightly dispersed. Patient D unstained. In other areas the packing of crystals is generally more compact.

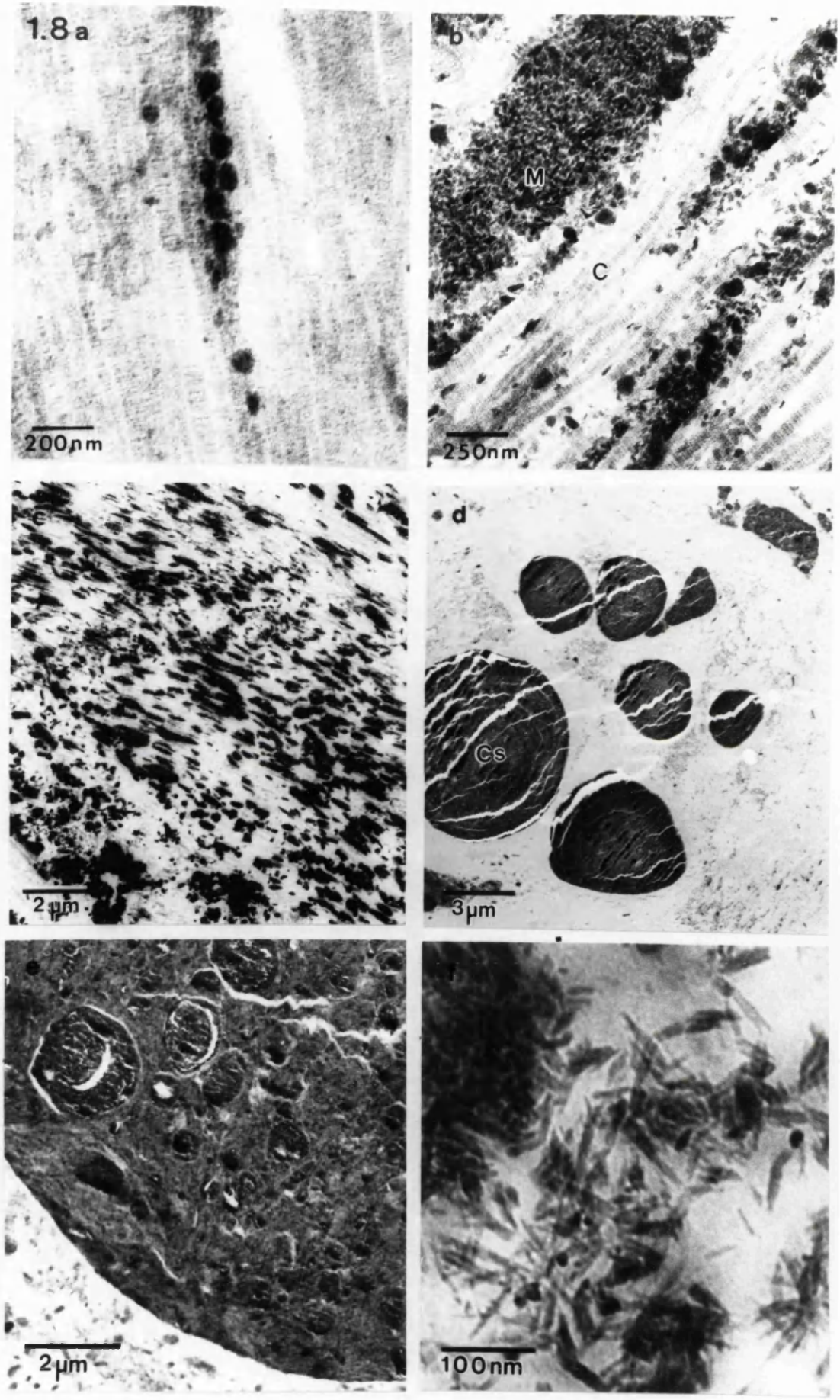

1.8a
200nm
b
M
C
250nm
c
2μm
d
Cs
3μm
2μm
100nm

Fig: 1.9a Mature deposit from patient D stained with Masson's trichrome. In this specimen bone (B) has been formed.

There appears to be cell necrosis indicated by the empty osteocyte lacunae (arrow).

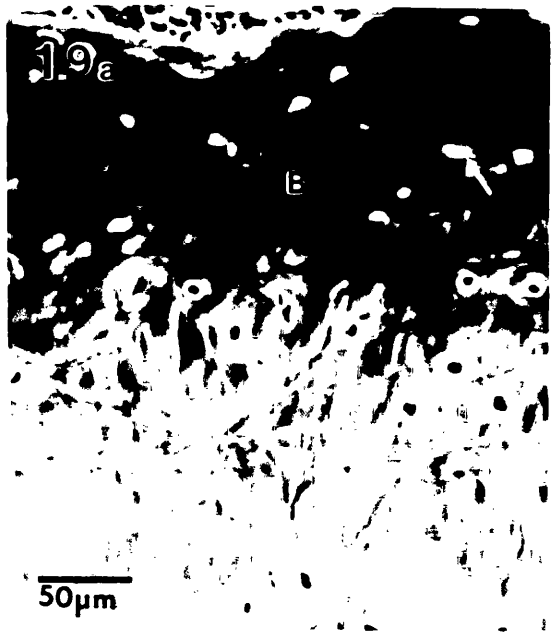
Fig: 1.9b Cells in the metachromatic tisssue have a more chondrocytic appearance whereas those within the bone are osteocytic (arrow), (H and E).

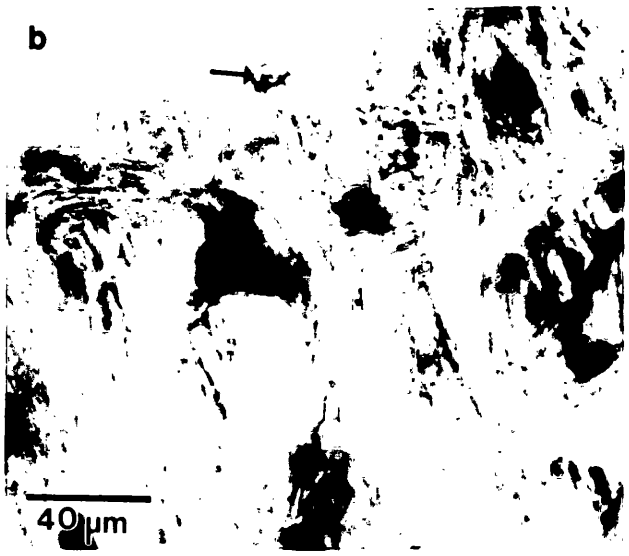
Fig: 1.9c In the same region as the bone, calcospherites (Cs) similar to those found in tissue sections from the other patients were also evident. There is also a spicule of bone (arrow) surrounded by red blood cells. This section was stained with Masson's trichrome stain.

Fig: 1.9d Micrograph showing presence of healthy osteoblasts (arrow) on the bone and cement lines (Cm) indicates that bone remodelling is ongoing (H and E).

Fig: 1.10a In all of the pathological specimens from patient H, and in areas of tissue sections from some other patients, there was an influx of inflammatory cells intermixed with nodules of mineral. The general appearance in these sections was that the nodules were confined in a highly cellular tissue in which foreign body giant cells and macrophages were observed (H and E). The arrowed deposit is enlarged in figure 1.10b.

Fig: 1.10b Higher magnification of foreign body giant cells (arrow) associated with a calcospherite, from patient H (H and E). Enlarged from an area in figure 1.10a.

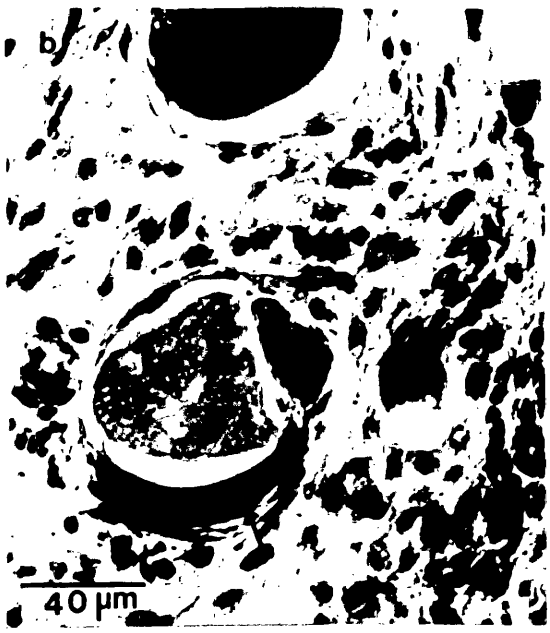

1.9a
B
50µm


b
40µm


c
C


d
Cm
50µm


1.10a
100µm

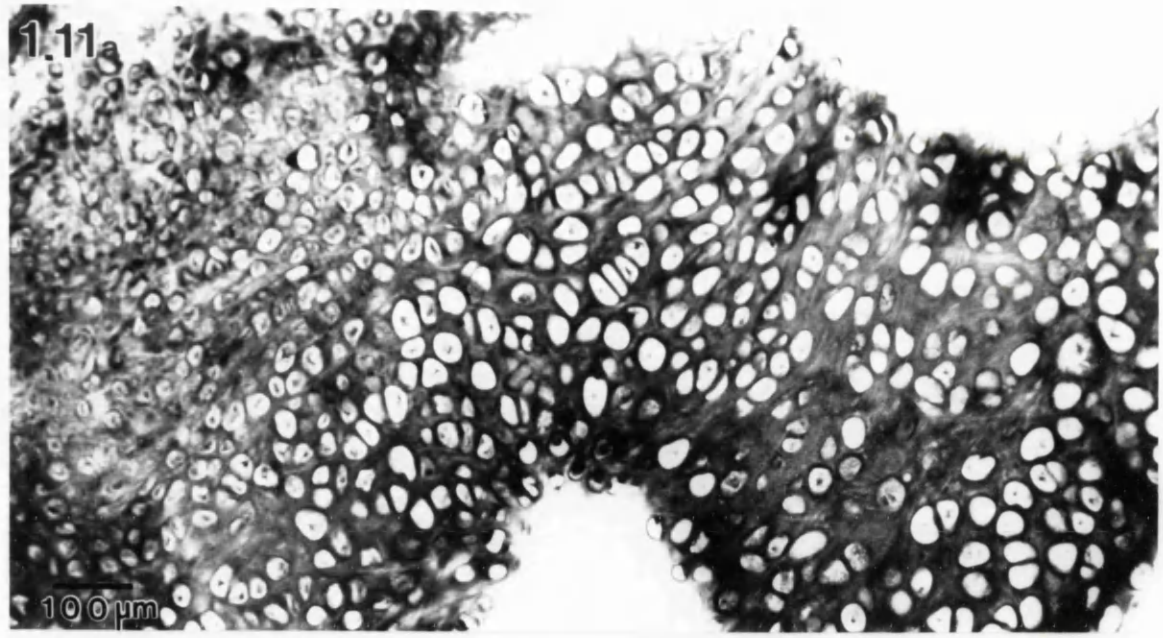

b
40µm

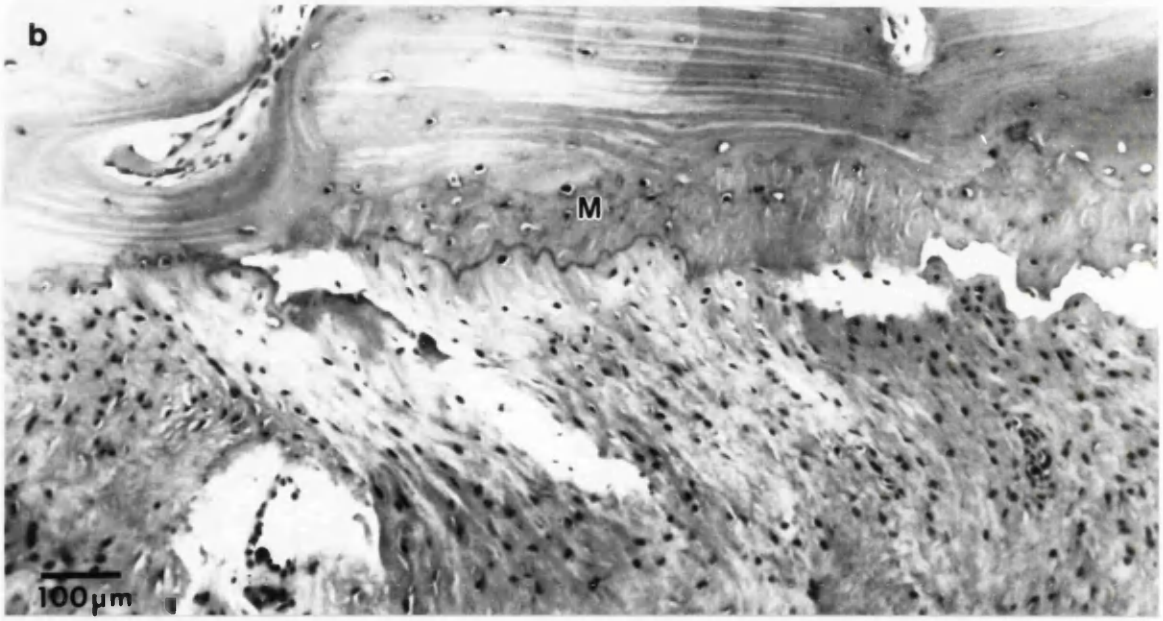
MYOSITIS OSSIFICANS TISSUE SECTIONS.

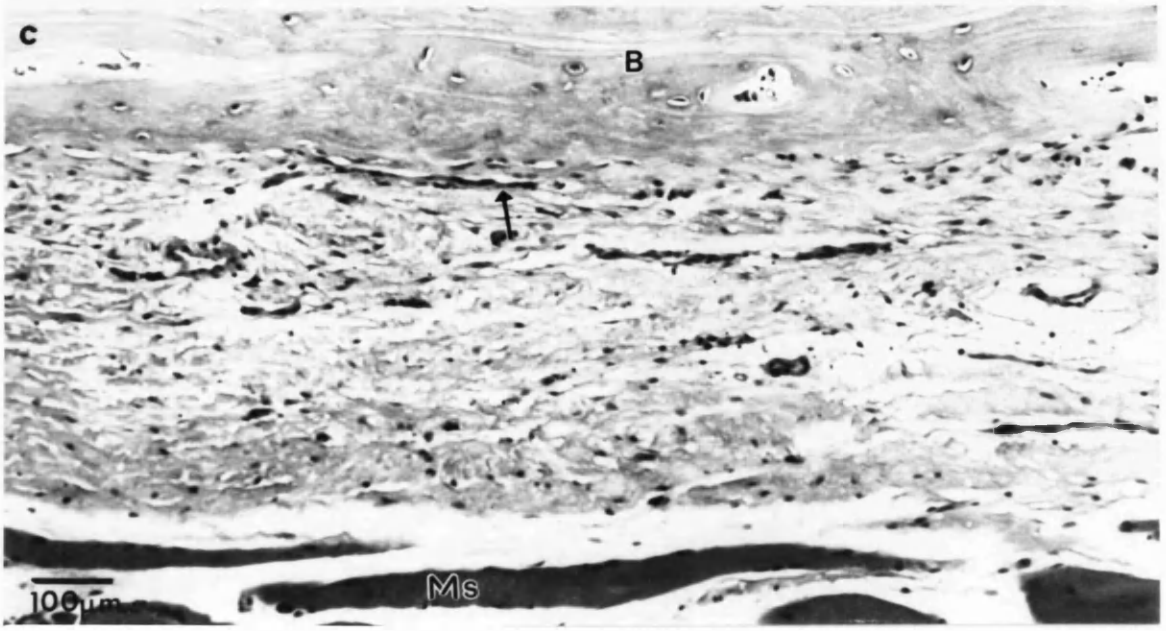
Fig: 1.11a Tissue specimen from a patient with myositis ossificans clearly shows chondrocytes in a cartilagenous matrix. Areas similar to this were never observed in the calcifying tendinitis specimens even in the region peripheral to the bone found in patient D.

Fig: 1.11b Other areas in the myositis ossificans specimens did have the same features as the bone deposit found in the tendon demonstrated by a metachromatic border containing similar 'chondrocyte-like' cells adjacent to the mineral front (M).

Fig: 1.11c Myositis ossificans does exhibit osteoblastic 'lining' cells (arrow) peripheral to the bone. There is also a fibrous layer between the bone (B) and muscle (Ms).


1.11a
100 µm


b
M
100µm


c
B
Ms
100µm

1:4. Discussion

The gross morphology of the supraspinatus tendon has been studied in two post-mortem specimens. The 'critical zone' where deposits are generally found is indicated in figures 1.1 and 1.3 which show the whole tendon, the latter in histological low magnification section. Structures to be observed include the abnormal presence of capillaries in the area assessed as being prone to calcific deposit formation. There is also evidence of some mucoid degeneration within the tissue matrix of the 'critical zone'. This specimen appears to be slightly degenerate which probably is not abnormal for tissue taken from a 55 year old, four days after death. Fig: 1.3c demonstrates by alizarin red stain for calcium salts, that the region of insertion of the tendon into bone contains fragments of mineral. None was observed away from this area. However, the presence of small amounts of mineral in human tendons may be quite common, and was observed during this study in non-pathological achillies tendon. Other important features shown by this section, which may have a role in the disease process are:

i. Muscle fibres, that merge into the tendon, were at no time seen within the pathological specimens despite the large overlap that occurs at the muscle/tendon junction.

ii. Fibrocartilage, as found at the insertion of tendon into bone, has been described as always accompanying signs of intratendinous calcification (Uhthoff, 1975; Uhthoff and Sarker,1978 and 1989; Refior et al., 1987). Blood vessels are sparse in calcified tendon increasing the evidence that avascularity may be a factor in the disease process. However, vascular proliferation is associated with the inflammatory response observed in some patients.

iii. Loose connective tissue which is often evident on the periphery of the pathological zone.

iv. Bone mineral into which tendon is anchored is similar to that deposited in the tendon.

From morphological observations it is not possible to deduce an answer to the 'chicken and egg' dilemma of which came first, the calcific deposit or the apparent

cellular and matrix changes. Obviously a proper time study is not possible using human tissue. The next best option is to compare specimens from different patients and in conjunction with their records attempt to establish a sequence of events of the time course of the disease.

Histological observations from the pathological tissue assume that all the samples can be grouped within one age category. This assumption is necessary for making comparisons between patients in an attempt to define the aetiology of the disease. This is especially true when consistent observations are made between specimens despite all of the possible variables.

In particular the amounts of deposited mineral vary. From an extreme case, patient J the whole large chalky deposit enclosed within a fibrous capsule was removed intact (fig: 1.2 a and b). In other cases, the area of calcium deposition was more diffuse and often there were numerous smaller nodules. In this morphological study the tissue could be catergorised in three main groups:

Group one: Some samples demonstrate a degree of metaplasia with rounded cells surrounded by comparatively large areas of disorganised matrix which lack cellularity and vascularisation (fig:1. 4.c to f). This is considered to be a non-inflammatory response probably a pre-resorptive stage and is equivalent to the initial two stages described by Uhthoff and Sarkar (1978) as precalcific and calcific.

Group two: In other samples, part or all of the tissue was found to be highly cellular, with abundant inflammatory cells. There is comparatively little extracellular matrix and often no residual tendon-like structure (fig:1.10 a and b). This stage is categorised as resorptive and restitutive by Uhthoff and Sarkar (1978). In both this and the first group, calcospherites, were present although in the second group, nodules were generally smaller and surrounded by inflammatory cells.

Areas demonstrating both of these stages were on occasion observed in a single tissue section and some over-lap as the pathological state of the tissue changes may be expected.

Group three: The third category included the rare observation of bone. This specimen also had some of the characterisitcs of group one and two tissue types.

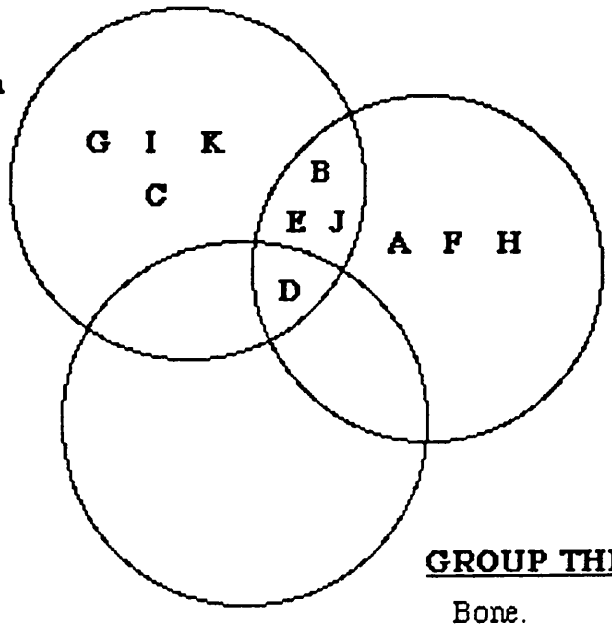
The grouping of the pathological sections examined can be represented in a Venn diagram giving the patient's reference letter. However, this assumes that the areas studied are representative of the whole pathology.

DIAGRAM 1.2

THE MORPHOLOGICAL GROUPING OF CALCIFYING TENDINITIS PATIENTS.

GROUP ONE

Rounded cells and an avascular, often metachromatic matrix.



GROUP TWO

Highly cellular, resorptive, may have small blood vessels.

GROUP THREE

Bone.

No coincident pattern is established to correlate this grouping with any of the factors known about the patients. It has been suggested that due to an increased frequency of HLA-A1 antigen in calcifying tendinitis patients that individuals may be genetically more susceptible to the condition (Sengar, McKendry and Uhthoff, 1987) however not enough information about these patient histories is available to assess this possibility.

Rounded cells in the area local to mineral deposition or surrounded by mineral have been frequently observed. These cells appear to have a mucin-rich lacunae as indicated by azur A staining. At the ultrastructural level, these cells are more similar to

chondrocytes as the mucin-rich halo is seen as an electron-lucent lacuna and they are large cells, have numerous short cell processes as well as a large nucleus. These cells are morphologically distinct from tenocytes (fig:1.5 a and b).

The chondrocyte-like cells described in this study do not store large islands of glycogen in their cytoplasm as described by Uhthoff and Sarkar (1978), but have a similar glycogen storage pattern to the more fibroblastic cells in the same tissue section.

When looking for evidence of an endochondral calcification mechanism vesicular structures were seen in the collagenous matrix of the samples studied in both 'normal ' and pathological tendon. The concentration of matrix vesicles in the pathological tendon and the distribution of those present appeared to be unrelated to the distribution of calcific nodules.

The calcific electron dense particles of vesicle shape and size were not observed to have a membrane and the membranous vesicular-stuctures were not seen to contain spicules of hydroxyapatite as has been described in endochondral ossification systems (Anderson, 1967; Bonucci, 1967). In addition, the chondrocyte-like cells did not appear to be in a state of hypertrophy as seen in a mineralising growth plate. It, therefore, seems less probable that matrix vesicles are the principal initiation mechanism for calcification in calcifying tendinitis. This does not necessarily contradict the findings of Jozsa et al. in 1980 who did not think that this pathology had the same initial stage as endochondral calcification. Jozsa and colleagues believe, however, that morphological evidence exists showing that calcification occurs within extracellular vesicles in the extracellular space.

As the abundance of 100-200nm mineralised nodules doesn't necessarily mean that these are membrane bound and hence vesicular it is important then to consider evidence for alternative mechanisms.

The subsequent growth of the calcific deposit is probably modulated by factors different from the initial stimulus (Ali, 1983) such as collagen precipitation. At the ultrastructural level, evidence was sought as to whether collagen type I serves as a template and that crystals may align along the collagen fibres. Some crystal alignment was observed in association with both the calcific deposit ultrastucture (fig1:8b) and in

the area peripheral to the bone from patient D (fig:1.8c). In the latter, the mineral appeared more organised. As mineral was also evident between, but not associated with the collagen fibres, the spread of crystal deposition does not appear to be solely dependent on direct contact with collagen fibres. Observations at this stage in the disease process do not enable comment on the above facets as possible initiating mechanisms.

When seeking initial mechanisms of mineralisation, the potential role of necrotic cells releasing degrading enzymes or the role of intracellular organelles, in a physically disrupted tissue acting as nuclei of mineralisation should not be overlooked. Mitochondria are the calcium stores for cells and have a high internal concentration of calcium ions. Some evidence of cell necrosis in the pathological specimens was observed and frequently mitochondria appeared swollen or to have burst (fig:1.7b). Mitochondrial calcium, released into the extra-cellular matrix that already may have saturation levels of calcium ions might be the trigger needed for initial hydroxyapatite formation. In addition enzyme activity associated with the cell remnants (ie. organelle and plasma membranes) could cause an accumulation of inorganic phosphorous.

Lipid content was not routinely investigated, as the chemicals used prior to wax embedding strip the tissue of its lipids. However, by electron microscopy, adipose tissue was observed in some pathological tendon, in the non-pathological specimens from the same patients, and in 'normal' post-mortem tendon. The adipose tissue was not closely associated with the calcific deposit.

The influx of inflammatory cells is a resorption phenomenon and therefore, although relevant to the course of the disease and results in both cellular and molecular changes in the tendon, is not a consideration in the search for causes and mechanisms of calcium deposition. The presence of inflammatory cells was often associated with a vascular proliferation in tissue closeby and repair may occur partly through the proliferation of synovial cells.

Previously metachromasia has been taken to indicate the establishment of a cartilaginous matrix. Uhthoff (1975) and also Uhthoff and Sarkar (1989) refer to fibrocartilage metachromasia as the precalcific stage. However, it is also possible that the disease

process is static, at a stage where mineral is no longer being deposited and perhaps the observable changes occur as a result of the adjacent mineral deposition. Metachromasia was also observed in the region of bone formation (fig: 1.9) in the case of patient D. However, because bone is presumably a rare occurrence in this pathology and the metachromasia was relatively common, this should not be taken as an indication that ossification is imminent.

Mature and acute phase deposits were removed from different patients and only in the case of patient D, where bone was discovered, were both types recorded in a single patient. Mature deposit proved to be highly collagenous and sometimes fibrocartilaginous in appearance with dispersed calcific deposit in areas through the matrix.

That bone forms in calcifying tendinitis patients due to a renewed blood supply to previously calcified tendon has been suggested by Uhthoff (1975) who has found two cases of ossifying tendons. Surrounding an isolated spicule of bone in the same section as calcospherites, red blood cells and fibrin were present indicating this might be the case. However, blood may be involved in calcification of tendon, as red blood cells were frequently observed intermingled with the calcific deposits (fig 1.4c).

Deposit laid down in the acute phase where oedematous inflammation is described (Faure and Daculsi, 1983) was of 'tooth paste consistency' (Bland et al., 1977). Acute deposits contained little collagenous tissue. This is more akin to the deposit that could be aspirated with a needle.

Calcific deposits varied considerably in size from the large nodule removed complete from patient K to the very minute individually disc-shaped deposits as observed with the electron microscope (fig:1.8a). Often the border between mineral and surrounding tissue was well defined and the deposits appeared spherical (fig:1.8d). The large islands of deposit were less homogeneous in texture having a 'puddingstone' appearance apparently formed by numerous small but inconsistently sized 'pebble-like' spherical deposits. Often these possessed a whirled infrastructure, and were crammed together with the spaces between filled by more of similar calcific deposit (fig:1.8e). These circular whirls appeared to be formed by the alignment of fine 'needle shaped'

particles (fig 1.8f) that are probably the plate-shaped particles reported in bone, but do not appear to be woven into a collagenous infrastructure. Taken in cross-section, the size of crystal varied, presumably according to the plane through the individual crystal that was sectioned or due to association of crysals. A comparison of the calcium to phosphorous ratios of the mineral from different patients is made in chapter four.

Interesting comparisons can be made by observing the morphology of myositis ossificans specimens. One mechanism of progression of myositis ossificans appeared to be endochondral as areas of cartilaginous tissue were observed (fig:1.11a). This tissue appears more similar to cartilage than that surrounding the calcific deposits in the calcifying tendon. This is evidence that calcifying tendinitis does not occur as a result of this type of chondrogenic modulation.

There were morphologically similar areas between the case of ossifying tendon and some myositis ossificans sections (compare figures: 1.9 a and b; 1.11b) where in both, a fibrocartilaginous type tissue occurred in apposition to the bone. This indicates that more than one mechanism causes the ossification of muscle and that at least one could be common to this and ossified tendon, but not necessarily in the more common calcifying tendinitis. In ossified tendon, osteoblasts were seen and there was evidence of osteoclast activity indicated by the pattern of the cement lines and hence bone remodelling, although osteoclasts were never observed in tissue sections (fig:1.9d). The bone was generally 'unhealthy' containing empty lacunae which osteocytes had occupied. Perhaps once established, the tendinous environment had proved unable to maintain viable bone.

Many complex changes both cellular and molecular can be observed in calcifying tendinitis and although a preliminary morphological characterisation of these changes is an important foundation for subsequent studies, conclusive evidence of a single mechanism inherent in these changes has not been established. It is apparent that, as with other pathological conditions, there are numerous contributory factors to the disease process.

CHAPTER TWO.

ALKALINE PHOSPHATASE IN CALCIFYING TENDINITIS

ALKALINE PHOSPHATASE IN CALCIFYING TENDINITIS.

2.1 INTRODUCTION

Phosphatases were first implicated in the mineralisation process in the 1920's by Robison (1923) with his discovery that alkaline phosphatase could cause precipitation of calcium phosphate. Subsequently, he initiated work that examined the distribution of the enzyme in various tissues including sites of mineralisation.

In mature bone the alkaline phosphatase reaction is restricted to the periosteum, endosteum and more superficially placed osteocytes (Bourne 1942; Lorch 1947), the matrix surrounding active osteoblasts was later also found to be positive (Morse and Greep,1951).

Alkaline phosphatase (AP) was shown to be associated with strands of connective tissue in membrane bone formation by Gomori (1943). In endochondral ossification three characteristic zones were described by Morse and Greep (1951). Alkaline phosphatase was found to be associated initially with cell nuclei in the proliferative zone, with the cells becoming progressively richer in the enzyme with maturation. Closer to the mineralised front where the cells are aligned, enzyme activity spreads from just the nuclei, to nuclei and cytoplasm, then into the surrounding matrix in the region of hypertrophy. Where the matrix becomes richer in alkaline phosphatase, the cells are less positive. In the zone of provisional calcification there is a decline in the enzyme activity. According to Kay and Robison (1924), phosphatases were always absent in cartilage that did not ossify.

Robison found bone to be twice as rich in phosphatases than kidney and twenty times richer than liver but the wide distribution of the enzyme in non-mineralising tissues raised questions about the specificity of the relationship between alkaline phosphatase and calcification. It is now known that the vertebrate alkaline phosphatases are isoenzymes with distinctive sensitivities to inhibitors, immune cross-reactivities and specific amino acid terminal sequences (see Wuthier and Register, 1985).

Keith (1928) described areas of ectopic bone production in a variety of unusual sites including laparotomy scars and the choroid coat of the eye which have since been histochemically shown to contain alkaline phosphatase. In some tisssues the enzyme AP may be restricted to specific sites, for instance, the capillary vessels. Early experiments (Huggins and Sammet 1933) demonstrated that areas of induced bone production were rich in phosphatases.

The regeneration of bone and skin occurring in wound repair is described by Bourne (1972). Repair begins with the migration of large numbers of alkaline phosphatase positive polymorphic cells, alkaline phosphatase negative macrophages, and fibroblasts, positive at first in the nuclei and around the cell membrane, into the injured area. After the first 24 hours, alkaline phosphatase positive fibroblasts are apparent and these produce phosphatase positive collagen fibres. Loss of phosphatase activity in the wound region signals the completion of the repair process. In bone there is a second cycle of phosphatase activity associated with the production of calcified matrix. Hence, under normal conditions alkaline phosphatase is associated with the development, turnover and repair of both membrane and endochondral bone.

The possible roles of alkaline phosphatase in calcification and the evidence for these have been reviewed by various authors including Bourne (1972), and Wuthier and Register (1985). There have been numerous different ideas about the function of alkaline phosphatase in mineralisation, some of which are summarised:

1) Production of phosphate ions which secure the precipitation of calcium as bone salt.

2) Acting as an inorganic phosphate transporter.

3) Increasing the local concentrations of inorganic phosphate (Pi).

4) Associated with the formation of the organic matrix of bone.

5) Concerned with the formation of a phosphate ester which acts as a template or part of a template for the catalytic crystallisation of bone salt.

6) Local destruction of mineral growth inhibitors (perhaps ester phosphate) via expression of phosphohydrolase activity, thus, keeping the surface of bone crystals free, permitting the continued growth of the crystals.

7) Acting as a Ca^{2+} binding protein

8) Acting as a Ca^{2+} pump (Ca^{2+}-ATPase) in cell or vesicle membranes.

9) Acting as a tyrosine-specific phosphoprotein phosphatase, hence, regulating cell division or differentiation. This assumes that AP plays an important role in the developmental process of skeletal tissues and is not merely involved in deposition of calcium phosphate in mineralizing matrix.

Although alkaline phosphatase is considered to be important in calcification the exact function of this enzyme remains unknown. Because of its presence throughout the body, alkaline phosphatase can in no way be taken as an indicator of mineralisation. Non-calcifying tissues must either lack other components necessary to initiate the process, have a much lower level of enzyme activity, or have inhibitory factors preventing their mineralisation.

There is some debate as to whether the serum alkaline phosphatase levels are significant as markers of ectopic calcification or ossification. In some diseases, such as myositis ossificans, where mature bone is formed rather than soft tissue calcification, an elevation of AP is coincident with the active calcification and follows the course of the disease (Furman et al, 1970). However, in calcifying tendinitis patients, there are no reports of elevated serum alkaline phosphatase levels. This may be because the degree of calcification is comparatively minute in relation to body mass, so any minor elevation may be undetectable against the normal background level of alkaline phosphatase in the blood.

Uhthoff and Sarkar (1978) speculated that due to the multiple foci of calcification, all the stages of the disease process; precalcific, formative, resorptive and restorative, could be present at one time in the pathological tendon. Despite serum levels of alkaline phosphatase not rising with ongoing calcification, a histochemically demonstrable local reaction of the enzyme, being elaborated by chondrocyte-like cells, in proximity to the sites of calcium deposition has, nevertheless, been described (Uhthoff, 1975). It is for this reason that the work described below was undertaken. In this investigation, different histochemical techniques were used to study the distribution

of alkaline phosphatase in samples taken from areas of calcium deposition and surrounding tissues.

Reaction mechanism

Detailed studies of a closely related alkaline phosphatase isolated from E.coli have shown that the native enzyme is a dimeric metalloprotein molecule with $4Zn^{2+}$ and $2Mg^{2+}$ present as functional ions. The enzyme can hydrolyse a wide range of phosphate esters catalysing their rapid dephosphorylation at alkaline pH. A phosphorylenzyme, stable at low pH, is formed during the hydrolysis of phosphate esters (Cocivera et al 1980; Caswell and Caplow, 1980). The rate-determining step in the reaction at high pH is the dissociation of tightly bound (although non-covalently) inorganic phosphate from the enzyme-product complex.

In this histochemical study, the alcoholic residue, formed by incubation of medium with tissue samples containing the enzyme is utilised. Alkaline phosphatase hydrolyses a substituted naphthol ester (naphthol AS-BI phosphate), yielding extremely insoluble naphthol derivatives which simultaneously couple with diazonium salts (new fuchsin) to give a red reaction product allowing light microscopical localisation of the sites of enzyme activity.

The ultrastructural histochemical investigation required an electron opaque reaction product so the incubation medium contained lead nitrate as the 'capture reagent' (Lewinson et al 1982) causing precipitation of lead phosphate at the site of enzyme activity.

2.2 METHODS

The samples shown on table 2.1 were used for the alkaline phosphatase investigations. For light microscopical investigations, frozen tissue cryostat sections were cut on to glass slides and were used unfixed. For ultrastructural studies a dilute fixative was used. Myositis ossificans specimens were used as positive controls. Histochemically negative control samples were incubated in the same reaction medium except for the

* Levamisole hydrochloride is a noncompetitive inhibitor of the liver, kidney, bone isoenzyme of alkaline phosphatase. In these experiments it inhibits the reaction, in known alkaline phosphatase positive tissue sections (myositis ossificans and rat kidney), therefore providing a negative control for the experiments.

addition of 2mM levamisole, a potent uncompetitive inhibitor of the liver, kidney, and bone isoenzyme (Van Belle,1976).

Alkaline phosphatase histochemical localisation by the Naphthol AS BI Phosphoric Acid method.

The cryostat sections were flooded with incubation medium or control medium and left for 20 minutes at 37°C. Incubating medium composed of 500µl of 4%(w/v) new fuchsin in 2M HCL with 500µl of 4%(w/v) sodium nitrite in distilled water, shaken together, then diluted to 40ml using fresh 50mM Tris buffer with 2ml of 0.1N HCl,adjusted to p.H. 9.0 (with 0.1N HCL) and containing 0.025%(w/v) Naphthol AS BI phosphoric acid (Sigma). For a negative control, 2mM Levamisole hydrochloride (Sigma) to was added to the incubating medium. Levamisole inhibits alkaline phosphatase activity. Some sections were counterstained with chloroform washed methyl green. The sections were then dehydrated, cleared and mounted.

Sites of alkaline phosphatase activity appear red. Sections were viewed and photographed on a Zeiss photomicroscope III.

Alkaline phosphatase electron microscope histochemistry.

Tissue was fixed by 1.5% glutaraldehyde in 0.085M sodium cacodylate buffer for 2 hours at 4°C and during this period the tissue was cut into small peices (0.1 x 0.1mm³) using a razor blade and after washing in cacodylate buffer (2x10ml for 5mins) incubated in 10ml of medium containing 40mM Tris/HCL, pH 9.0, 9mM sodium *B*-glycerophosphate, 5mM magnesium chloride, and 3.6mM lead nitrate for 30 minutes at 37°C (Lewinson et al 1982; Rees and Ali, 1988). Control samples included levamisole hydrochloride to a concentration of 2mM in the incubation medium. Tissue was then washed in 2x10ml of buffer and post-fixed in 1% osmium tetroxide and processed as for standard electron microscopy (see chapter one).

TABLE:2.1 SAMPLES TREATED FOR HISTOCHEMICAL LOCALISATION OF ALKALINE PHOSPHATASE.

REGIONS SAMPLED	PATIENTS A	B	C	D	E	F	G	H	J	K
Tendon junction										
Supra-infraspinatus	X	-	X	X	-	-	-	-	-	-
Sub-scapularis										
without deposit	-	-	-	-	-	-	-	-	-	X
With deposit	-	-	-	-	-	-	-	-	-	X
Infraspinatus										
Without deposit	-	X	-	-	X	X	-	-	X	-
With deposit	X	-	-	-	-	-	-	-	-	-
Supraspinatus										
Without deposit	-	-	X	-	-	-	-	-	X	-
Adjacent to deposit	-	X	-	-	-	-	X	X	-	-
Roof of the deposit	-	-	-	-	X	-	-	-	-	-
Wall of the deposit	-	X	X	X	-	R	X	X	-	-
Floor of the deposit	-	-	-	-	X	-	X	X	X	-
Bursal calcification	-	-	-	-	-	X	-	-	-	-

KEY:

Sample	X
No sample	-
Resorbed sample	R

Samples studied by electron microscopic histochemistry: Patients A to F and K.

Samples studied by light microscopic histochemistry: Patients G, H and J.

2.3 RESULTS.

The results obtained from calcifying tendons were frequently negative but occasionally demonstated a sparse distribution of reaction product that indicated some enzyme activity. For this reason a strong positive control that demonstrated the elaboration of alkaline phosphatase due to a pathological mineralising condition in human connective tissue was required. Tissue samples processed by the same methods as the calcifying tendinitis specimens from patients suffering from myositis ossificans were used for this purpose.

Histochemically, the reaction product has been easily demonstrable on cryosections for light microscopy using specimens of myositis ossificans (fig: 2.1a). However, using eight different regions from within and around the mineralising nodules from three patients with calcifying tendinitis, minimal alkaline phosphatase reaction product was achieved (fig: 2.1 b and c). The area shown in figure 2.1c was the maximum red reaction product seen in the calcifying tendon sections. There was no red reaction product from the levamisole treated sections.

Electron microscope histochemistry, confirmed the light microscopical results. At no time could alkaline phosphatase be described as abundant in the calcifying tendinitis specimens. Specimens from a total of 18 areas from seven patients were incubated in the reaction medium, sectioned and viewed by electron microscopy (see table 2.1). Eight samples were from non-pathological areas of tendon and in only one patient had the calcific deposit resorbed to such an extent that mineral could only be found in association with the bursa. In all these specimens, minimal extra-cellular alkaline phosphatase was found. Only in samples from the deposit walls from patients D and C and the roof of the lesion from patient E was the enzyme associated with the outer cell membrane (figs: 2.2a, 2.3c and d) (See chapter 1.1 for a definition of the areas surrounding the deposits). Many calcifying tendinitis specimens demonstrated a fine intracellular reaction precipitate on the cytoplasmic membranes in apparently normal regions of tendon but not more strongly in cells close to the mineral (figs: 2.3 a and b; and 2.4 a). This very fine precipitate could also be observed in some of the levamisole controls the worst case of non-specific precipitation was seen in the levamisole controlled reaction of a myositis ossificans specimen (fig: 2.4 b) and this precipitate was different in

nature to the dark crust of reaction product seen around alkaline phosphatase rich cells. In control specimens, the tissue was incubated in the same medium but with the addition of 2mM levamisole. Apart from occasional fine precipitate this completely abolished the already minimal reaction in the calcifying tendinitis specimen and greatly reduced the reaction in the case of myositis ossificans tissue.

Using the same methods, the myositis ossificans test specimens gave very strong reactions, demonstrating the presence of alkaline phosphatase in the matrix of the osteoid layer close to the bone (figs: 2.5 b and c). Away from the mineralising front, the cells embedded in the soft connective tissue had a different intra-cellular reaction to those in the calcifying tendinitis specimens (fig: 2.5 a) since in some cells the reaction appeared to be associated with the cell nucleus.

FIGURES.

FIGURE LEGENDS

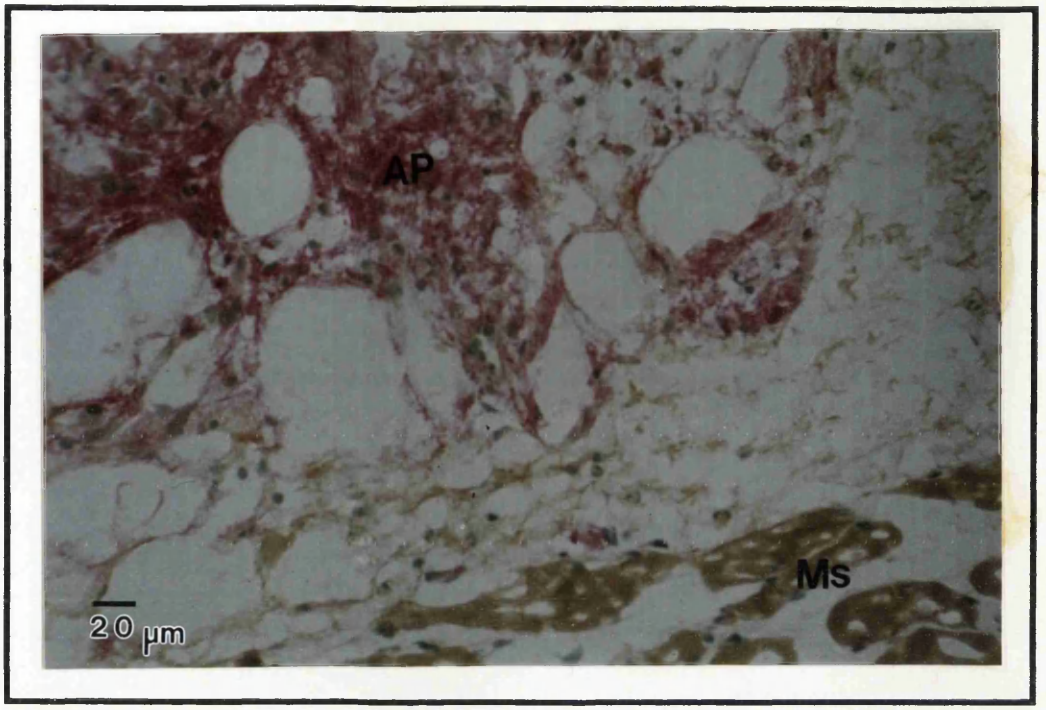
Light histochemistry

Fig: 2.1a Red reaction product demonstrates the site of alkaline phosphatase activity (AP) at the muscle bone interface in a cryostat tissue section from a patient with myositis ossificans. Muscle bundles have been labelled (Ms).
Counterstained with chloroform washed methyl green.

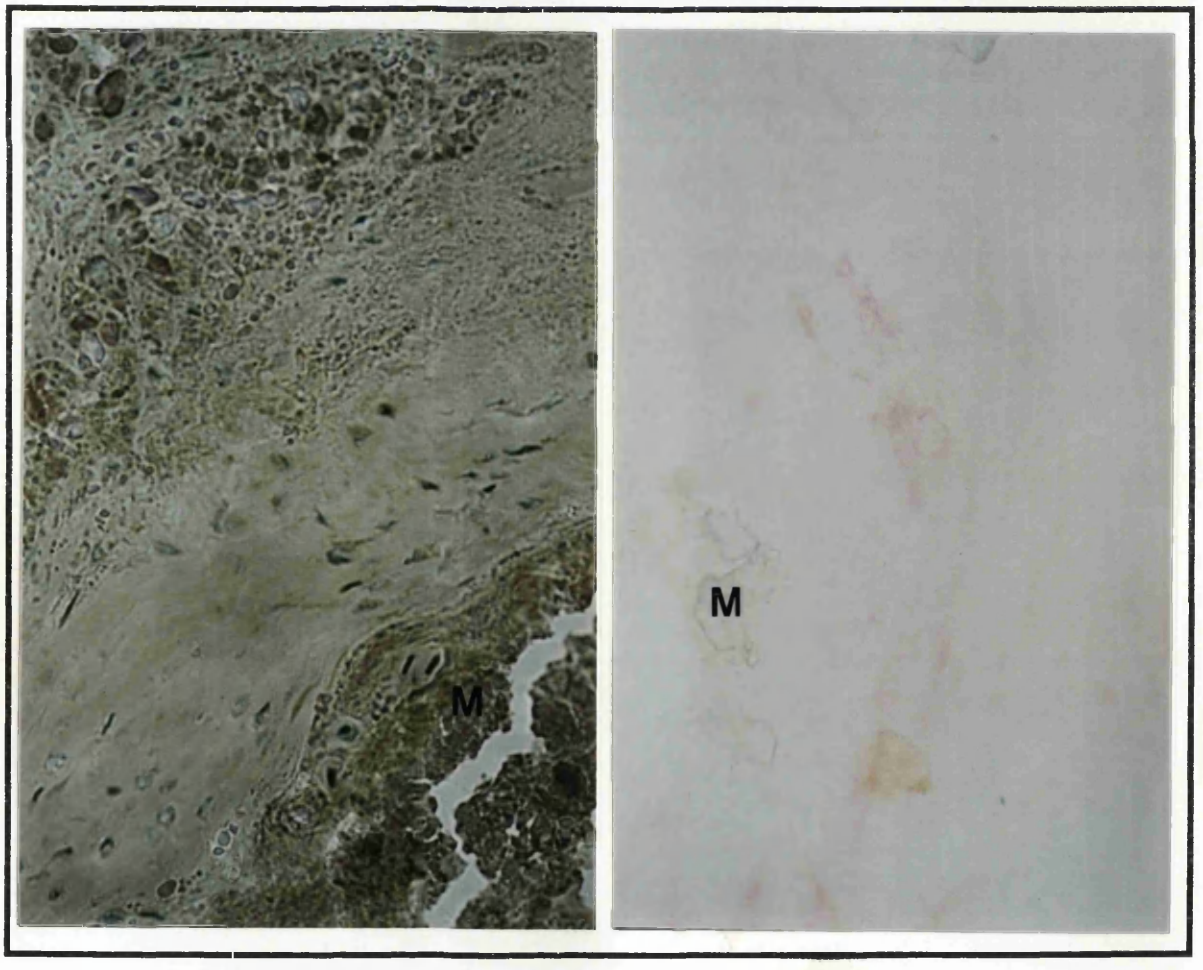
Fig: 2.1 (b and c) Micrographs of tissue samples from around the calcific deposit in patients G and J. These represent the maximum distribution of reaction product found in any of the tissue sections from calcifying tendinitis patients examined. Mineral has been labelled (M).
Section 2.1b (from patient G) is counterstained with chloroform washed methyl green.
Red reaction product was absent from control sections where the incubation medium contained levamisole.

Micrographs are magnified to the same scale.

2.1a



2.1 b and c



Ultrastructural histochemistry

Calcifying tendinitis: This series of micrographs are all from tissue incubated in alkaline phosphatase reaction medium.

Fig: 2.2a Section of the deposit wall taken from patient D. Low magnification picture of a highly cellular area. This area of tisssue is not tendinous in appearance. Some of these cells have the reaction product associated with their plasma membranes (AP), however, not all the cells from this area are alkaline phosphatase rich. There was no mineral in close proximity to this area of phosphatase activity. Rarely in these specimens was there this much indication of alkaline phosphatase activity. Stained with UA and LC.

Fig: 2.2b Patient D, from the deposit wall and the same tissue section as fig:2.2a. This area containing calcific crystals is not rich in alkaline phosphatase. Intra- and extracellular mineral can be seen in this micrograph (M). Mitochondria (m) and a cell nucleus (n) are labelled. As was usually found there is no evidence of alkaline phosphate reaction product in this area. Stained with UA and LC.

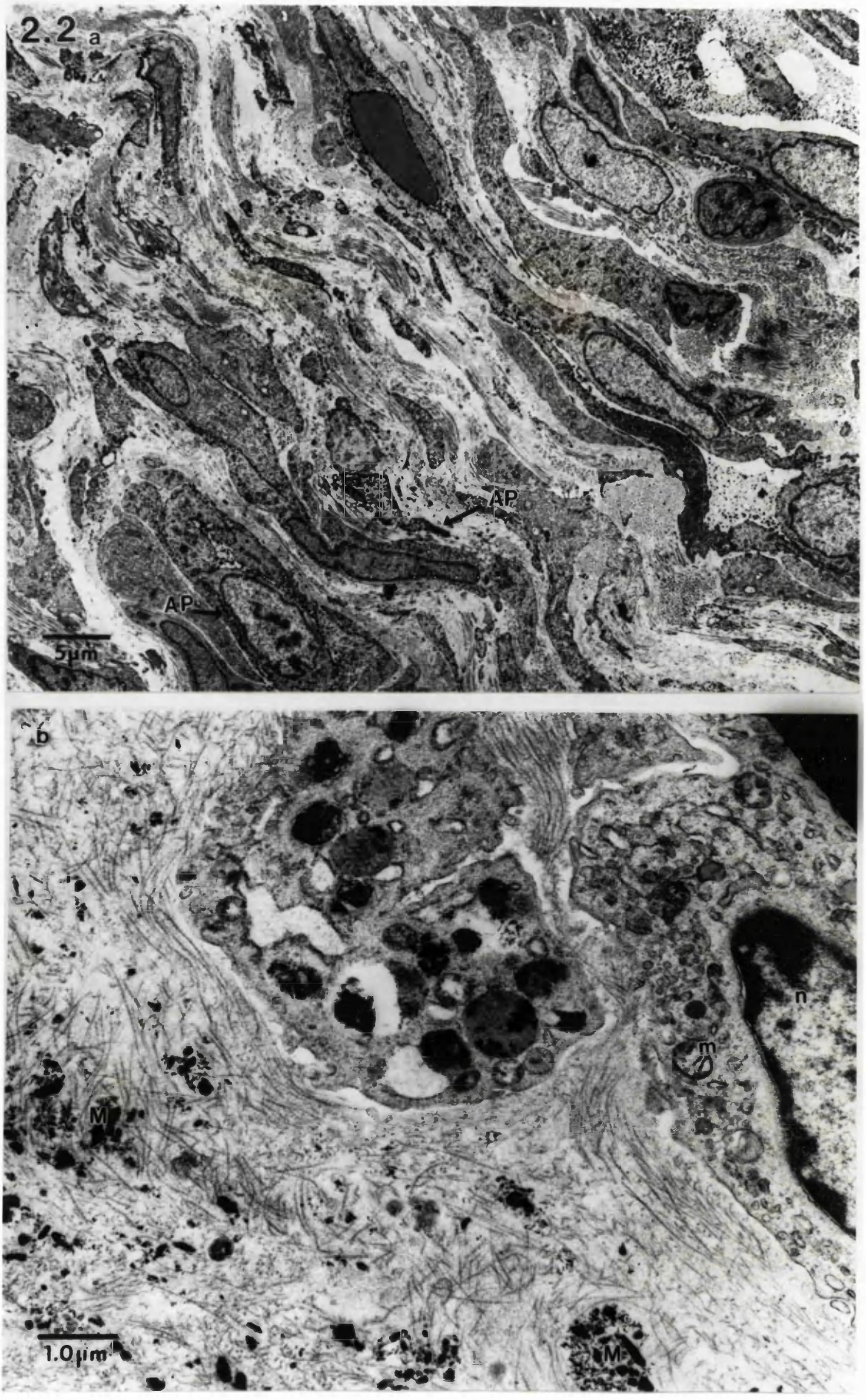

2.2 a
AP
AP
5μm
b
n
m
M
M
1.0μm

Fig: 2.3 (a and b) Patient C. Apparently normal regions of tendon showing fibroblastic-like cells in transverse and longitudinal section. The increased contrast of the organelles is probably due to a non-specific fine electron dense deposit of lead phosphate and was also observed in levamisole controlled incubation specimens. Stained with UA and LC.

Fig: 2.3c Patient B, from the deposit wall. A chondrocyte-like cell is distinguished by being more rounded, possessing numerous short processes (p) and appearing to be set into a finer matrix rather than that of the course collagenous (C) tendon. Small nodules of deposited mineral (M) are close to the cell but there appears to be only a minimal alkaline phosphatase reaction product associated with the tips of the cell processes. This was the maximum reaction seen in a mineralised area. Stained with UA and LC.

Fig: 2.3d Patient E from the roof of the calcific lesion. A highly necrotic cell with nucleus (n). Here a large precipitation of reaction product (AP) has occurred. Again this was not characteristic of the amount of reaction product demonstrated throughout the tissue, but there did appear to be an association of reaction product with cell fragments. (UA and LC).

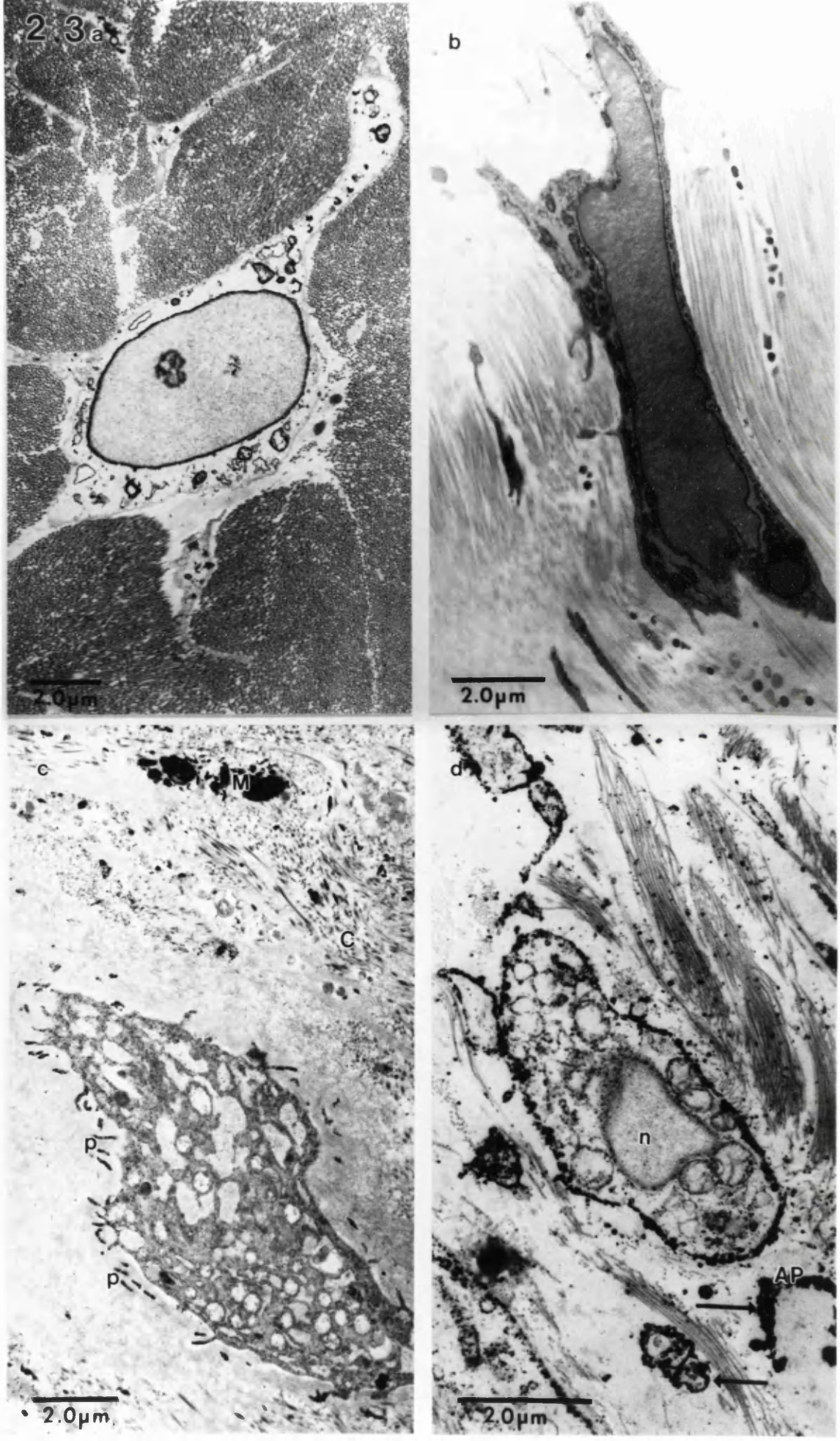
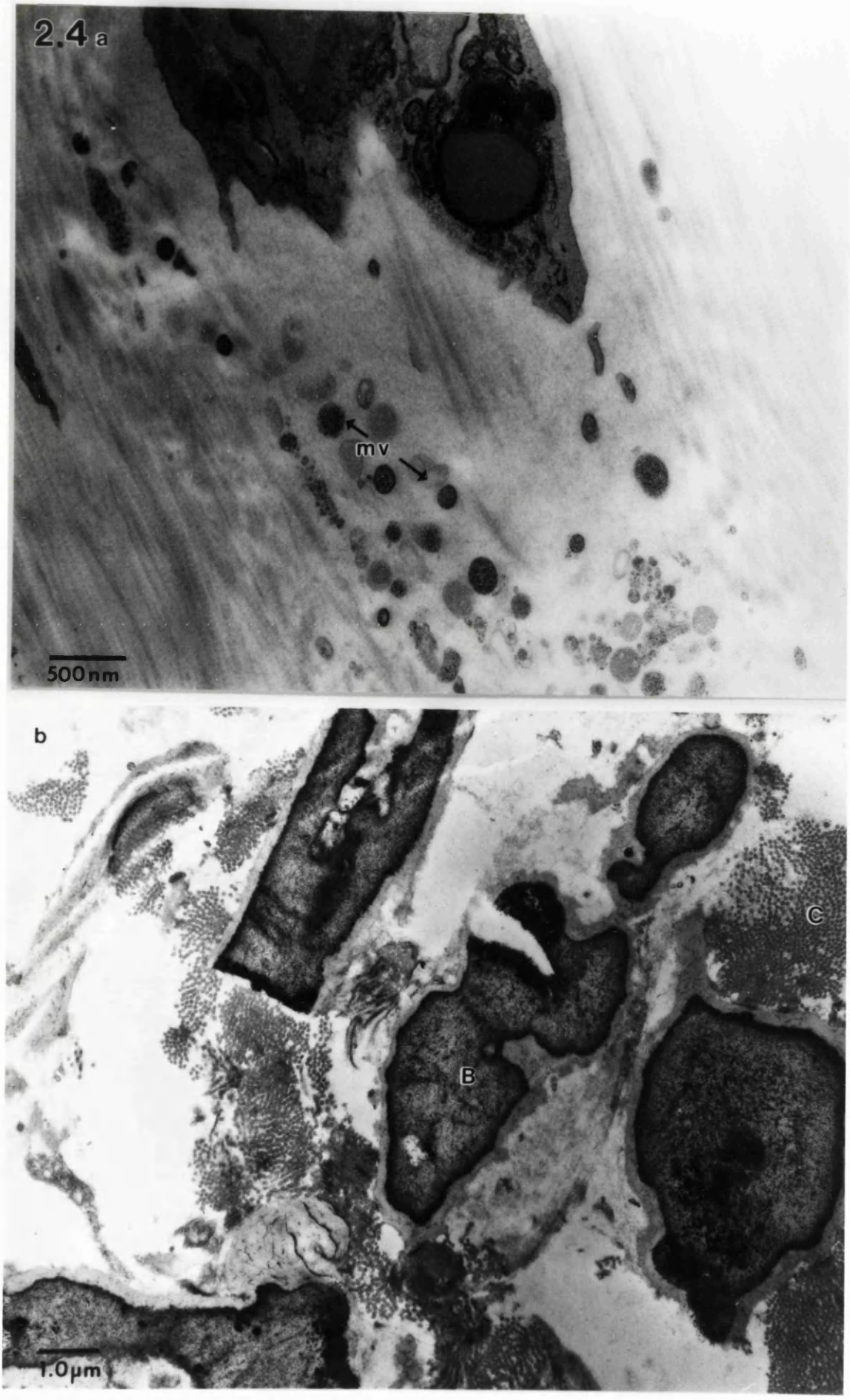

2 3a
2.0μm
b
2.0μm
c
M
A
C
p
p
2.0μm
d
n
AP
2.0μm

Fig: 2.4a Enlargement of part of figure 2.3b. Patient C, fibroblastic cell from a relatively normal area of tendon. The cell processes or matrix vesicles (mv) demonstrate a very fine lead precipitate also located on membranes within the cell. This was also occasionally observed in levamisole control tissues (UA and LC).

Myositis ossificans tissue sample: Levamisole control.

Fig: 2.4b This area of bone (B) and collagen (C) demonstrated the maximum amount of reaction product observed in a levamisole control sample. The very fine lead precipitate was not dissimilar to that seen intracellularly in fig: 2.4a above(UA and LC).

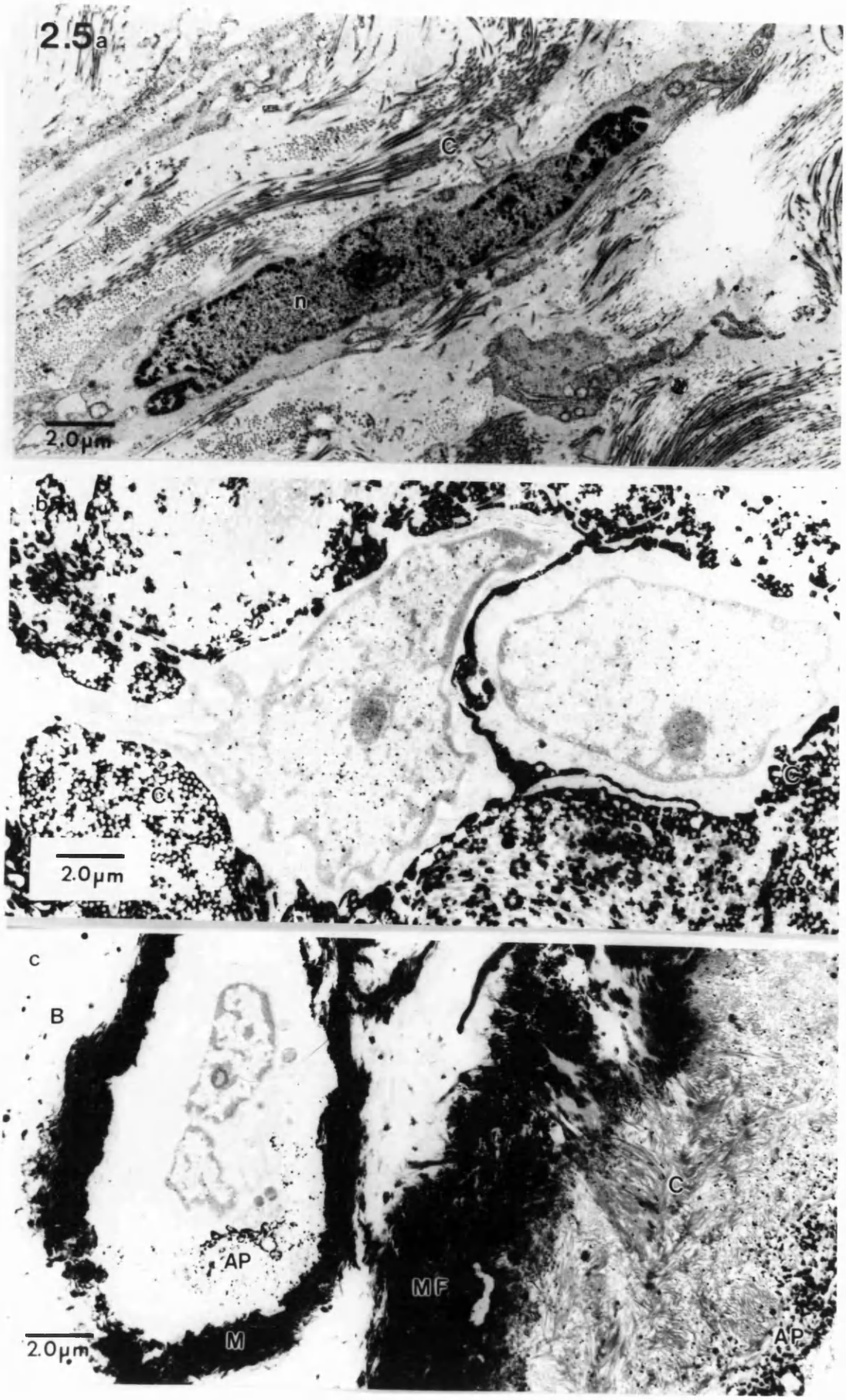

2.4 a
mv
500nm
b
C
B
1.0μm

Myositis ossificans tissue sample: Alkaline phosphatase incubation medium.

Fig: 2.5a Fibroblastic-like cell and disorganised collagenous matrix (C). Cells that are remote from the area of mineralisation but within the same section gave this demonstration of intracellular enzyme activity associated mainly with the cell nucleus. (UA and LC).

Fig: 2.5b Osteoid lining cells located close to the mineralised front. The enzyme product is not found within the cells whereas the surrounding matrix is dense with precipitate of lead phosphate. The collagen fibres (C) are apparent in transverse section by virtue of their comparative electron lucency. (UA and LC).

Fig: 2.5c An osteocyte still associated with alkaline phosphatase activity (AP) within the space enclosed by newly formed mineral (M) and surrounding bone (B). There appears to be a border region between the mineralising front (MF) and the osteoid layer where the collagenous matrix (C) is not rich in alkaline phosphatase (AP) activity. This is consistant with observations made by scanning along the mineralising front. (UA and LC).


2.5a
C
n
2.0μm
b
C
C
2.0μm
c
B
AP
M
MF
C
AP
2.0μm

2.4 Discussion

Selected micrographs have been shown and the figure legends describe whether,i) the picture is characteristic of the results throughout ii), it shows a maximum reaction product, or iii) is an atypical region.

The light microscopical histochemical results were not as expected. The calcifying tendinitis specimens demonstrated minimal alkaline phosphatase activity (figs: 2.1b and c), but, confidence in the technique was maintained by observing the strong red colour obtained by incubating in test medium cryostat sections of human tissue undergoing pathological ossification (fig: 2.1a).

Ultrastructural histochemistry confirmed the light microscopical results from calcifying tendon. Not suprisingly, the ultrastructural method was far more sensitive to enzyme located within the tissue. Care is required in interpretation of the results obtained from this technique as non-specific precipitation can cause false positive results to be recorded. For this reason, very finely divided tissue samples (minced) were used and when observing sections by electron microscopy, only regions of activity away from the edge of the tissue block were accepted when determining the presence of genuine reaction product. The edges of tissue blocks may give abnormally strong reactons and were avoided for this reason. After taking these precautions it was assumed that the tissue was fully infiltrated with the incubation medium and that some lead would precipitate out of solution causing a very fine non-specific precipitate that could be visually differentiated from the more granular specific accumulation of reaction product by alkaline phosphatase. The major disadvantage of this technique is that due to prior fragmentation of the tissue, there was little orientation of ultrastructure within a sample. Records of which area of tendon in relation to the calcific deposit sampled and whether or not calcific deposits were situated in the same section were important for identifying the relative location of precipitates.

In figure 2.2a where a dark reaction product was demonstrated, the cells were not tenocytic nor chondrocytic, but appeared to be endothelial or synovial in morphology. Areas of this specimen did demonstrate resorptive features. The area was highly cellu-

lar and not tendinous in nature, although collagen bundles were apparent between some cells (see chapter 1). These cells, although demonstrating some reaction product, cannot be described as rich in AP when compared to the myositis ossificans tissue (figs: 2.5 b and c). On the observed evidence it seems unlikely that this activity is initiating calcification.

In figure 2.3 d the tissue is necrotic and AP activity is associated with cell debris. Tissue necrosis, was initially believed to be the mechanism of calcifying tendinitis. This would be a self-perpetuating process as the gross deposition of mineral in the shoulder could cause mechanical damage accompanied by chemical toxicity due to the high levels of calcium in the tissue and perhaps enzymatic attack due to the release of cytokines and lymphokines from necrotic cells. Arm movement could cause further necrosis and associated AP activity. The resulting release of calcium ions due to the destruction of mitochondria and cellular damage has been considered as a possible initiating factor for ectopic calcification (see chapter 1). As it has already been concluded by Kim (1983) that enzyme activity (e.g.alkaline phosphatase) is not required for the calcification of cell debris. That the phospholipid composition of the extracellular membranes is sufficient cause to initiate calcification. Any associated alkaline phosphatase activity would be an extra factor to promote mineralisation.
It would be instructive if further studies into the pathological mechanisms of calcifying tendinitis incorporated data relating to enzymes associated with cell death, eg: acid phosphatase, ATPase or endonuclease. Micrograph 2.2b typifies the region where crystal is sparsely scattered. Here the mineral is partially intracellular which is more likely to be due to resorption than mineral production. There is minimal or no alkaline phosphatase activity associated with areas that contain mineral.

In figure 2.3c a chondrocyte-like cell with mineral close by, similar to those described by Uhthoff 1975, is seen. Whilst the cell processes are darker than the cytoplasm indicting that the AP is concentrated in membrane budding away from the cell, Uhthoff's observations from proximal to the site of calcium deposition, that the chondrocyte-like cells were hypertrophied and elaborated alkaline phosphatase, are not sub-

stantiated by this study. However, Uhthoff used the Gomori method (1952) for demonstrating this enzyme, a technique which is known to give a false positive reaction if calcium is already present in the tissue (Drury and Wallington, 1980).

Tenocytes observed in tendon that was removed from an apparently normal area do not have any enzyme activity associated with the outer cell membrane. However, the fine precipitate associated with normal organelles such as lysosomes and mitochondria has made them into sharply defined cytoplasmic entities (fig: 2.3 a and b). This did occasionally occur in the levamisole control specimens and therefore should be regarded as non-specific lead precipitate.

There is no doubt that AP is involved in the initial mineral formation in matrix vesicles. Matrix vesicle mediated mineralisation has been reviewed by Ali (1983), Anderson (1985) and Wuthier (1985). Although occasional vesicular structures can be seen in the collagenous matrix of our samples (chapter 1) these were not associated with alkaline phosphatase activity unlike the reaction product demonstrated in maturing cartilage when incubated with the same medium (Lewinson et al, 1982). Matrix vesicles were found in the pathological tendon but the distribution of those present appeared to be unrelated to the distribution of calcific nodules. It is, therefore, improbable that matrix vesicles are the principal initiation mechanism for calcification in calcifying tendinitis, unless it is accepted that the formation of deposit is complete in all the patients studied or that our ultrastuctural sampling was not from the correct regions of tendon to identify the on-going process.

Interesting comparisons can be made by observing the ultrastructural detail in myositis ossificans specimens treated in exactly the same manner. The mechanism of mineralisation in myositis ossificans is not just endochondral ossification although areas of calcified cartilaginous tissue may be observed (see chapter 1), but areas of osteoid compatible with a membrane bone type mineral formation can also be observed. Here, the elaboration of alkaline phosphatase into the matrix was apparent and caused the collagen fibrils to become negatively stained by virtue of being comparatively electron lucent. The cells of the osteoid layer did not demonstrate intracellular alkaline

phosphatase activity which suggests that the enzyme is no longer being produced by the cell and the enzyme has been pushed out into the surrounding matrix now rich in enzyme activity. Alternatively, this could be a diffusion artifact. If the limiting component is the quantity of enzyme substrate present in the incubating medium, this may have been used in the matrix reaction thereby not leaving any substrate, or not allowing substrate to remain in solution for long enough, to penetrate the cells.

The observation of a seam directly adjacent to the pre-mineralising front that does not demonstrate AP activity (fig: 2.5c) supports the observations from endochondral calcification of Morse and Greep (1951). This seam of inactivity could be due to product inhibition of the AP by inorganic phosphate from the mineralising front. Thus, the enzyme would be present in this inactive seam, butbe fully inhibited by the presence of phosphate. Alternatively, the rate determining step of the enzyme activity is the second part of the reaction mechanism, so the enzyme may already be active to maximum capacity and, therefore, unable to further precipitate histological substrates. Immunocytochemistry could resolve this issue further as the antigenic determinant should still be localised in the inactive seam if the enzyme were present, even in a latent form.

There is support for the findings of Majno and Rouiller (1951) who described osteocytes enclosed within bone, still associated with alkaline phosphatase, but as the bone substance encloses them they become less active as they get more deeply embedded (see fig: 2.5c). Also, away from the mineralised region but still within the same section, cells have been found to possess enzyme rich nuclei, which unlike the normal tenocytes do not have the fine non-specific precipitate associated with organelles (fig: 2.5a). These could be equivalent to the cells of the endochondral cartilage proliferative zone described by Morse and Greep (1951).

The calcifying tendinitis specimens contained significantly less AP activity than myositis ossificans specimens whilst the controls were virtually clear of granular reaction product. In the levamisole inhibited samples the non-specific reaction was sparse even in the phosphatase-rich myositis ossificans specimens. The nature of any background deposit differed by being more diffuse, as shown (fig: 2.4b) in a micrograph

that represents the maximum reaction observed amongst control specimens. This is consistent with the inhibition of AP by levamisole, indicated by the lack of reaction product produced by the enzyme action.

In conclusion studies conducted here have demonstrated that if calcium deposition was being initiated at different focal points, at the time of sampling, with associated low levels of AP activity then the association of this enzyme was mainly with fragments of cell debris. It is highly unlikely that AP activity can be considered to be a major factor influencing the progress of the disease once initiated.

CHAPTER THREE.

IMMUNOLOCALISATION OF MATRIX MACROMOLECULES.

IMMUNOLOCALISATION OF MATRIX MACROMOLECULES

3.1 INTRODUCTION

This investigation compared some of the matrix components found in calcifying tendon to those located in normal tendon and some components of human articular cartilage. By establishing which components are specific to the pathological matrix, evidence can be accumulated about the nature of changes that have occurred. Hence, the presence of a range of macromolecules was evaluated by immunolabelling techniques in both the non-pathological and diseased tendon samples. Human cartilage was used as a positive control tissue, since locating cartilage-specific proteins could lend support to the theory of chondrogenic modulation in pathological tendon (Uhthoff 1975).

Collagen type II is found normally in cartilage and in the vitreous humour of the eye. However, it is synthesised by numerous cell types in the developing embryo. The abundant presence of this molecule in the pathological tendon would support the proposed mechanism of chondroid metaplasia (Uhthoff, 1975).

Collagen type VI is a matrix component of cartilage and other connective tissues, but has not yet been demonstrated to be present in normal human tendon nor has it been associated with pathological tendon. Collagen type VI is not a marker for chondrogenesis but was of specific interest as it has been implicated in various disease processes including tumour pathology (see Keene et al., 1988).

Hyaluronan binding region (BR) is a molecule attached to proteoglycan core proteins that facilitates the formation of aggregates in the presence of hyaluronan. The aggregation of proteoglycans improves water retention by the tissue and so might be expected to be found in in connective tissues that experience compressive loading such as cartilage. To date, there are no references available associating this molecule with human tendon, however, Scott et al. (1981) find that both chondroitin sulphate and hyaluronan preponderate in foetal rat tail tendon. A study comparing proteoglycan synthesis by proximal and distal regions of bovine flexor tendon (Koob and Vogel, 1987), demonstrated a

concentration of larger proteoglycans in the distal region of the tendon, an area normally subjected to compressive loading.

Sulphated glycosaminoglycans are side chains of proteoglycan molecules and components of both cartilage, bone and all other tissues. Chondroitin sulphate inhibits calcification of bone formed in vitro (Tenenbaum and Hunter, 1987) however, it is not known whether it prevents mineralisation in vivo.

Chondroitin has been found in cartilage of calluses formed during fracture healing (Page and Ashhurst, 1987). The antibody (1B5) recognises delta unsaturated disaccharides of chondroitin or hyaluronan occurring due to digestion with chondroitinase or streptomyces hyaluronidase Caterson et al. (1985).

Chondroitin-4-sulphate is generally abundant in embryonic tissues and probably those undergoing repair. The proteoglycans of bone matrix are small and contain mostly chondroitin-4-sulphate chains (Page and Ashhurst, 1987).

Chondroitin-6-sulphate molecules are side chains of the largest proteoglycans, which are normally abundant in adult cartilage. The proteoglycans in cartilage, fibrous tissue and cavities of cancellous bone are large and have chondroitin-4-, chondroitin-6- and keratan sulphate chains dependant on species (Page and Ashhurst, 1987).

Chondroitin-6-sulphate (non-digested sections). The antibody (3-B-3) that recognises chondroitin-6-sulphate in normal cartilage will also recognise a chondroitin-6-sulphate epitope which has a glucuronic acid terminal sequence, in the absence of chondroitinase pre-digestion of the tissue. It thus represents a novel native epitope and has been found preferentially in osteoarthritic cartilage (Hardingham et al., 1989).

By investigating the absence or presence and the distribution of these molecules in the tendon, more information can be derived about the pathological conditions surrounding the calcific deposits and may throw some light as to the pathological mechanisms which lead to crystal formation.

3.2 METHODS

Three immunolabelling methods were used in this investigation. Immunofluorescent labelling, immunoperoxidase labelling and at the ultrastructural level, immunogold labelling.

Frozen Tissue.

The tissue sections were taken from both normal and pathological regions of tendons from patients G and H (see table 2.1, samples studied for light microscopic histochemistry). Non-pathological achilles tendon, specimens from three regions of supraspinatus tendon taken at post-mortem, and normal human articular cartilage were also sectioned and used as controls. Each piece of tissue was orientated on a disc of cork in cryomountant and immersed in liquid nitrogen for rapid freezing. The blocks of tissue and later, the 7μm thick cryostat sections on glass slides were well wrapped in parafilm or clingfilm and stored at -70°C.

Pre-treatment of tissue sections.

(For pretreatment rationales, see page 98).

To expose selected epitopes some cryosections were pre-digested with hyaluronidase (1.45 iu/ml) or chondroitinase ABC (0.25 iu/ml) or a hyaluronidase (1.45 iu/ml) and chondroitinase ABC (0.25iu/ml) cocktail diluted in phosphate buffered saline (PBS) for 45 min at 37°C. For collagen type II and some collagen type VI experiments, a bacterial collagenase (Worthington) digestion (0.6 iu/μl) for 20 min at 37°C was used instead of the other digestion procedures to try partially digesting the collagen structure to expose epitopes that might otherwise be concealed. All the subsequent steps were carried out at room temperature whilst sections were contained within a light-tight, wet box.

Immunofluorescence techniques on cryostat sections.

After digestion and after each incubation step the sections were washed in 3 x 10 min. changes of PBS, pH 7.4. The primary antiserum was diluted with PBS, to the required concentration (see table.3.1), the sections were flooded and incubated for 45 min at room temperature in the wet box. Goat anti-primary polyclonal serum IgG rhodamine-conjugate (Dakopatts) was diluted to 1/40 in PBS. For monoclonal primary antisera

For further details about the antibodies used in these immunolocalisation studies, see appendix three (in the back pocket).

TABLE:3.1

PRE-DIGESTION OF TISSUES, ANTIBODY DILUTIONS AND LABELS USED.

MOLECULE ON WHICH THE EPITOPE IS LOCATED.	PRE-DIGESTION	PRIMARY ANTIBODY & DILUTION	LABEL USED
COLLAGEN TYPE II	ChondroitinaseABC and Testicular hyaluronidase Cocktail; Or Collagenase	Monoclonal 1/100 (C1C11)	Rhodamine & Peroxidase
COLLAGEN TYPE VI	Testicular hyaluronidase collagenase	Polyclonal 1/500 (BR)	Rhodamine, Peroxidase & Gold particles
HYALURONAN BINDING REGION PROTEIN	Chondroitinase ABC	Polyclonal 1/25 (VI.A)	Rhodamine & Peroxidase
PROTEOGLYCAN CHONDROITIN SIDE CHAIN	Chondroitinase ABC	Monoclonal 1/100 (1-B-5)	Rhodamine
PROTEOGLYCAN CHONDROITIN - 4-SULPHATE SIDE CHAIN	Chondroitinase ABC	Monoclonal 1/100 (2-B-6)	Rhodamine
PROTEOGLYCAN CHONDROITIN -6-SULPHATE SIDE CHAIN	Chondroitinase ABC	Monoclonal 1/100 (3-B-3)	Rhodamine
PROTEOGLYCAN CHONDROITIN -6-SULPHATE SIDE CHAIN	No Pre-digestion	Monoclonal 1/100 (3-B-3)	Rhodamine

Pretreatment rationales.

Collagenase digestion was used to break up the collagen molecules in an attempt to expose antigens that might otherwise have been concealed.

Hyaluronidase digestion was used to disorganise the tissue surrounding collagen fibres, hopefully aiding antibody penetration and access to the epitopes.

Chondroitinase digestion was necessary to generate the antigenic determinants where the epitope was integral in the proteoglycan structure.

* Blocking serum is normal serum of the host species in which the secondary antibody was raised. This is applied to inhibit highly charged tissue components from non-specifically binding antibodies.

rabbit anti-mouse IgG was diluted to 1/40 in PBS. Sections were flooded with this for 45 min. at room temperature in the dark.

Control sections were treated in the same manner as the test sections except for the incubation with primary antibody where non-immune serum diluted 1/20 in PBS was substituted instead. In the collagen type II experiments a pre-absorbed antiserum control was also used.

Sections were mounted under glass cover slips in glycerol/PBS (1:9) containing 1,4-diazobicyclo [2.2.2.] octane (DABCO 25mg/ml supplied by Sigma) as this has been found to reduce fading during fluorescence microscopy (Johnson et al., 1982). Sections were then photographed on Ilford HP5 film using a Ziess Photomicroscope III with an epifluorescence attachment.

Peroxidase anti-peroxidase immunolabelling on cryostat sections.

Specific tissue digestion procedures for each epitope to be localised (table 3.1) were carried out. All incubation steps were carried out in a wet box at room temp.and at no time were the sections be allowed to dry. Sections were first treated with methanol containing 0.3% hydrogen peroxide (Sigma) for 10min, then washed in PBS, 3 x 10min..

*Blocking serum was applied for 15 min.; normal goat serum was used as blocking serum in the localisation procedure for a polyclonal primary antiserum and normal rabbit serum for a monoclonal primary antiserum, both diluted 1/ 20.

Specific primary antiserum or control antiserum was diluted in PBS (see table 3.1) and applied for 45min, sections were then washed in PBS, 4 x 10min. Control serum was non-immune serum from the same animal species as the primary antiserum used, diluted 1/20 in PBS or pre-adsorped antiserum in the case of collagen type II.

The bridge antibody was goat anti-rabbit IgG (Sigma no. R-2004) for a polyclonal primary antiserum and rabbit anti-mouse IgG (Sigma no. M-9637) for monoclonal primary antiserum, both were whole molecule preparations diluted to 0.2mg/ml in PBS and applied for 45min. Sections were then washed in PBS, 4 x 10min.

Peroxidase anti-peroxidase (PAP) was diluted to 40µg/ml in PBS and applied for

45min. This was PAP labelling antibody (Sigma no. P-2026) for polyclonal anti-rabbit bridge antibody and (Sigma P-2416) for monoclonal anti-mouse bridge antibody. Sections were then washed as described above.

To obtain a colour reaction 0.5g/l diamino-benzidine in PBS with 0.5ml/l of 30% stock hydrogen peroxide was filtered and used fresh to flood on to the sections. Staining took about 1min. and the reaction was stopped by washing with tap water. Some sections were counterstained with haematoxylin prior to dehydration and mounting in DPX. Sections were viewed and photographed on a Ziess photomicroscope III.

Cryo-ultramicrotomy and immunogold labelling for collagen type VI.

Small pieces of frozen tissue from the wall of the deposit from patient G were cut to a size of about $1mm^3$ and fixed with 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2 at 4°Cfor 6 h. These were then placed in 2.3M sucrose dissolved in 0.1M phosphate buffer and infused at 4C for 24 h, during this time the tissue pieces sank through the sucrose indicating that infiltration was adequate to cryoprotect the specimen.

Cryosections 100nm thick were cut on a Reichert ultracut E* with FC4 cryoattachment* and were placed on pioloform coated, 200 mesh nickel grids and then floated, sections downwards, on PBS containing 0.5% BSA and 0.05% Tween 20 (PBT). The sections were stored floating on buffer, at 4°C, overnight. PBT was used for washing the sections and diluting antisera during immuno-labelling. The grids were floated on drops of PBT and then transferred to rabbit anti-collagen type VI polyclonal serum diluted 1/500 and 1/1000 for 1 h at room temperature. This was followed by 3 x 10 min. washes on PBT. Protein-A 15nm gold conjugate was diluted to 1/50 and sections were floated on drops of this for 2 h at room temp. Grids were washed for 3 x 10min. in PBT followed by 3 x 10min. on millipore filtered, distilled water. Control sections were treated in the same way except that they were incubated on drops of PBT not collagen type VI anti-serum. Contrast was added to the sections by floating grids on 2% aqueous neutral uranyl acetate for 5 min at room temp, the grids were then placed on drops of 2% polyvinyl alcohol and dried in a wire loop. Sections were examined on a

Philips CM12. T.E.M.

* Equipment that was lent to this laboratory for a limited period by Cambridge Instruments Ltd.

Pre-embedding labelling for collagen type VI.

Cryostat sections approximatly 7µm thick were cut (from the wall region of patient G), and were stored for up to several months at -70°C. When required, sections were fixed in 0.5% glutaraldehyde and 2% paraformaldehyde for 2 h. The sections, whilst in fixative were scraped from the glass slides with a razor blade and floated in fixative, in small Petri-dishes. The subsequent immuno-labelling was performed in these dishes. Sections were washed in several changes of PBS before removal of all the fluid and drying the dishes around the sections, prior to immersing the sections in drops of hyaluronidase/chondroitinase cocktail (see pre-treatment of tissue sections) and incubating at 37°C for 30 min. The sections were then washed in several changes of PBS over 3 h, before again drying the main area of the dish (surrounding the section) and incubation of sections in small drops of rabbit anti-collagen type VI serum diluted to 1/250 in 2% BSA-PBS, overnight at room temperature. The sections were washed in PBS six more times over 8h before incubating overnight at room temperature in buffer containing 5nm gold particles conjugated to goat anti-rabbit serum (Janssen pharmaceutica) and diluted 1 in 10 in PBS. The sections were then washed every hour with PBS over 3h before dehydration through an alcohol series (see chapter one), transferring out of plastic dishes into 2ml eppendorf tubes before further dehydration with propylene oxide, and infiltration with resin (Araldite CY212, Agar Scientific). The resin was placed at 65°C for 5min before pelleting the sections using a micro-centrifuge at 8500g for 5 min. The blocks were then polymerised by incubating at 65°C for 18h.

Sources of primary antisera.

The collagen type VI polyclonal antiserum was a gift from Shirley Ayad at Manchestter University. The collagen type II (C1C11) antiserum came via Dr. Charles Archer, from the Developmental Hybridoma Bank, University of Iowa, USA. Hyaluronan binding region protein, chondroitin (1-B-5) , chondroitin-4-sulphate (2-B-6) and chondroitin-(6-

sulphate (3-B-3) antisera were all donated by Dr. Mike Bayliss (Kennedy Institute, London) and the latter three came originally from Dr. Bruce Caterson, Chapel Hill, North Carolina, U.S.A.

3.3 RESULTS

The presence of strong or weak immunolabelling patterns or the absence of detectable antibody binding on the tissues used in this study have been summarised (table 3.2). The immunofluorescence technique gave some variable and inconsistant results as well as convincing data. No evidence for the presence of collagen type II was found. However, when trying to demonstrate the presence of collagen type II, a low-dose collagenase digestion was adopted in an attempt to improve access to epitopes that may have been masked. Again, fluorescence was not observed.

In collagen type VI and collagen type II studies, a distinctive pattern was seen in both test samples and in the non-immune serum control of the mineralised tendon sections (fig:3.1a-d). Digestion with a chondroitinase/hyaluronidase cocktail, then later bacterial collagenase, proved that the fluorescence pattern obtained was artefactual and made re-assessment of previous results necessary, since similar fluorescence was observed when looking for other antigens. The brightness was misleading due to the focal nature of isolated spots that were similar in size to the area expected to fluoresce from a strongly positive cell and its lacunae. This pattern was never observed in non-pathological tissue and was therefore considered to be due to the calcific deposits. Repetition of the light microscopy investigation using the permanent method of PAP had an added advantage of permitting sections to be viewed under Kohler illumination, thus allowing a more detailed examination of the features local to the regions of antibody localisation. Small dense granular structures were noted in control and test sections not associated with the main nodules of mineral but sequested within the surrounding matrix. It is these that were probably the source of the artefactual fluorescence (fig: 3.3j). Expected artifacts of autofluorescence from the bulk of mineral or adsorption of the fluorescent conjugate directly and non-specifically onto the hydroxyapatite did not interfere with

TABLE:3.2 The tissue distribution of the matrix molecules investigated.

TISSUES EXAMINED	MOLECULES RECOGNISED						
	Collagen Type II	Collagen Type VI	Binding Region	Chondroitin 0-sulphate	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Chondroitin 6-sulphate non-digested
CARTILAGE	+	+	+	-	+	+	-
NON-PATHOLOGICAL TENDON	-	+	+	-	-	+	-
		-				-	
TENDON WITH CALCIFIC DEPOSITS	-	+	+	-	+	+	-
			-				

KEY

STRONG LABELLING +

WEAK LABELLING +

WEAK LABELLING -

NO LABELLING -

the results.

Collagen type II localisation observed by both immunofluorescent and immunoperoxidase labels.

Using this monoclonal antibody (C1C11) it was not possible to find collagen type II in non-pathological tendon nor sections of mineralised tendon that contained masses of large rounded 'chondrocyte-like' cells (figs: 3.2a and b). The cartilage sections used as positive controls demonstrated the specificity of this antibody, whilst the control section that had been incubated with pre-absorbed collagen type II antibody was clear of label, apart from the articular surface where there was a strong edge effect that can be attributed to non-specific binding.

Collagen type VI localisation by immunofluorescent and immunoperoxidase labels.

This polyclonal antiserum clearly demonstrated a pericellular distribution in the articular cartilage and control sections were free of non-specific label apart from an edge effect (figs:3.3a-d). There was sparse but demonstrable collagen type VI in the achilles tendon as demonstrated by the PAP technique (fig 3.3g and h) and faint staining in the post-mortem specimens of supraspinatus tendon as shown by immunofluorescence (figs: 3.3e and f). In the pathological tendon there were cellular areas near to the deposited mineral that were clearly positive (fig: 3.3 i). Type VI collagen appeared in focal areas around groups of cells and control sections showed some PAP staining in association with densely brown oval structures (fig: 3.3j). These were assumed to be the artefacts already demonstrated by fluorescent techniques. The control picture shown is of the worst area of PAP control section staining observed with respect to this antibody (fig: 3.3j). The evidence obtained initally from immunofluorescence studies with the collagen type VI polyclonal antiserum was confirmed by applying the immunoperoxidase technique in order to avoid artefacts.

Collagen type VI localisation by immunogold labels.

The light microscopy results encouraged the ultrastructural investigation using cryosections. The results confirmed the light microscopical findings as cells within the

mineralised areas were surrounded by an electron-lucent area that proved to be positive for collagen type VI antigens by the immunogold labelling technique (fig 3.4 a and b). There was no apparent interference with the labelling pattern by the mineral in this ultrastructural study. PBS controls were clear of non-specific labelling (fig: 3.4c). However, because of the unique nature of these tissue samples, thawing of frozen tissue was necessary prior to processing for immunogold labelling, which explains the poor ultrastructural preservation of the cryosections shown.

The pre-embedding labelling method for cryostat sections, although confirming a positive result was less successful in demonstrating the distribution of antigenic determinants in the pathological tendon than those results obtained from the cryo-ultramicrotome sections (fig: 3.5 a and b).

Hyaluronan binding-region localisation by immunoperoxidase.

In normal human achilles tendon the presence of this molecule was evident and control sections showed low background labelling (fig: 3.6a and b). The distribution of this molecule was uneven in the pathological tissue but its presence was demonstrated (fig: 3.6c and d).

Chondroitin localisation by immunofluorescence.

There was no evidence to suggest that this molecule is located in pathological or non-pathological tendon. The molecule was not demonstrated to be present in adult human articular cartilage either.

Chondroitin-4-sulphate localisation by immunofluorescence.

This molecule was located in digested cartilage and gave a similar distribution pattern to those shown for chondroitin-6-sulphate in this tissue.

Chondroitin 4-sulphate was not evident in 'normal' tendon. However, in the pathological tendon there was a very strong positive fluorescent signal that bore no resemblance to the artefactual fluorescence observed earlier in the study (fig: 3.7a-d). The matrix around cells in the mineralised area appeared to be packed with antigenic determinants. Even though the cells themselves were not fluorescent, the highly cellular nature of the tissue was evident as cells were outlined by the reaction in the surrounding

matrix. This only occurred in areas around the mineral deposits.

Chodroitin-6-sulphate localisation by immunofluorescence.

Non-digested tissue: In non-digested human cartilage and in both 'normal' and pathological tendon there was no fluorescence (fig: 3.8c and d).

Digested tissue: In chondroitinase treated cartilage there was strong fluorescence from the deep zone (fig: 3.8a and b) and non-immune serum controls were negative (fig: 3.8e and f).

Fluorescence in achillies tendon demonstrated that the molecule was sparsely distributed in non-pathological tendon. The area of calcification demonstrated strong positive fluorescence that showed selected cells were forming a chondroitin-6-sulphate rich matrix. The fluorescence was in rings and loops that appeared to be about the expected size to enclose single or groups of cells (fig: 3.9a-d).

Controls exhibited the artefactual fluorescence as did the experimental sections. This could be distinguished from the authentic label because of the specific nature of the pattern observed.

The strongly fluorescent pattern in the pathological tendon is dissimilar to the diffuse spread of label observed in cartilage or throughout the non-pathological tendon.

FIGURES.

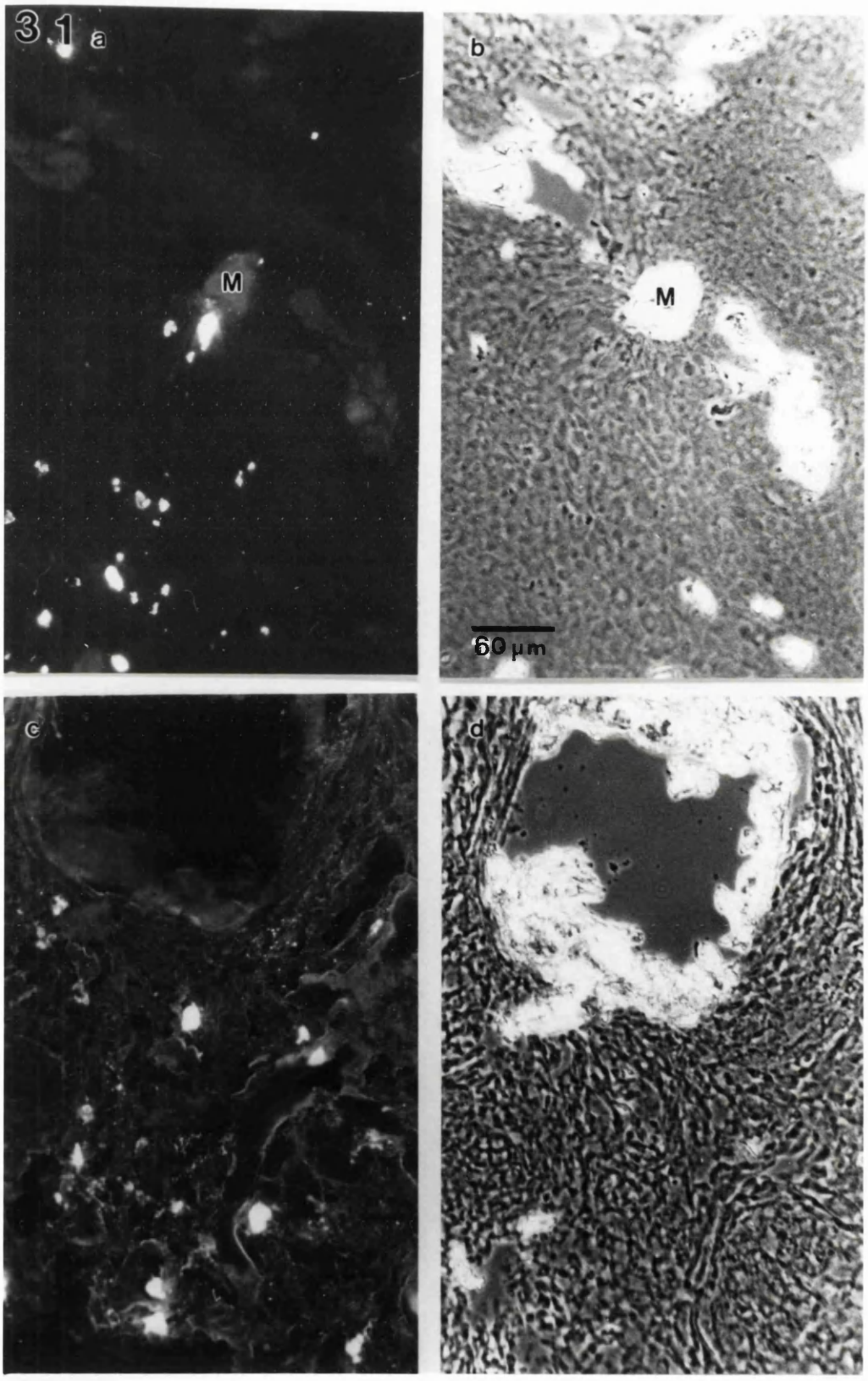
FIGURE LEGENDS

Artefactual fluorescence.

Fig: 3.1 (a and b) Artefactual fluorescence demonstrated on collagenase digested tissue sections with collagen type II monoclonal antiserum and corresponding phase micrograph. Note that the bulk of the calcific deposits (M) do not appear to fluoresce.

Fig: 3.1 (c and d) Artefactual fluorescence demonstrated on collagenase digested tissue sections with collagen type VI polyclonal antiserum and corresponding phase micrograph.

Micrographs are magnified to the same scale.


3.1 a
M
b
M
60 µm
c
d

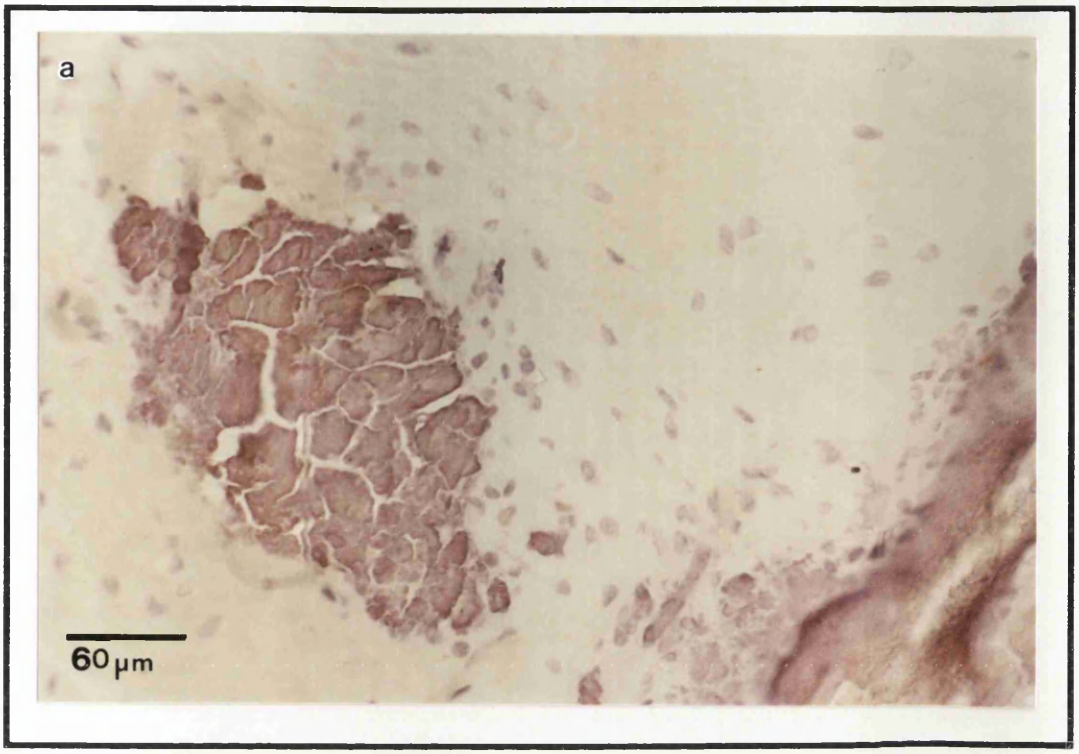
Collagen Type II

Fig: 3.2a Absence of collagen type II staining using a monoclonal antibody by PAP method in a mineralised region of pathological tendon from patient H.

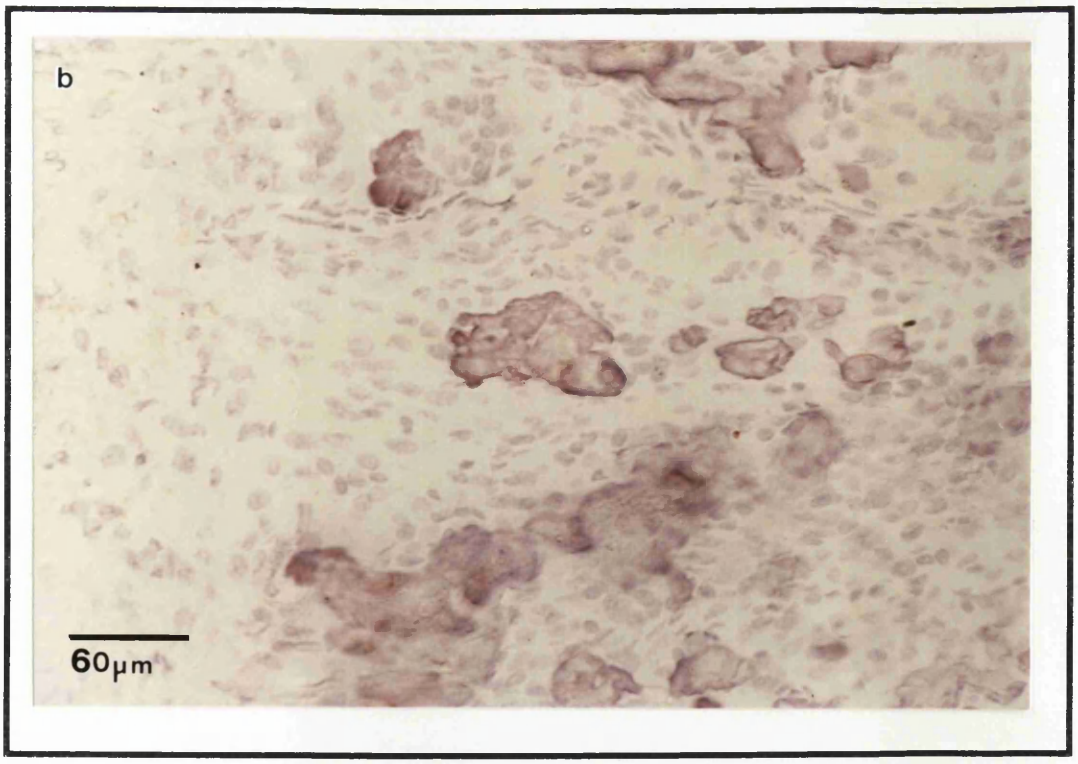
Fig: 3.2b Control for collagen type II staining using pre-adsorbed monoclonal antibody by PAP method in a mineralised region of pathological tendon from patient H.

Note the lack of reaction product in both the labelled and control sections. Micrographs are magnified to the same scale.

3.2 a.



3.2 b.

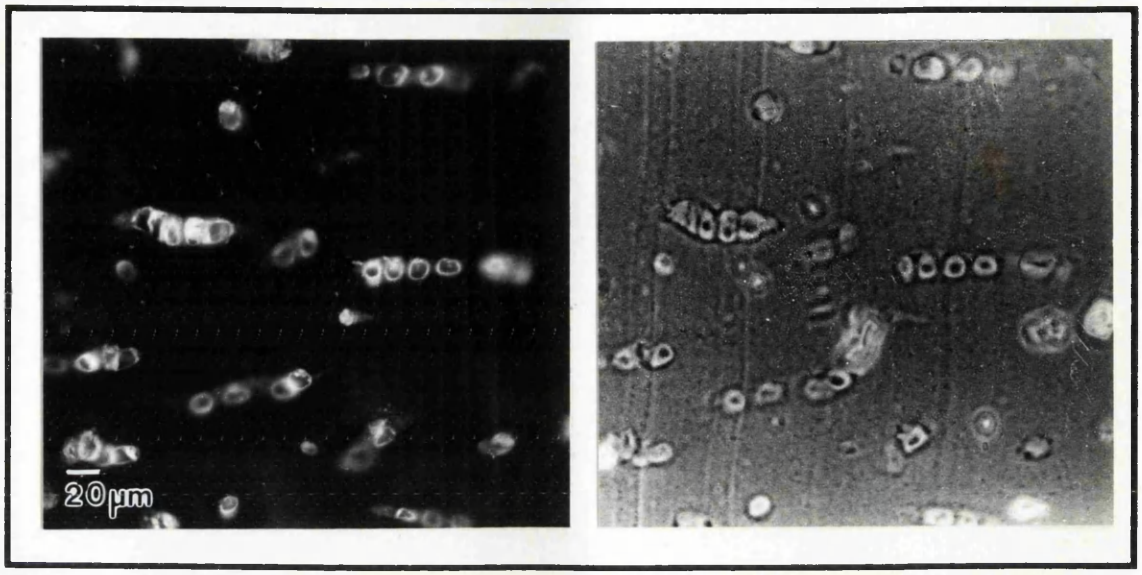


Collagen Type VI

Fig: 3.3 (a and b) Fluorescence demonstrated on human cartilage sections with collagen type VI polyclonal antiserum and corresponding phase micrograph. Micrographs are magnified to the same scale.

Fig: 3.3 (c and d) Cartilage sections with collagen type VI, PAP immunocalisation and non-immune serum control. There is brown reaction product (fig:3.3c) in the cell lacunae (arrow) demonstrating the presence of collagen type VI. Sections have been counterstained with haematoxylin. Micrographs are magnified to the same scale.

3.3 a and b



3.3 c and d.

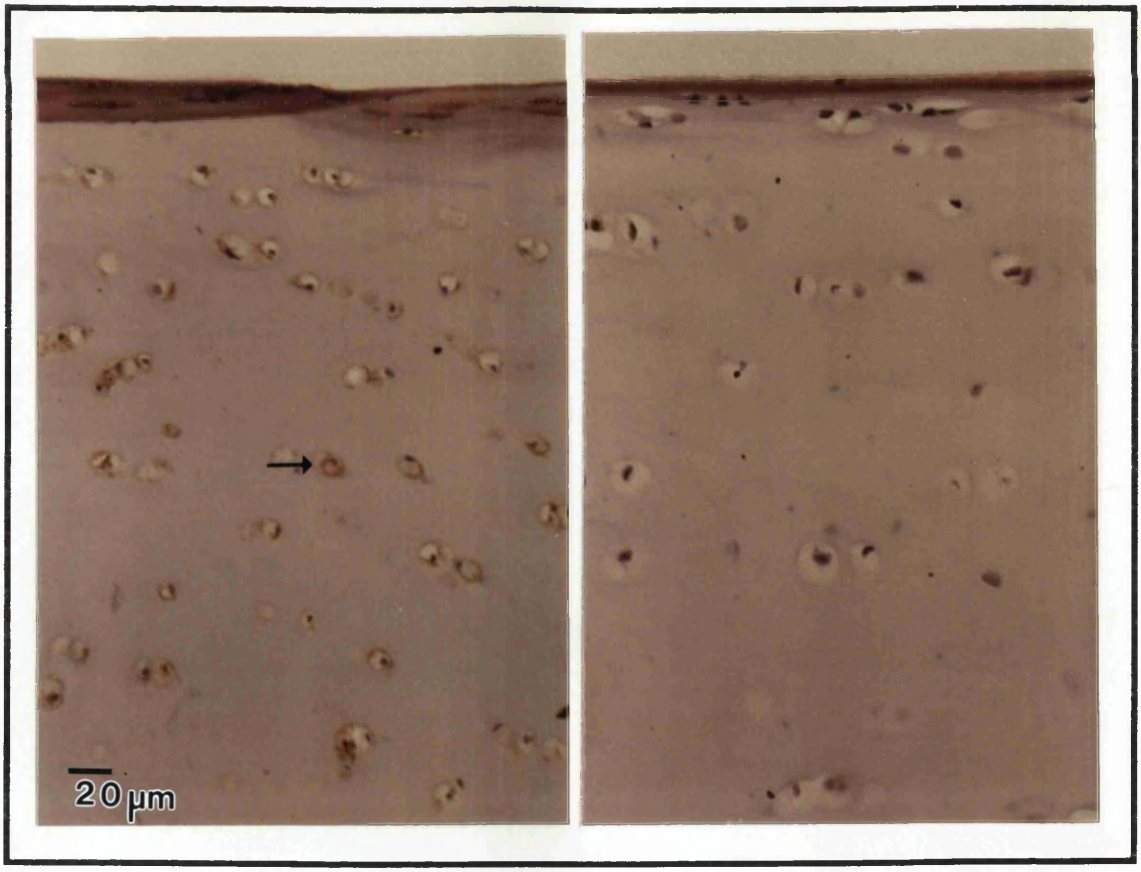
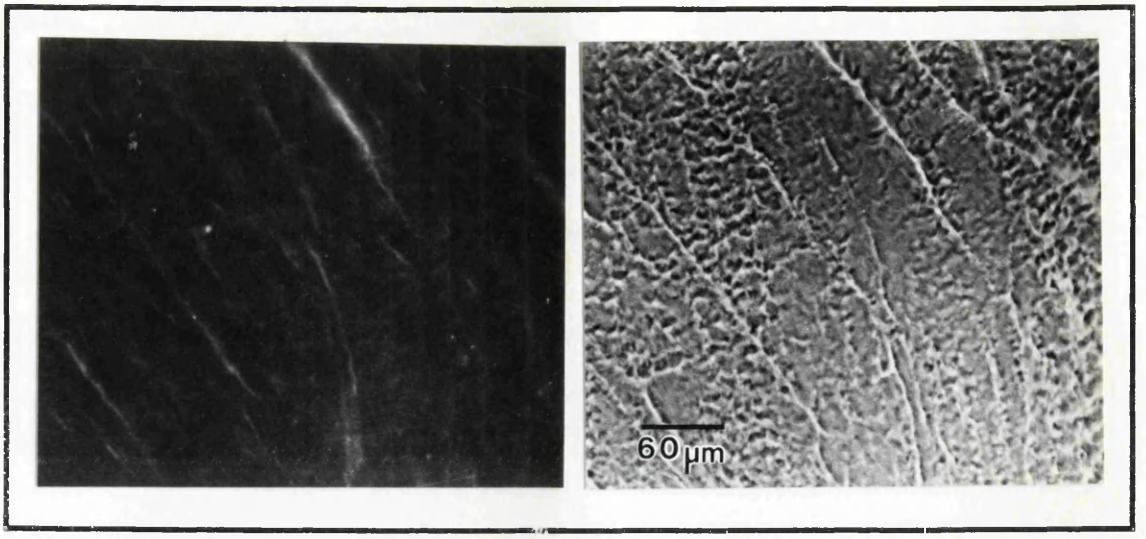


Fig: 3.3 (e and f) Immunofluorescent pattern of collagen type VI in post-mortem 'normal' supraspinatus tendon and corresponding phase micrograph.
Micrographs are magnified to the same scale.

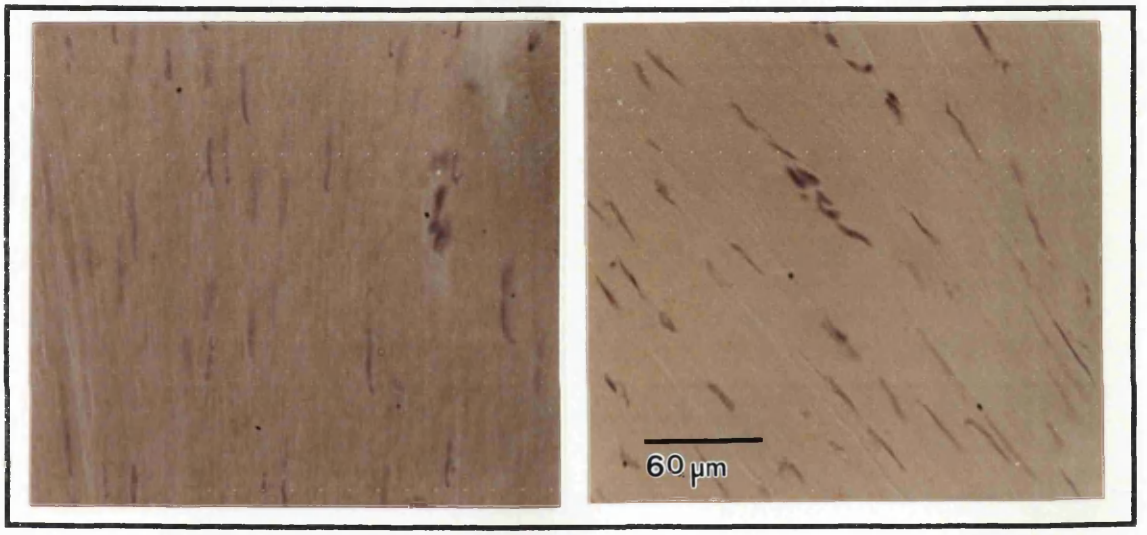
Fig: 3.3 (g and h) PAP immunolocalisation of collagen type VI in non-pathological human achilles tendon and non-immune serum control (h). Note the slight reaction product in the collagenous matrix.
Micrographs are magnified to the same scale.

Fig: 3.3 (i and j) Pathological tendon, collagen type VI by PAP and non-immune serum control (j) which is the worst area of control staining observed and demonstrates the dense ovoid structures believed to be responsible for the artefactual fluoresence (arrows) seen in figures 3.2 (a to d).
Sections have been counterstained with haematoxylin.
Micrographs are magnified to the same scale.

3.3 e and f.



3.3 g and h.



3.3 i and j.

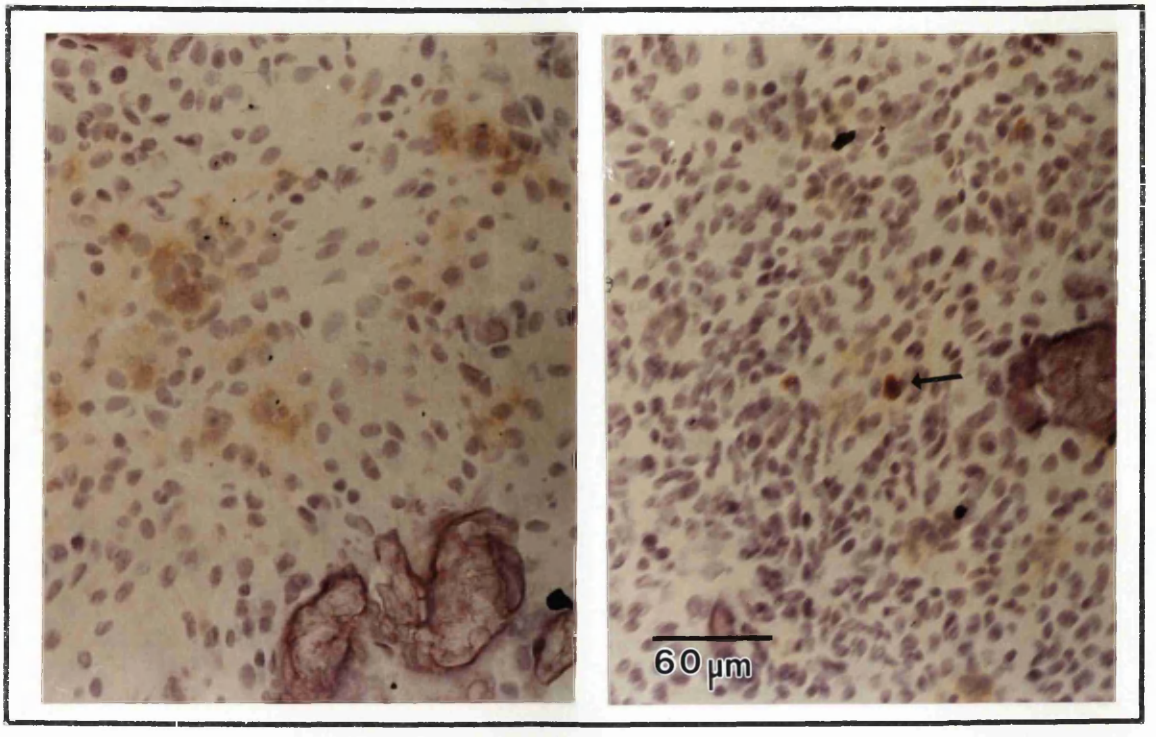
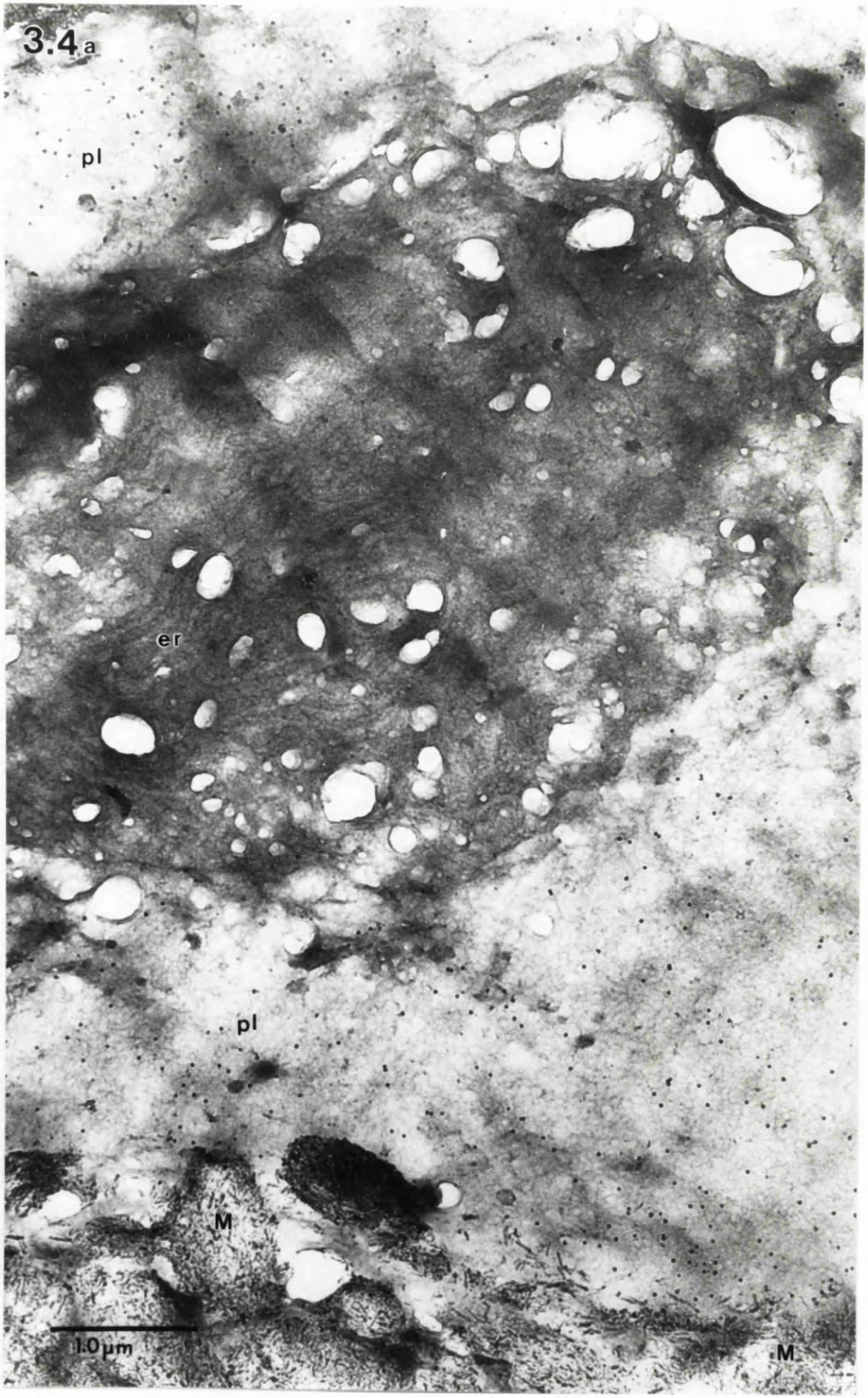


Fig: 3.4a. Micrograph of a cryosectioned chondrocyte-like cell, found in calcified tendon and labelled for collagen type VI . 15nm gold particles are most densly distributed in the pericellular lacunae (pl). There was little intracellular non-specific labelling and the antigen was more sparse in the mineralised tissue (M). Despite poor ultrastructural preservation endoplasmic reticulum (er) can be observed within the cell.

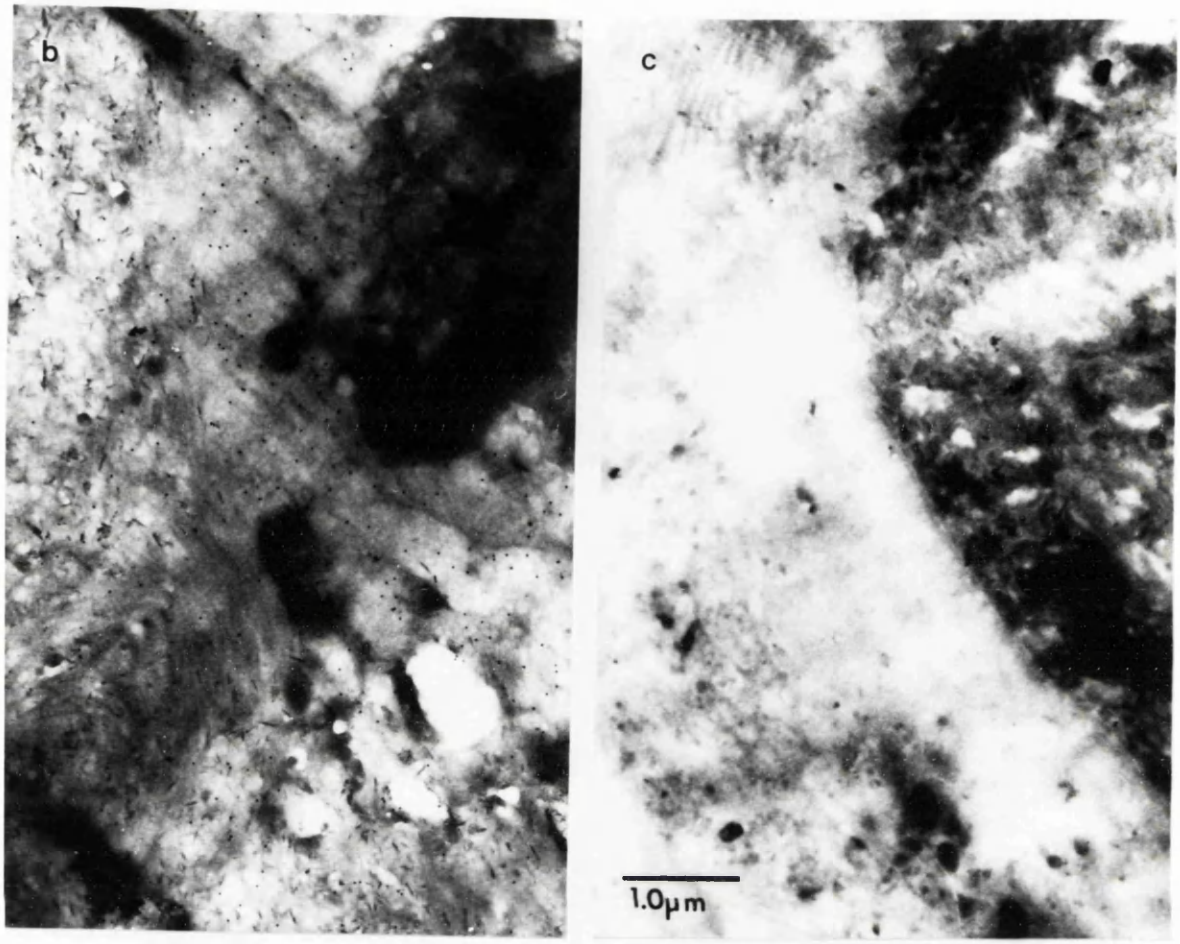

3.4a
pl
er
pl
M
1.0 µm
M

Collagen Type VI

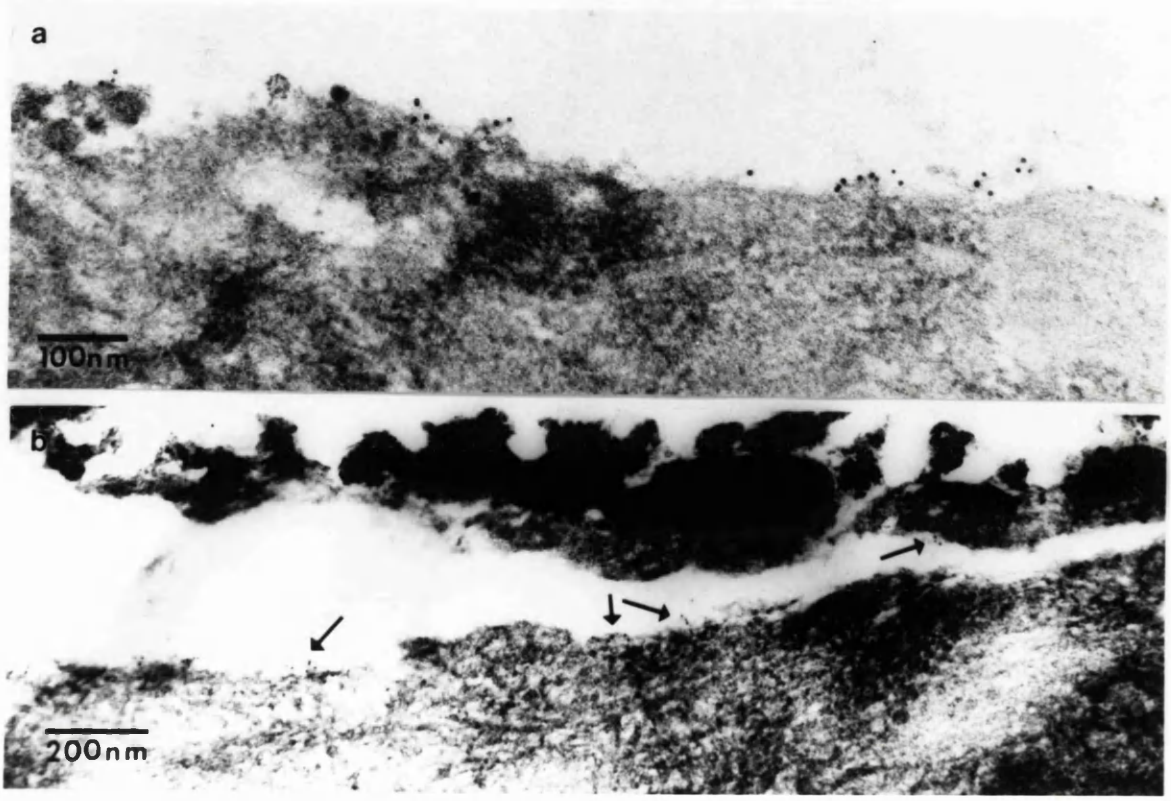
Fig: 3.4 (b and c) Micrographs of immunogold labelled cryosections of chondrocyte-like cells from calcified tendon. Labelled for collagen type VI (b) and PBS control (c). Typically the Protein-A 15nm gold conjugated markers were concentrated in the pericellular lacunae. The PBS control demonstrated little contamination of the section. Fig. 3.4c shows a similar area to those shown from the test sections.
Micrographs are magnified to the same scale.

Fig: 3.5 (a and b) Pre-embedding labelling pattern obtained with collagen type VI antiserum and 5nm GAR gold conjugates. The controls were consistantly clear of gold label. These micrographs demonstrate the most densly labelled areas of the the pathological tendon using this technique. There appears to be no penetration of label into the section. Figure 3.5b shows a split in the tissue at the mineral edge where there appears to be areas of accumulation of label (arrows).

3.4



3.5



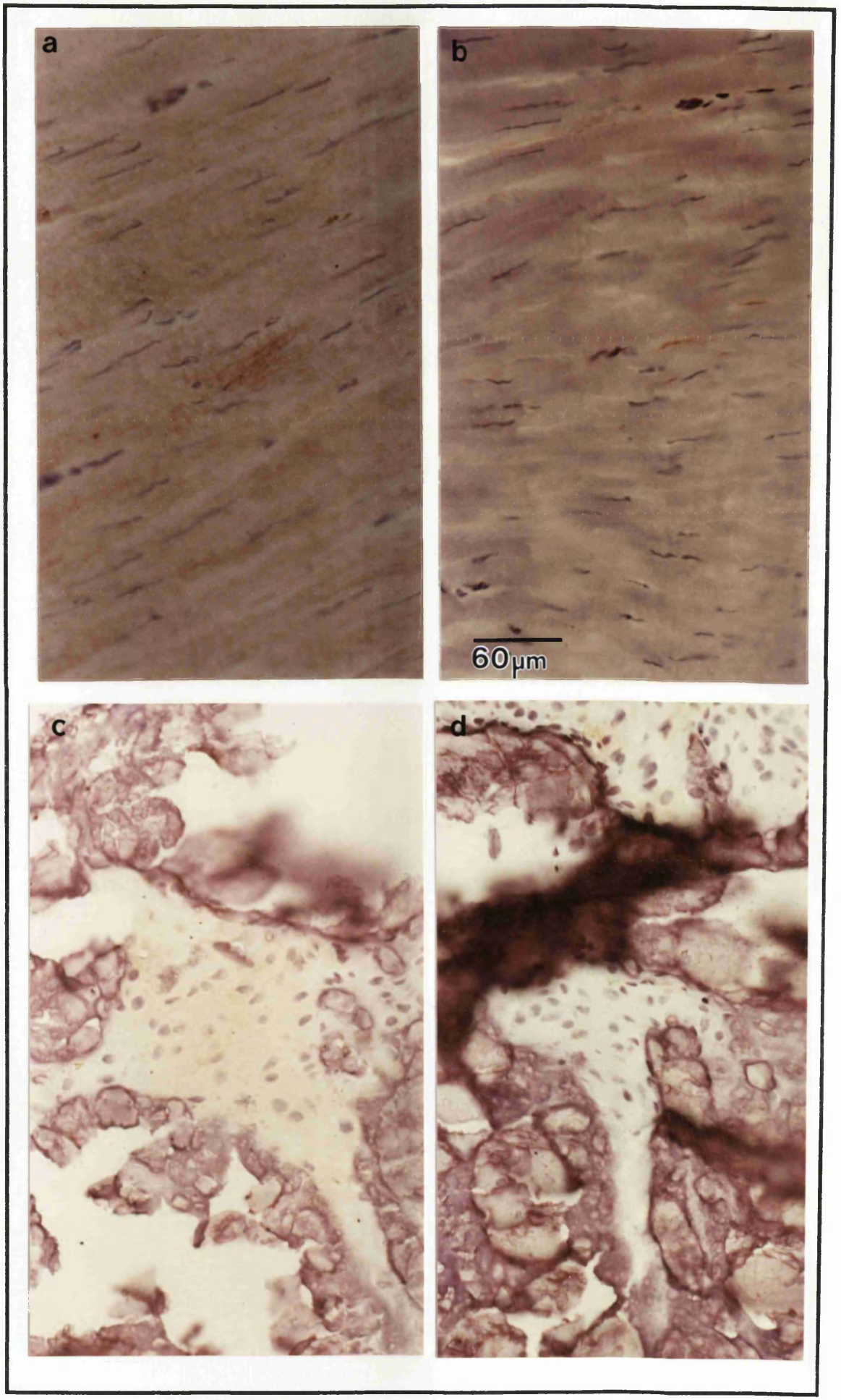
Hyaluronan Binding Region.

Fig: 3.6 (a and b) Hyaluronan binding region protein in normal human achilles tendon by PAP method and control section. Note the brown reaction product on the labelled section. Sections have been counterstained with haematoxylin.

Fig: 3.6 (c and d) Pathological tissue showing hyaluronan binding region by PAP and control. Sections are of tissue taken from patient H and have been counterstained with haematoxylin. Comparative areas are shown.

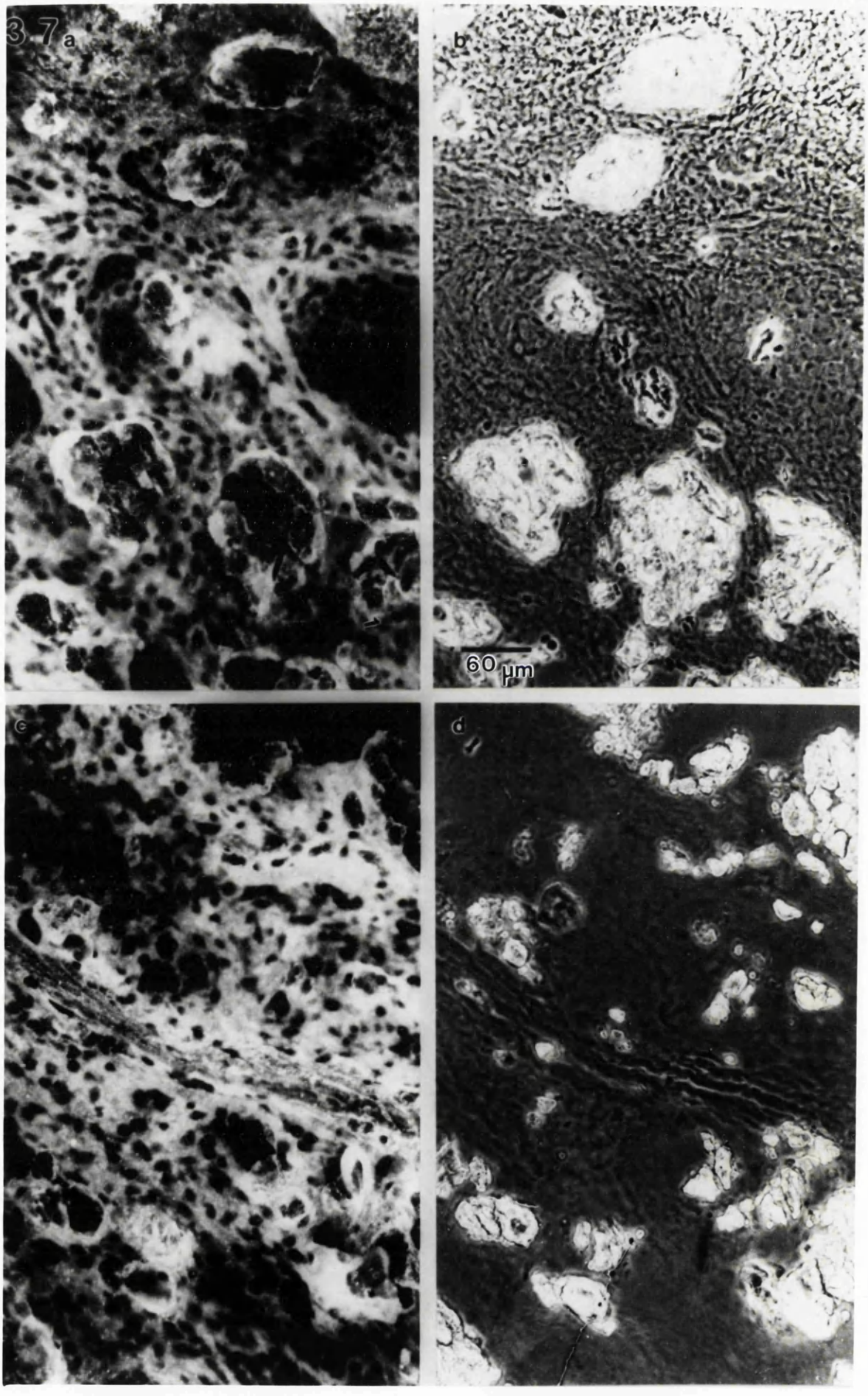
Micrographs are magnified to the same scale.

3.6 a to d.



Chondroitin-4-sulphate.

Fig: 3.7 (a to d) Fluorescence micrographs of areas of pathological tendon (patient H) and corresponding phase micrographs demonstrating the elaboration of chondroitin-4-sulphate in this tissue.
Micrographs are magnified to the same scale.


3.7a
b
60 µm
c
d

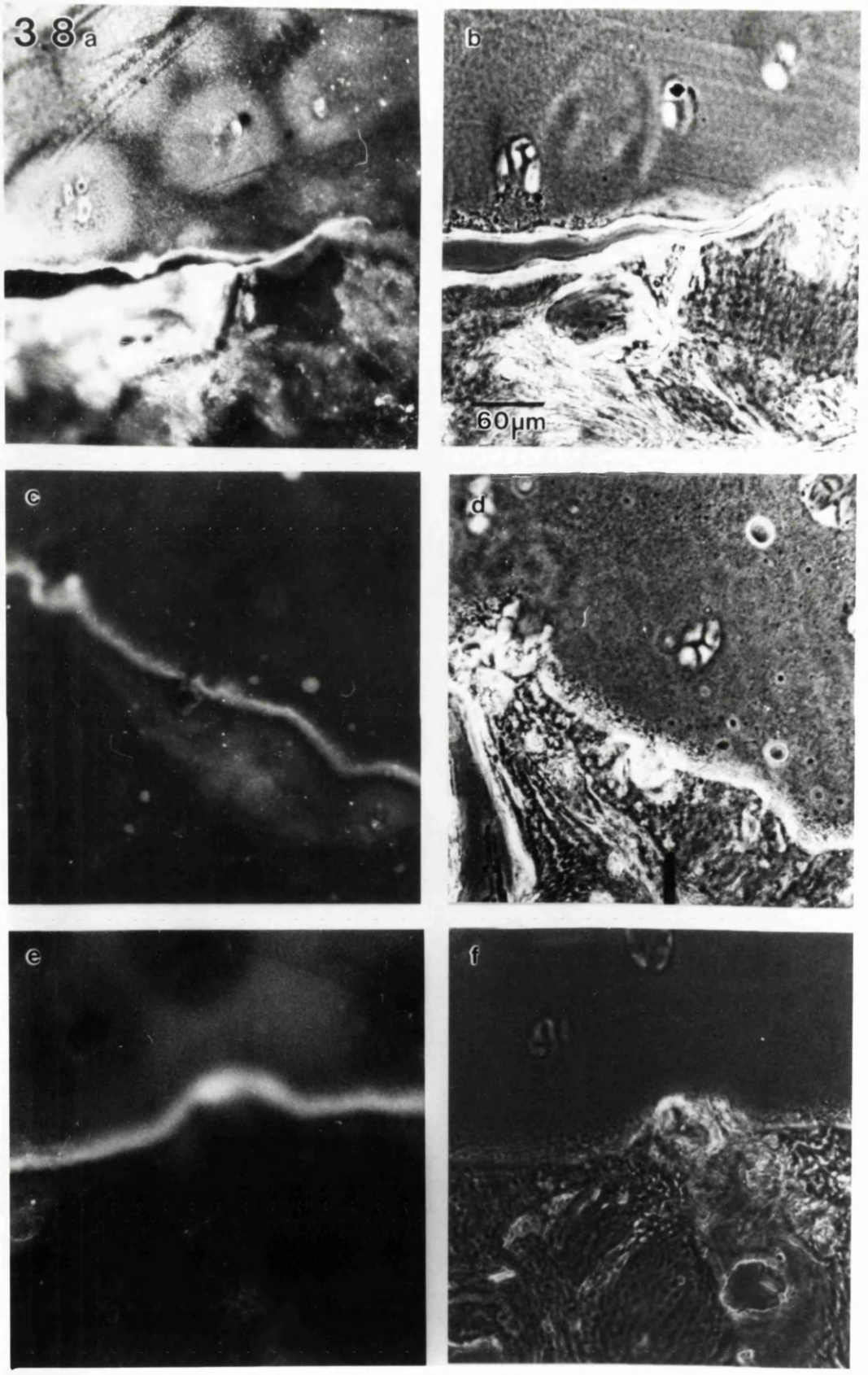
Chondroitin-6-sulphate.

Fig: 3.8 (a and b) Chondroitinase digested cartilage and corresponding phase micrograph showing the normal distribution of chondroitin-6-sulphate in human cartilage.

Fig: 3.8 (c and d) Non-chondroitinase digested cartilage and corresponding phase micrograph.

Fig: 3.8 (e and f) Control section, chondroitinase digested then incubated with non-immune serum and corresponding phase micrograph.

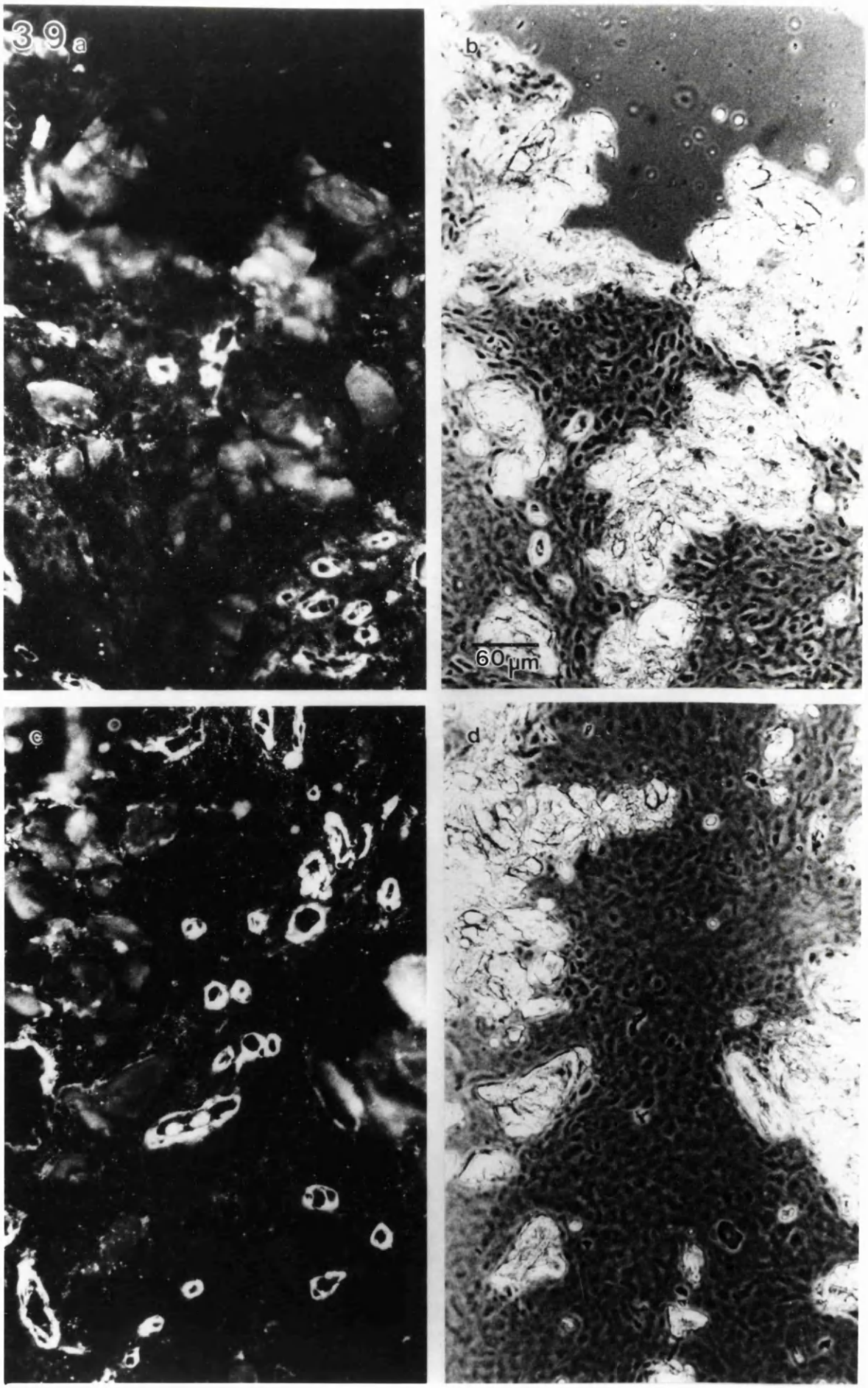
Micrographs are magnified to the same scale.


3.8a
b
60μm
c
d
e
f

Chondroitin-6-sulphate.

Fig: 3.9 (a to d) Micrographs of areas of pathological tendon (patient H) and corresponding phase micrographs demonstrating the pericellular distribution of chondroitin-6-sulphate in this tissue.

Micrographs are magnified to the same scale.


39a
b
60 µm
c
d

3:5 Discussion

Immunofluorescence proved to be a useful and reliable technique where a strong positive reaction was obtained or where a completely negative result was found in non-mineralised tissue. The plus/minus results given on table 3.2 gave a less clear-cut pattern of antigen distribution in the relevant tisssue suggesting that small amounts of the epitope were probably present. In calcified tendon the initial results using this technique proved misleading, however, by using the immunoperoxidase system, satisfactory evidence was obtained, to show that the non-specific glow from dense bodies in the mineralised matrix was artefactual. Ignoring these highly fluorescent structures (fig: 3.1) the technique could still be applied and interpretation of specific and very different fluorescent patterns was possible.

It is possible that these artefactual structures are small newly formed calcospherites that absorb protein (in this case the fluorescent marker) onto their surfaces. Perhaps the larger deposits were formed sometime before these and their exposed surface is already well coated with protein. Garnett and Dieppe (1990) suggest that hydroxyapatite crystals selectively absorb serum proteins onto its crystal surface and further show that serum components, such as human albumin, have an inhibitory affect on seeded HA crystal growth (Campion et al. 1990; Garnett and Dieppe, 1990). However, in an avascular tissue there could be depletion of inhibitory molecules within the calcifying matrix formed by the delay between formation of the deposits and the seepage of proteins through the tissue to coat the mineral from the nearest blood vessels. This might account for the very small deposits becoming highly fluorescent. Unlabelled cryosections of mineralised areas of tendon were observed in the fluorescent microscope to confirm that this artefact was not autofluorescence.

Collagen type II immunolocalisation.

This monoclonal antibobody was made and characterised by Holmdahl et al.in 1986. The main structural component of tendon is collagen type I. Although there is no reason to expect to find collagen type II in normal human tendon, if chondrometaplasia

does occur and gives rise to tendon calcification, then collagen type II produced by chondrocytes should be evident around or within the mineralised matrix. In fact, no evidence was found for the prescence of collagen type II by cells in the observed areas (fig: 3.2). This was the conclusion after a detailed examination of cryosections from patients G and H. Although these sections included both fibrocartilaginous and inflammatory areas, data from many more patients are needed before assuming that calcifying tendinitis never occurs via a fibrochondrogenic modulation. If type II collagen had been produced prior to calcium deposition it would be reasonable to assume that some residual type II collagen would still be within the tissue. Collagen types I and II do occur in hypertrophic growth cartilage of the chick prior to calcification (von der Mark and von der Mark, 1977; Oshima et al., 1989) and also where there is chondroid tissue that never ossifies in normal human mandibles (Goret-Nicaise, 1984).

The absence of type II collagen labelling may rule out true chondrogenesis as a mechanism leading to calcification. However, this does not preclude a fibrocartilaginous transition in the absence of type II collagen.* Indeed, fibrocartilage seems to be heterogenous tissue whose constituents vary according to species and location, and more precise documentation is required in order to distinguish differences that may exist between normal and pathological varieties. One may also question the term fibrocartilage if there is no type II collagen present and argue that 'fibromucoid' tissue may be a less misleading term.

Collagen type VI immunolocalisation.

Collagen type VI is a molecule that forms a meshwork within many connective tissues (Keene et al., 1988) and may have an anchoring role within and between various connective tissues. In the current study collagen type VI appeared to be a minor component of non-pathological tendon which was dispersed throughout the matrix (fig: 3.3 e to h). However, in regions of pathological tendon the distribution of type VI collagen was patchy (fig: 3.4i and j). The reasons for this are unknown, but the influx of inflammatory cells may have disrupted a formerly more even distribution of this molecule.

* See end of chapter (p132) for more information about the known collagen content of fibrocartilage.

In cartilage type VI collagen labelling is largely of fine pericellular fibrils within the cell lacunae (fig: 3.3). Similarly, in areas of the pathological tendon, type VI collagen was also most evident within the cell lacunae, particularly around the rounded 'chondrocyte-like' cells. Again, whether this apparent distribution was coincidental (perhaps because of limited antibody penetration into the dense matrix of the cartilage and tendon) or is indicative of a similar phenotypic trait remains to be resolved.

The pre-embedding labelling experiments were less sucessful. The results, however, did confirm that there appeared to be antigen recognised amongst the collagen fibres and peripheral to mineral (fig: 3.5b). There appeared to be less labelling in the non-pathological tendon. Non-immune serum controls were virtually free of label. A much better approach to this form of cryostat section labelling for electron microscopy may be to use the same protocol but larger gold particles (a minimum of 40nm) or to silver enhance smaller (1nm) gold particles, and then to process the section flat and view the specimens by scanning electron microscopy to examine the surface labelling pattern. This approach may circumvent the problem of limited antibody penetration into dense extracellular matrices.

Hyaluronan binding region protein (BR) immunolocalisation.

Hyaluronan binding region protein indicates the presence of proteoglycan monmers which have the ability to form macromolecular aggregates with hyaluronan, an interaction which is stabilised by a link protein. Aggregated proteoglycans are found throughout articular cartilage and their presence in non-pathological tendon has been reported in bovine flexor tendon in regions that are subjected to compressive forces (Vogel et al., 1986, Koob and Vogel, 1987).

The BR antibody did not label more intensely in the pathological tissue than in the non-calcified tendon. This was surprising but suggested that the proteoglycans distributed throughout the tendon have aggregating abilities. The pathological area had more similarity in histological appearance to distal tendon that encompasses the fibrocartilaginous insertion and may be found to contain similar larger proteoglycan species if stud-

ied biochemically. It is quite likely that the bovine flexor tendon, studied extensively by Vogel and colleagues, also contains a small percentage of aggregating proteoglycans which are distributed throughout the tissue. Whilst in regions of compression a role for aggregating proteoglycans can be easily proposed, i.e. they provide the tissue with compressive stiffness derived from osmotic pressure generated from a high fixed charge density of the molecules, their role in regions of tension is more obscure. However, in any region of tension, there must be a compressive element. For example, if a tendon is placed under tension longitudinally, then there must be a degree of compression across the transverse plane. This is likely to be most apparent around the cell lacunae which often possess fine extracellular elements. Therefore, it follows that a degree of compressive stiffness will be desirable throughout the tissue.

Chondroitin sulphate.

The antibodies to the chodroitin sulphate molecules recognise specific epitopes at the linkage region of the chondroitin sulphate chain to the core protein. These antibodies have been used previously for the identification of specific glycosamioglycans in tissue sections (Caterson et al., 1985; Page and Ashhurst, 1987).

In the current study large amounts of chondroitin-4-sulphate appeared to be produced in the pathological tissue sections (fig: 3.7a to d). Chondroitin-4-sulphate is a side chain found on smaller proteoglycan molecules. There was a lesser but more specific indication of cells synthesising chondroitin-6-sulphate (fig 3.9a to d). In non-pathological tendon the presence of chodroitin 6-sulphate was more prominant than the chondroitin-4-sulphate. As this tissue was obtained from patient H, the antibody reaction and cellular nature of the matrix confirmed that the tissue was in the process of a strong inflammatory response. The large quantities of chondroitin sulphate produced by cells in the area of calcium deposition is assumed to be a repair phenomenon.

As chondroitin sulphate has also been shown to have an inhibitory effect on crystal growth (Hunter et al., 1985; Tenenbaum and Hunter, 1987) this would aid repair by inhibiting further calcification and is perhaps a factor in reversing the course of the

disease. In these experiments no association between chondroitin sulphate molecules and the crystals was observed despite a strong reaction from specific cells in the area of mineralisation. The distribution of antigenic determinants was not studied at the electron microscope level. It seems probable that cells surrounded by calcific crystals would produce a non-mineralising matrix in an attempt to survive.

In a study of connective tissue matrices during fracture healing, Page and Ashhurst (1987), summarize the available data on the progression of macromolecules produced in healing wounds and draw attention to similarities in this pattern with that of the developmental process. Hyaluronan is the first glycoaminoglycan to appear in healing tendon followed by the chondroitin sulphates (Reid and Flint, 1974) which may be accompanied by keratan sulphate. Keratan sulphate has already been found in compressed regions of normal bovine tendon (Vogel and Thonar, 1988) and in developing chick tendon (Craig et al, 1987).

It may be that in the repair process in calcifying tendinitis, the production of these glycosaminoglycans is staggered with some overlap. In the sections examined, both are being produced but the ratio of one to the other is likely to vary over the course of time and, towards the end of the repair period, the ratios may well be reversed. A way of assessing this may be to take the calcified samples of tendon and treat them as explants for culture purposes, then after a period of time assess the proteoglycan content of the tissue. Whilst it is almost certain that explanted cells would not behave in the same manner as the cells in vivo , useful information might be obtained about the sequence of production of different molecules.

Chondroitin sulphate inhibits calcification of bone formed in vitro (Tenenbaum and Hunter, 1987). However, it is not known whether it prevents mineralisation in a cellularly controlled environment. In epiphyseal cartilage and bone, a decrease in proteoglycan content is associated with provisional calcification and mineralisation respectively. In a culture system, a moderate decrease in alkaline phosphatase activity was recorded when chondroitin sulphate was added to the system. No massive elaboration of alkaline phosphatase activity was detected in our pathological tissues (see chap-

ter two) which may be connected to the inhibitory presence of chondroitin sulphate. However, it is more likely that the enzyme was not present at histochemically detectable levels due to other considerations discussed in chapter two.

The immunolocalisation in these tissue sections of chondroitin-4-sulphate and chondroitin-6-sulphate was not unexpected due to their association with reparative processes. Chondroitin-0-sulphate (chondroitin) was not found in any of the tissues studied nor was the over-sulphated chondroitin-6-sulphate associated with diseased cartilage. This is a glycosaminoglycan associated with osteoarthritic cartilage and neoplastic soft connective tissues (Hardingham et al.,1989) and may have been expected to be present in pathological tendon. Although inhibitors of mineralisation, chondroitin sulphates are present in bone, these factors are not necessarily contradictory, as these glycosaminoglycans may have a mediating role to play in the organisation of mineral relative to the collagen type I fibres of bone.

In animal models the characterisation of matrix components has been important in evaluating the sequence of events in fracture healing. Here we confirm the histological picture of a repairing tissue in samples from patient H, by the presence of the glycosaminoglycan molecules. Specimens from patient G were also used for most of the collagen studies. In the pathological areas this tissue appeared to be more fibrous (see chapter one) and may indicate a different phase of the pathology. Ideally, the characterisation of matrix molecule components in the walls of calcific nodules from larger numbers of patients, including Alcian blue staining for confirming glycosaminoglycan types (Page and Ashhurst, 1987), combined with other histological results to assess the stage of the disease process would be the best approach for tackling further studies.

In 1975 Eyre and Muir published a study of collagen in different cartilages of the pig. In this they could not detect collagen type II in fibrocartilage of the semilunar meniscus of the knee. However, another fibrocartilage, the annulus fibrosus was found to contain a significant amount of collagen type II, and indeed, microanalysis of serial samples is reported to show a smooth graduation in collagen composition from virtually all type I collagen, at the extreme outer edge of the disc, to type II collagen on reaching the nucleus pulposus.

CHAPTER FOUR.

MICROANALYSIS OF CALCIFIC DEPOSITS.

MICROANALYSIS OF CALCIFIC DEPOSITS.

4.1 INTRODUCTION.

In spite of much work on the identification of the crystalline form of hydroxyapatite, little has been done to establish the limits of biological variation of the calcium to phosphorous ratio of pathological deposits between patients.

Faure et al., (1982) examined calcified material from three patients suffering from shoulder tendon sheath calcification and a fourth subcutaneous calcific deposit taken from a patient's forearm. The techniques used by this group were scanning electron microscopy, X-ray diffraction, infrared spectrometry, scanning and transmission wave-length dispersive X-ray spectrometry, high resolution transmission microscopy and electron microdiffraction. Their conclusion was that the deposits were made of carbonate containing apatite, were heterogenous and composed of at least two separate phases, the crystallinity of which appeared different. Quantitative microanalysis gave values between 1.61 and 1.73 Ca/P molar ratios. The carbonated nature of the apatite crystals confirmed the results of Saez-Clavere et al., (1980) who used infrared spectrometry to study two calcific deposits.

Hydroxyapatite and associated compounds of calcium and phosphate can exist with a range of Ca/P ratios when observed outside the complex human body. Neuman (1980) describes a variety of forms in which compounds of calcium and phosphorous exist and is an invaluable source of information on their structures:

Table 4.1

Formula	Name	Molar Ca/P	Abbreviation
$Ca(HPO_4).2H_2O$	Dicalcium phosphate dihydrate	1.0	DCPD
$Ca_4(HPO_4)_3$	Octacalcium phosphate	1.33	OCP
$Ca_9(PO_4)_6$ (var.)	Amorphous calcium phosphate	1.3-1.5	ACP
$Ca_3(PO_4)_2$	Tricalcium phosphate (Orthophosphate).	1.5	TCP
$Ca_5(PO_4)_3OH$	Hydroxyapatite	1.66	HA

These are listed in order of decreasing acidity, decreasing solubility and increasing thermodynamic stability (Neuman 1980). DCPD at acidic pH is relatively stable, but above pH 7.0 all the compounds except HA are relatively unstable and in an aqueous environment will recrystallise or undergo solid state conversions to form the thermodynamically stable HA. Although local changes in pH probably contribute to pathological calcification (physiological pH is 7.4) an increase in alkalinity would result in the stability of HA.

Neuman suggests that due to the grouping of phosphate molecules in the various compounds it could be possible to have "sandwich" mixtures or "surface" compounds, for instance a layer of DCPD or OCP on a base crystal of HA. This would correspond with the observations of Faure et al., (1982) that there is more than one crystalline form of hydroxyapatite.

HA has a microcrystalline structure and under physiological conditions it never forms crystals larger than thirty to forty nanometers in any dimension. Thus, HA is calculated to have an enormous specific surface area of 50-250m^2/g (Neuman 1980). A coating of more soluble calcium phosphate compounds on less soluble mineral would enable HA to exhibit some of the chemical and solubility properties of those compounds contributing to the overall structure.

Another important feature of HA is that it cannot be formed directly from its constituent ions in solution. The thermodynamically stable end product has the formula $Ca_{10}(PO_4)_6(OH)_2$ which is the smallest crystalline unit. By adjusting conditions, the formation of HA from calcium and phosphorous goes through a combination of the compounds given in the table, starting with DCPD which at alkaline pH is not stable and may be transient, and not precipitated. Whether every member of the sequence given is a necessary step is not known, but in supersaturated solutions HA can catalyse its own formation. The extent of solid state transition (i.e. dissolution and recrystallisation) that occurs in this system is also unknown. The nature of the solid phases of bone and cartilage have caused great controversy but there is agreement that HA is present and that in low density fractions of very newly deposited bone mineral there is DCPD.

Calcium pyrophosphate has been associated with calcific periarthritis (Gerster et al., 1983) although mixed crystal deposition is probably more common in other crystalline deposition diseases (Dieppe et al.,1988). Two of the patients in the current study were treated for carpel tunnel syndrome (see appendix one) which suggests some predisposition to abnormal calcification. This particular co-occurrence of diseases associated with respect to mixed crystal deposition has been reported by Lagier et al., (1984). This group found the carpel tunnel syndrome deposits to be mixed calcium pyrophosphate dihydrate with apatite crystal deposition occurred in the tendon synovial sheath.

Serum is undersaturated with respect to de novo apatite formation and supersaturated with respect to dissolution. However, as there is a substantial amount of HA in the human skeleton it must be concluded that there are physiological mechanisms that prevent the whole body from becoming calcified.

X-ray microanalysis makes it is possible to compare calcium to phosphorous ratios but provides no data on the carbonate content of the crystals. Details of the standard used to calibrate the microanalytical system and variations in the preparation protocols with their corresponding effect on the results obtained are given.

This chapter assesses the variation in the molar calcium to phosphorous (Ca/P) ratios between samples from eight patients. There is also an assessment of the variation in calcium to phosphorous ratio between calcospherites and bone observed in patient D.

4.2 METHODS.

The hydroxyapatite standard value used to calibrate all subsequent results was obtained from sintered HA prepared for medical implantation and characterised by its X-ray diffraction pattern (Klein et al.,1988). Calcium pyrophosphate and orthophosphate standards were prepared from commercially available chemicals. Fragments of human cortical bone were obtained from a freshly removed femoral head taken from a patient with osteoarthritis. The same buffer, fixative and resin mixtures were consistently used throughout processing of these specimens and in the preparation of standards. When

ultrathin sectioning into a boat, it was found that sodium cacodylate was a better medium than distilled water to float sections away from the block face (see results). Consequently, a single batch of sodium cacodylate was used throughout these series of experiments. Aliquots were dispensed and stored at - 20 °C prior to use.

The variation of deposit ratios between eight different patients was examined. Cryosections (for method see chapter three) were also studied from one patient, thus enabling a comparison with a part of the same specimen that had been dehydrated and resin embedded. A comparison was also made of the calcium to phosphorous ratios of bone and calcific deposits located in one tissue section (patient D).

Energy dispersive x-ray microanalysis.

The tissue was processed as for the standard protocol for TEM specimens, only omitting the secondary fixation with 1% osmium tetroxide. The hydroxyapatite standard used to obtain ratios was provided by Dr.C. Klein (1988) and is ordinarily used as a bioceramic. X-ray diffraction patterns after sintering demonstrate a hydroxyapatite structure and this standard was treated in the same way as specimens. Commercial standards of hydroxyapatite (Sigma: $Ca_{10}(OH)_2(PO_4)_6$), calcium pyrophosphate (Sigma: $Ca_2P_2O_7$) and precipitated calcium orthophosphate (BDH: chiefly tricalcium phosphate $Ca_3(PO_4)_2$) were processed in Eppendorf tubes and centrifuged between wash, dehydration and resin step changes (resin was heated for 10 min. at 60°C prior to changes and mineral was centrifuged to the bottom of the Eppendorf tubes before polymerisation). Thin sections of 100nm were cut as described previously (chapter one) and mounted on copper grids precoated, for support, with 0.45% pioloform in chloroform.

Unstained sections were viewed on a CM12 transmission electron microscope at an accelerating voltage of 100Kv and analysed using an EDAX facility. After setting the eucentric height, specimens were manually tilted by 20° toward the detector giving a total "take off" angle of 40°. Normal TEM allowed selection of an area suitable for analysis. The probe spot size (between 500 and 30nm) and a 'top hat' condenser aperture (usually 70nm) were selected to give an optimum initial count rate of between 2500

and 3000 counts per minute after removal of the objective aperture. The counts were gathered for a total of 200 live seconds. X-ray spectra were analysed using ' the quantitative analysis of thin sections' software package (EDAX PV9800). The background intensity was subtracted from the characteristic peak intensity for each value.

A minimum of ten analyses was carried out on each sample.

Molar ratios

Microanalysis is made quantitative by presenting ratios of elements found in the area analysed. However, no absolute value is obtained for a particular element. Where values are low there is comparatively less calcium and comparatively more phosphorous. High values indicate that there is comparatively more calcium and less phosphorous. The empirical value of 1.66 for HA means that there are 1.66 moles of calcium to 1.0 mole of phosphorous (or 0.6 moles of phosphorous to every mole of calcium).

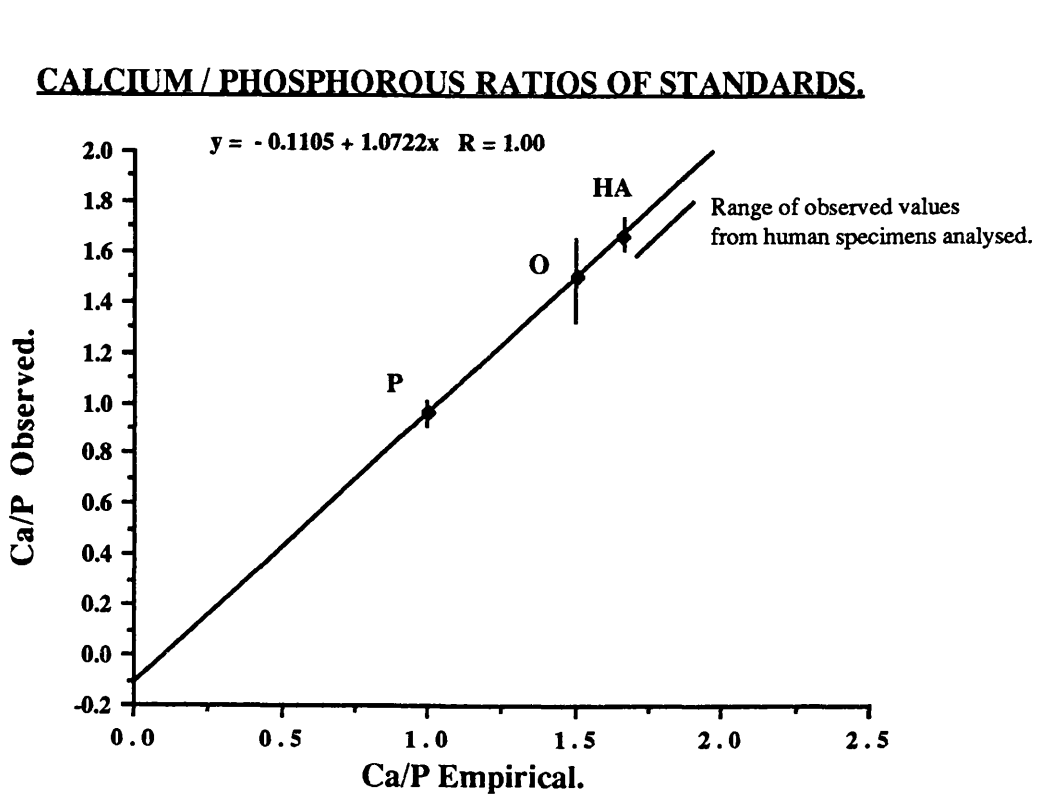
4.3 RESULTS

Statistical analysis

Where comparisons are made between two populations which show a normal distribution, a student's unpaired t-test can be applied. To compare each sample against the HA standard an unpaired t-test was used to establish whether there was a significant difference. When more than two populations were being compared (for the comparison of variation between patients) results were ranked in ascending order according to their mean values and by applying the students unpaired t-test to consecutively ranked samples, the grouping of patients into those with significantly different values was possible.

In figure 4.5 the ultrastructure of the calcospherites and bone microanalysed from the same section are compared. Figure 4.6 demonstrates that there is a significant difference between the Ca/P molar ratios of these two calcified tissues.

FIG: 4.1



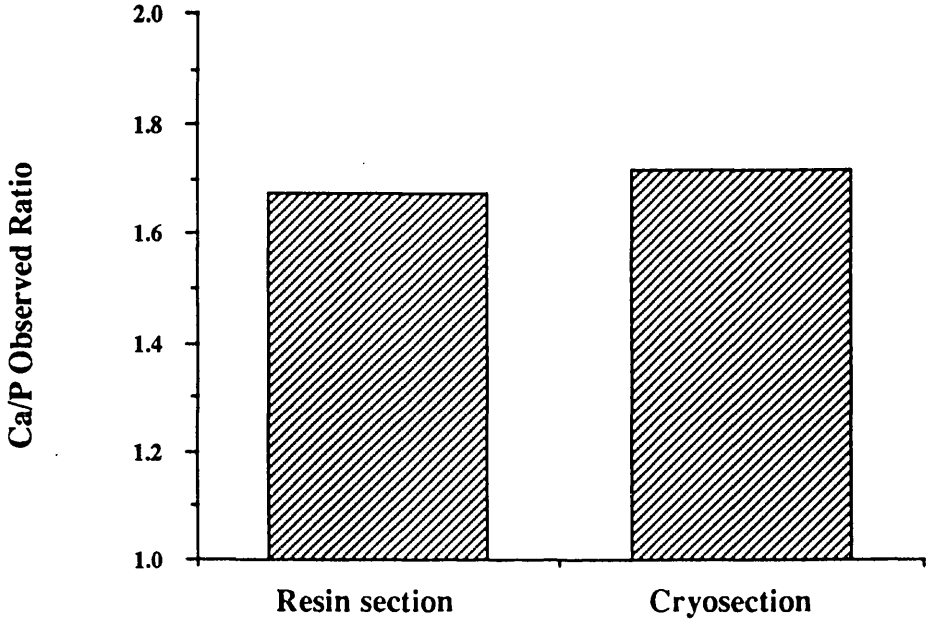
STANDARDS

This scatter plot shows the observed mean calcium to phosphorous ratios plotted against the known empirical value for each standard. The ceramic hydroxyapatite was chosen for calibration of the system rather than the commercial HA. The values tabulated below include the unplotted means obtained from the commercial HA and that given by the microanalysis of human bone.

Type of Calcium Phosphate	Symbol	Ca/P ratio observed
Pyrophosphate (commercial)	P	0.960 ± 0.038
Orthophosphate (commercial)	O	1.505 ± 0.259
Hydroxyapatite (ceramic)	HA	1.664 ± 0.062
Other Values		
Hydroxyapatite (commercial)		1.569 ± 0.062
Human Bone		1.670 ± 0.042

FIG: 4.2

RESIN SECTION VERSUS CRYOSECTION



Cryosections were cut and examined from sucrose infiltrated specimens containing deposit from patient G. The same area (wall) was examined after sections from resin embedded tissue were cut onto sodium cacodylate buffer. The difference in microanalysis results obtained by these two methods of processing, were at the limits of significance, when analysed by the student's unpaired t-test.

Type of section	Ca/P ratio observed
Resin embedded	1.676 ± 0.044
Cryocut	1.718 ± 0.046

Unpaired t-test X : Resin Embedded. Y : Cryosection.

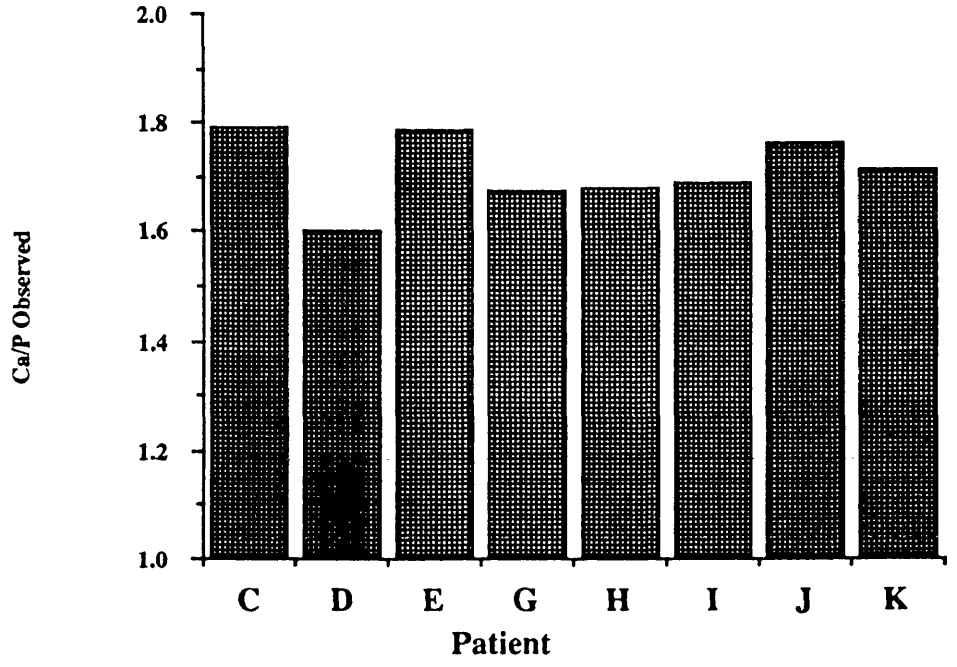
DF:	X Count:	Y Count:	Mean X:	Mean Y:	Unpaired t Value:
18	10	10	1.676	1.718	-2.039

$0.025 < p \leq 0.05$

Values are considered to be significantly different where p is less than 0.05.

FIG: 4.4

VARIATION IN CALCIUM TO PHOSPHOROUS RATIOS BETWEEN PATIENTS



Areas of calcific deposit were analysed from eight of the patients. The mean values from these are ranked into ascending order and the significant differences between consecutive sets of data were calculated by the student's unpaired t-test.

Patient	Ca/P Observed mean value of ten readings.	p	Sub-group.
D	1.602 ± 0.069		a
		0.0005 < p ≤ 0.005	
G	1.676 ± 0.044		b
		p > 0.4	
H	1.679 ± 0.099		b and c
		p > 0.4	
I	1.687 ± 0.035		b and c
		0.05 < p < 0.1	
K	1.712 ± 0.030		c
		0.01 < p ≤ 0.025	
J	1.764 ± 0.068		d
		0.1 < p ≤ 0.375	
E	1.786 ± 0.072		d
		p > 0.4	
C	1.793 ± 0.068		d

– – – – – – – – – – Not significant.
——————— Significant.

Values are considered significant where p is less than 0.05.

FIGURES.

FIG: 4.5

Fig: 4.5a Unstained section of the calcospherites found and analysed in tendon taken from a mature area of deposit (patient D).

Fig: 4.5b Enlargement of an area of calcospherite. Note the fine needle-like HA crystals do not appear to be bound to a collagenous matrix.

Fig: 4.5c An area of bone taken from the same unstained and ultrathin section as the calcospherites observed in (a).

Fig: 4.5d Enlargement of an area of the bone. Note the collagenous matrix and HA crystals.

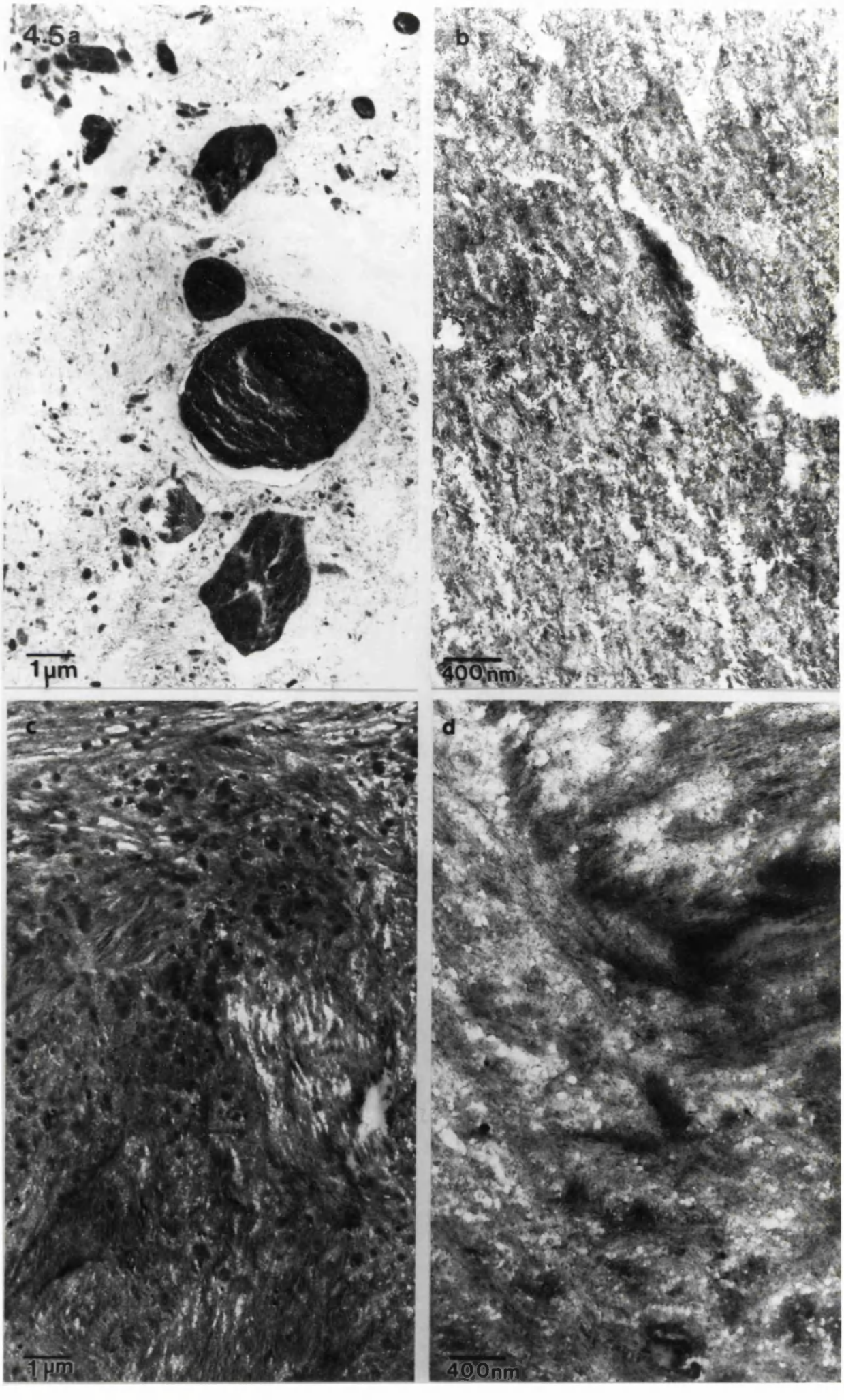
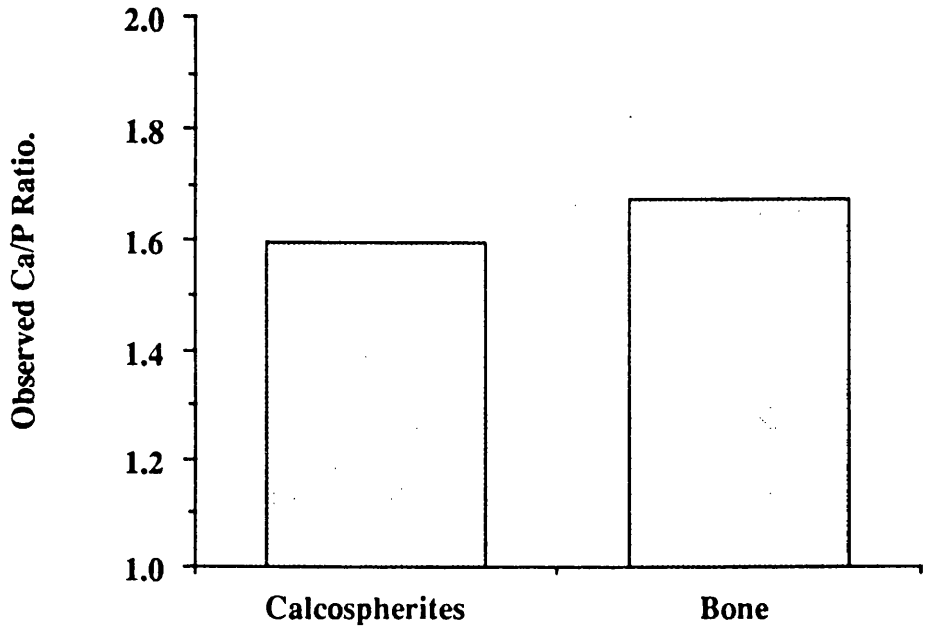

4.5a
1μm
b
400nm
c
1μm
d
400nm

FIG: 4.6

CALCIFIC DEPOSIT VERSUS BONE.



In a single ultrathin section of tissue from patient D, calcospherites similar to those observed in other patients could be seen close to bone found within the tendon. The distance at the closest point was measured to be 700nm on the T.E.M. using an image measuring device. The calcium to phosphorous ratios of both were analysed and compared statistically using the student's unpaired t-test.

Area analysed	Ca/P ratio observed
Calcific deposit	1.599 ± 0.03
Bone	1.673 ± 0.03

Unpaired t-test X : Calcific deposit. Y : Bone

DF:	X Count:	Y Count:	Mean X:	Mean Y:	Unpaired t value:
28	15	15	1.599	1.673	-6.64

$p \leq 0.0005$

Values are considered to be significantly different where p is less than 0.05.

4.4 DISCUSSION.

To obtain valid microanalytical data the correct reference values for the substance to be analysed need to be installed into the software package. By using hydroxyapatite the assumption is made that this is the primary component of the analysed specimens. Sintered HA was chosen as the best standard as this sample had been well characterised (Klein et al. 1988) and when plotted with values obtained for calcium pyrophosphate and calcium orthophosphate (fig: 4.1) against the known empirical molar ratios, a line of perfect correlation was obtained. The HA ceramic value was also virtually the same as that obtained from human bone removed from the base of a femoral head. The commercial HA gave a lower calcium to phosphorous ratio. This ratio in commercial calcium orthophosphate showed a much larger standard deviation than for any other substance measured in this study, although the observed mean ratio was close to the empirical molar ratio (1.505 ± 0.259). This variation was assumed to be either, due to the instability of the crystal in the processing buffer (pH of 7.2) causing some dissolution and recrystallisation to occur or, due to the original commercial sample consisting of 'chiefly' calcium orthophosphate as described by the manufacturer's (BDH).

The range of observed results from pathological specimens demonstrates that where deviation occurred the tendency was for the Ca/P ratios to be higher than the mean HA value.

Figure 4.2 represents a comparison of the Ca/P ratio between a resin embedded pathological tissue section and a cryo-processed section, both containing deposit from the same area of the same patient (G). The cryosection was not dehydrated and was processed with phosphate buffered fixative and infiltrated with 2.3 M sucrose in phosphate buffer. If the phosphate had altered the result from the cryosection the expected increase in phosphorous would have reduced the molar Ca/P ratio. However, the ratio obtained in the cryosection was slightly higher, which suggests that dissolution and loss of calcium due to processing of tissue into resin is probably a greater source of error as it is not possible that either sample could have gained calcium ions. The results ob-

tained are on the limits of significant difference, $0.025 < p \leq 0.05$, with a 95% confidence limit and therefore the significance of this variation is debatable. All the other specimens (including the standards) were processed into the resin using sodium cacodylate buffer.

Once a resin block was obtained, 100nm thick sections had to be floated onto a boat to be picked up onto copper grids. For ultrastructural studies the boat is filled with water with a pH of around 6.0. When the Ca/P ratio (fig: 4.3) obtained from the HA standard (ceramic) using buffer was compared with water, a significantly lower ratio was obtained from the water medium. This suggests that either there was probably greater dissolution of the calcium salts into the water, HA being less stable at a more acidic pH, or alternatively, the phosphorous may have selectively leached out of the sections cut onto the buffer. As the buffer mean was closer to the expected empirical value, all subsequent sectioning for microanalysis was carried out onto sodium cacodylate buffer. Studies have been carried out on the problems of demineralisation in thin sections of fully calcified bone by Boothroyd, 1964 and Thorogood and Craig-Gray, 1975.

Figure 4.4 shows the range of variation in Ca/P ratio between patients, the lowest mean being 1.602 ± 0.069 and the highest 1.793 ± 0.068. These extremes are outside those values obtained by Faure et al., (1982) however, the number of samples is greater. The ranking shows that the results fit into three significantly different groups although four sub-groups (a to d) occur. Some specimen fit into two sub-groups due to significant difference that occurred between the lowest and highest means within the middle group. The calcific deposit from the wall region of patient D is grouped alone and is discussed further below. Patients GHI and K form a second group whilst patients JE and C gave the highest Ca/P ratios. Unfortunately the sample size is too small to contemplate a possible correlation of these groups with morphological observations. These samples were all treated in the same manner with the same solutions for the same length of time, so variation should be due to the differences in the deposit. The differences suggest that there may be variation in the dissolution of the deposits from different

patients and that there may be variation in the ratio of Ca/P deposited. Either or both of these phenomena are probable but from these results it is not possible to establish whether or not one is dominant .

Samples from patient D have proved unusual throughout this study. The calcific deposit from the wall region of the deposit gave a significantly lower mean value from any of the others. In the mature area of the deposit bone has been found (chapter one). In this investigation bone and the more usual calcospherites were demonstrated in one section (fig:4.5). When 15 spots from each area were analysed the Ca/P ratio was again found to be low in the calcific deposit whereas the value for bone was close to the HA standard. Selective leaching from one area of the specimen is unlikely, although the mineral in the bone is probably bound more tightly being woven into the tissue structure of collagen fibres (fig:4.5). The possible movement of calcium ions from the calcific deposit into the area of bone, leaving the deposit with a comparatively lower Ca/P ratio than those deposits normally found in other patients (not associated with bone) should be considered in this instance. However, the mean given by analysis of the bone is close to those of other calcific deposits and the values were not significantly different from the bone standard.

The anomalous data associated with patient D, together with the unique pathology, leads to the suggestion that this may not be a case of calcifying tendinitis in its classical form. On the other hand whether it represents a rarer form of calcifying tendinitis or indeed be a quite different pathology altogether, remains to be resolved.

None of the individual microanalytical values from any of the calcific deposits suggest that pyrophosphate could be present in the mineralised areas. The lowest mean value obtained, 1.599 ± 0.03, is closer to the that of hydroxyapatite than calcium orthophosphate, but does suggest that some dissolution of HA may have occurred.

GENERAL DISCUSSION.

GENERAL DISCUSSION.

The surgical removal of calcifying human tendons is infrequent, so that the sample size in any three year study cannot be large. Nevertheless, all of the observations in this thesis were made on fresh human tissue and so are directly and validly applicable to the disease. Where possible it is the intent of physicians to use non-surgical methods to dissipate the calcific deposits. If this fails, it is impossible to assess the effects treatments may have had on the course of the disease by subsequent examination of tissue samples. A common feature of the patients used in this study was that non-surgical intervention had been considered unsuccessful. Generally there was a considerable time-lapse (three weeks minimum: see appendix one) between the final treatment and the time of surgery.

Any study of human pathological tissue removed at surgery is observing an advanced stage of the disease. There is no way of knowing the events that took place in the earlier stages of the disease process. This point has to be kept in perspective in the interpretation of observations on specimens of calcifying tendons, more so, because there is no relevant animal model for this disorder.

Calcifying tendon undergoes cellular and molecular changes. In specimens from seven of the eleven patients morphological observations show 'fibrocartilage' (an avascular tissue containing large rounded cells) to be associated with the area of mineralisation. This confirms previous observations of fibrocartilage in calcified rotator cuff tendons (Uhthoff, 1975; Uhthoff et al., 1976; Uhthoff and Sarkar 1978; Sarkar and Uhthoff 1978; Refior et al., 1987; Uhthoff and Sarkar, 1989). However, Jozsa et al., 1980 did not observe a fibrocartilaginous transformation, inflammation or necrosis in other calcified tendons that had spontaneously ruptured.

Whilst some individual cells seen in calcifying tendinitis specimens have a chondrocyte-like appearance (fig: 1.4f; 1.5b; 1.6b) there is no evidence of endochondral ossification.

Fibrocartilage is normally formed where tendon meets bone (fig. 1.3a and b), either at

the tissue interface, or where tendon is sliding on a hard bony surface. Consequently, the presence of fibrocartilage in a mineralised area of tendon may be expected and might be secondary to the calcification, not a cause. The presence of inflammatory cells is certainly associated with resorption of the calcific deposit (fig: 1.10a and b).

The absence of alkaline phosphatase activity and collagen type II in areas of fibrocartilage further suggest that the rounded cells are not chondrocytes. However, the absence of alkaline phosphatase activity in this study, does not mean that this enzyme was not involved in the mineralisation process, merely that this enzyme was not active at the time of surgery.

The absence of collagen type II shows that the chondrocyte-like cells are not situated in a truly cartilaginous matrix and therefore, despite appearances, should not be classified as chondrocytes.

Collagen type VI is almost certainly produced by tenocytes as it is found dispersed through non-pathological tendon (fig: 3.3e and g). This molecule forms a meshwork within many connective tissues (Keene et al., 1988) and may have anchoring role within and between areas of the tendon studied. The controversial rounded cells also appear to synthesize collagen type VI, but not collagen type I. The absence of visible collagen type I fibres in the cell lacunae makes collagen type VI appear concentrated in the immediate vicinity of the large rounded cells (fig: 3.3i; 3.4a and b). Unfortunately the localisation techniques used were not quantitative.

The hyaluronan binding region protein is present throughout normal cartilage and tendon and its distribution appeared to be unaffected by the pathological calcification. Although an elaboration of chondroitin sulphate occurred in the pathological tendon, this appeared to be associated with the repair process. It may be the case that these molecules are inhibiting cystallisation of HA and hence reducing further mineralisation. A moderate inhibitory effect of chondroitin sulphate on alkaline phosphatase activity in culture has been observed by Tenenbaum and Hunter (1986), which may also account, to some extent, for the lack of alkaline phosphatase activity found in some of the pathological tissue samples.

Another possibility is that cells local to the mineralising environment perhaps become protected by synthesizing an inhibitory matrix and create lacunae. These lacunae contain collagen type VI (not collagen type I), proteoglycans which may be chondroitin sulphate rich, and certainly many other molecules not yet identified. Athough the pericellular matrix in mineralising tissue appears cartilaginous, this seems likely to be due to the similarity of composite molecules to those in chondrocyte lacunae rather than because an endochondral process is driving the pathological calcification.

Ruling out chondrocyte-mediated calcification other possibilities have to be considered.

Vesicular structures and small particles of mineral were observed within the calcifying matrix (fig: 2.4a; 1.8a) but a definite association between either of these and the large rounded cells was not recognised and the vesicles may have been produced by other cell types. Landis (1986) reported vesicular structures within the early stages of turkey leg tendon calcification, a tissue that undergoes non-pathological mineralisation in the absence of fibrocartilage formation. Membranous cell fragments and unhealthy cells were seen on occasion suggesting that cell necrosis may also be occurring local to the calcific deposits. Alkaline phosphatase activity was observed to be associated with some cell fragments at an ultrastructual level (fig: 2.3d). Therefore, calcification due to release into the tissue matrix of the mitochondrial calcium stores from dying cells is also a consideration.

Seams of mineral were frequently found packed between collagen type I fibres (fig: 1.8b and c), while apparently inert calcospherites were often observed within the collagenous matrix (fig: 1.8d).

Among the many molecules which could be expected to play a role in promoting or inhibiting pathological calcification in tendon but which have not been considered in this thesis is osteonectin (SPARC). Osteonectin RNA is known to be widespread particularly in tendon as well as in bone tissue. Osteonectin is a glycoprotein that has affinity for both collagen and hydroxyapatite mineral and in vitro causes mineralisation of type I collagen (see Triffitt, 1987). This, along with a battery of structural molecules,

hormones, cytokines and serum proteins, may all be involved in the process of tendon calcification. The increased availability of specific monoclonal antibodies and advances in in-situ hybridisation techniques may help to establish the prominant factors responsible for tendon mineralisation in the future.

Microanalytical data demonstrated variation of the calcium to phosphorous ratios from different patients and also that the substance deposited is mainly hydroxyapatite, the mineral in bone. Variation in the Ca/P ratio between patients may be influenced by the stage of the disease. More data would be necessary to correlate these parameters. This technique was highly susceptible to alterations in sample preparation. Thin sectioning of resin embedded specimens onto sodium cacodylate buffer (pH 7.2) was shown to be preferable to water alone (fig: 4.3).

HA is microcrystalline with massive surface area and hence enormous opportunity for adsorption and surface exchange. Recent work by Garnett and Dieppe (1990) suggests that hydroxyapatite crystals specifically adsorb onto their surface different serum proteins and these can have an inhibitory effect on crystal growth. In the matrix soup, serum proteins are not the only molecules exposed to the crystals but also those matrix molecules being produced by cells proximal to the deposit. Some or all of these molecules may be held within the mineralised matrix in vivo. The presence of adsorbed molecules on the crystal surface could affect the Ca/P ratio if, for instance, the quantity of phosphorylated molecules was varied between patients and also between areas within the tendons. The identification of the proteins found coating the HA crystals from in vivo samples of hydroxyapatite would form an interesting study.

Specimens from patient D were very unusual as bone was found in a sample of mature deposit. The calcific deposit from this patient gave the lowest Ca/P molar ratio. The stability of calcium in the deposit proximal to bone may have been less due to increased solubility - perhaps because of movement of ions associated with bone formation. The bone sample was found to have the same Ca/P ratio as other deposits and cortical human bone. Due to its different aetiology, the rare morphology of this specimen and the lower Ca/P ratio indicate that there may be a need for a seperate disease category for the classification of patient D. This might be called ossifying tendinitis.

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APPENDICES.

APPENDIX ONE.

Details from the Patient's Records.

Ref. Letter.	Occupation, sex, hand preference and age.	Side of pain.	Pre-op. duration of pain.	Site of dep. and surgical description.	Injections into shoulder.	Time prior to op. of last injection and types.	Other relevant information.
A	Policewoman. F. R. 39y.	R	3 y	Infraspinatus tendon, no visible deposit seen, some scuffing.	4x (LA and HC) over 12w.	9 m, LA and HC into sub-acrom.space.	Bilateral dep. (Other done 2y and 5m previously).
B	Personnel director. M. NK. 54	R	9m	Supraspinatus.	1 x LA and 2 x HC over 9 w.	3 w, HC.	Bilateral, R-felt pain 30y prior. Carpal tunnel synd. 7y prior. Mild hypertension.
C	School secretary. F. R. 49	L	4-5y	Supraspinatus. Cuff at Supra-Infra Junction swollen.	3 x HC and 1x LA.	6m, LA.	Chronic calcific deposit.
D	NOTES UNAVAILABLE F. 54.	R		Supraspinatus tendon and some in the bursa.			Both mature and acute phase deposits removed. Bone found.
E	Works in stockmarket. M. NK. 44	R	3y +	Supraspinatus at insertion. Thick fibrotic bursa, synovial injection.	6 x (LA and HC).	LA and HC. more than 2 m prior.	Chronic calcific deposit.
F	NOTES UNAVAILABLE F. 41.	R		Resorbed from the supraspinatus, remnants of mineral in bursa.			No deposit found in tendon, only in bursal tissue.
G	Housewife. F. R. 53	R	4y	Dep. throughout whole supraspinatus Grossly thickened bursa.	3 x (LA and HC).	7m, LA and HC.	Arthroscopy and needling 14m prior caused consolidation of dep.
H	Housewife. F. NK. 50	R	3y	Very small dep. at insertion of supraspinatus.	3 injections over 12m.	6m, LA into sub-acrom. space.	Inflammed bursal tissue, with thickened Odeamatous cuff. Degen. osteophyte.
I	Haulage man M.R. 35	R	3y	Supraspinatus insertion.	4 x (LA and HC).	5m , LA and Lederspan	Deposit was inspisated and powdery. Previous carpal tunnel decompression.
J	Floor layer. M. R. 40	R	4y	Supraspinatus insertion 2 x 1 cm dep.	LA.	1m, LA.	Photographed (see fig: 1.2 a and b).
K	Housewife. F .NK. 42	L	3-4y	Subscapularis-upper part. Bursal hypertrophy.	LA and HC.	2m, LA into sub-acrom. space.	Large inspisated dep.

Abbreviations: Op. Operation; Dep. Deposit; F. Female; M. Male; R. Right; L. Left; NK. not known; y. years; m. months;w.weeks; acrom. acromial; LA. Local anaesthetic; HC. Hydrocortisone; Degen. Degenerative.

APPENDIX TWO.

CASE REPORT: Patient G.

History

A 53-year-old, right-handed, housewife had no history of shoulder problems until age 50 years, when sawing wood, she noticed a gradual ache in the epaulette region of her right shoulder. The pain remained in this region but also spread to the front of the shoulder and became worse on internal rotation. The pain was especially bad at night or in response to heavy work but no movement of the shoulder was lost. The complaint did not respond to steroid injection, physiotherapy and ultrasound, or non-steroidal anti-inflammatory (NSAI) agents. She had no parasthesie in the arms or history of dislocation, her general health was good and no other joint was affected.

On examination

The patient was a fit looking, slim woman, however, the right shoulder was noted to be carried lower than the left. There was wasting of the supraspinatus with slight wasting of the infraspinatus. The range of movements in both shoulders, active and passive, is the same. Abduction is restricted to 160°. Resisted adduction of the right shoulder reproduced the pain, whereas, resisted biceps action did not. There was no clinical evidence of instability and no local tenderness.

X-rays

Calcification of the supraspinatus tendon on the right was apparent. The well defined edges indicated that the deposit is probably mature.

Arthroscopy/Bursoscopy

An absolutely quiet bursa of average size with occasional non-pathological folds. The surface of the supraspinatus tendon at its insertion was smooth but rucked up as though the the surface was unstable. No surface changes to indicate the site of deposit were apparent but a bulge at the posterior limit was needled and found to be gritty. Despite repeated needling of this area no calcium escaped.

Arthroscopy: Apart from mild general tear the joint was normal and gave the impression that there were no signs other than the calcific deposit to explain the patients symptoms.

Two months after arthroscopy: the pain eased after arthroscopy and the patient was asymptomatic for a whole week, until lifting a baby at a christening which triggered more pain. Further X-ray indicated spreading of calcification. A bone scan proved to be entirely normal. Local anaesthetic injection relieved symptoms for two months during a period when the patient was more active with the shoulder, but then developed some recurrence of pain. On re-examination there was no pain on rotation and the patient was then symptomatic with little evidence of a painful arc. More injections with local anaesthetic and hydro-cortisone gave temporary relief.

Four months after arthroscopy: the pain was maximal on carrying heavy weights. On examination the patient was minimally tender to palpitation over anterior aspects of the greater tuberosity and had a full active and passive range of movement. Futher X-ray showed that the calcific deposit had consolidated and her symptoms remained the same.

Ten months after arthroscopy: Patient admitted for operative excision of the calcific deposit from the right shoulder.

Operation report: Antero-superior deltoid split with anterior acromioplasty. The bursa was opened formally. The deep surface of the bursa was grossly thickened over supraspinatus. It was formally reflected to expose a grossly enlarged tendon in which the whole of the supraspinatus tendon upto the myofascicle junction was completely taken over. The tendon was formally opened longitudinally and a massive deposit curetted out.

Specimens were taken from:-
- adjacent to deposit
- root, floor and lateral margin of the deposit
- round supraspinatus from RI
- round infraspinatus.

Four months after operative excision of calcific deposit: Patient is completely pain free with full range of movement but has been advised to buy an electric saw.

APPENDIX THREE: DETAILS OF THE ANTIBODIES USED.

Molecule on which the epitope is located.	Antibody and class.	Location of the epitopes.	Source of immunogen and species immunised
Collagen type II.	C1CII IgG.	αI(II) Chain	Rat chondrosarcoma. Raised in mouse.
Collagen type VI.	VI. A	Polyclonal antibody which recognises more than one epitope.	Bovine. Raised in rabbit. Not affinity purified but does not cross-react with other collagens.
Hyaluronan binding region protein.	BR	Polyclonal antibody which recognises more than one epitope.	Human: trypsinized proteoglycan monomer, further HPLC purified. Raised in rabbit.
Proteoglycan chondroitin side chain.	1-B-5 IgG.	After chondroitinase digestion, this monoclonal antibody recognises a non-sulphated n-acetyl-galactosamine next to an unsaturated hexuronic acid. (Also recognises hyaluronan after hyaluronidase digestion).	Rat chondrosarcoma. Raised in mouse.
Proteoglycan chondroitin -4-sulphate side chain.	2-B-6 IgG.	After chondroitinase digestion, this monoclonal antibody recognises a -4-sulphated N-acetylgalactosamine next to an unsaturated hexuronic acid.	Rat chondrosarcoma. Raised in mouse.
Proteoglycan chondroitin -6-sulphate side chain.	3-B-3 IgM.	After chondroitinase digestion, this monoclonal antibody recognises a -6-sulphated N-acetyl-galactosamine next to an unsaturated hexuronic acid. Without digestion, 3-B-3 recognises a native epitope at the non-reducing end of the glycosaminoglycan chain (abolished by periodate oxidation).	Rat chondrosarcoma. Raised in mouse.

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DOES CALCIFYING TENDINITIS OCCUR VIA A CHONDROGENIC MODULATION ?

Hann SR, Bayley JIL and Ali SY.
Department of Experimental Pathology, Institute of Orthopaedics (University College), R.N.O.H., Stanmore, Middx. HA7 4LP.

Calcifying tendinitis can be a debilitating disease occurring where mineral becomes deposited in tendon. The pathogenetic mechanism is unknown but Uhthoff (1975) proposed that calcifying tendinitis occurs via a chondrogenic modulation (Virchows Arch. A Path. Anat. Histol. 366, 51-58).

Cellular, molecular and enzymic features of pathological tendon have been compared and contrasted with normal tendon and articular cartilage in addition to ossifying muscle. Fresh tissue samples were examined by light and electron microscopy. Pathological specimens from patients showed variable morphological changes. Some areas were fibrocartilaginous, however, the tissue was dissimilar to that exhibiting pathological endochondral ossification. In mineralised human tendon alkaline phosphatase activity (normally associated with mineralisation) was not evident.
Immunohistochemical labelling studies could not detect the presence of collagen type II in pathological tendon. The distribution of other matrix molecules (e.g. type VI collagen) was also examined.
Using x-ray microanalysis, calcium to phosphorous ratios of calcific deposits were compared with bone.

The results show that the fibrocartilaginous region associated with the pathological calcification is morphologically dissimilar to pathological endochondral ossification and the matrix components differ to those found in human articular cartilage. From these comparisons, evidence suggests that calcifying tendinitis does not occur via a chondrogenic modulation.

315 TYPE X COLLAGEN IN SKELETAL GROWTH AND REPAIR.

Gary Balian, University of Virginia, School of Medicine, Box 374, Charlottesville, VA 22908.

Hypertrophic chondrocytes synthesize type X collagen during the transition of cartilage to bone. This protein is a major component of cartilage extracellular matrix. Type X synthesis is restricted to the hypertrophic zone of the growth plate and appears in the areas of provisional calcification of cartilage. Although it does not appear in bone, type X can be induced during fracture repair and occurs predominantly in the areas of hypertropic chondrocytes with mineralized matrix. Monoclonal antibodies were prepared to identify the structure and synthesis of type X. Using antibodies that recognize the amino terminus of the type X polypeptide, the sequence of a non-triple helical region containing the epitope was determined. Monoclonals to the triple helical and non-triple helical domains were used to demonstrate that the non-triple helical extensions at the ends of type X are not proteolytically cleaved during synthesis and secretion. These extensions may be important in the assembly of molecules in the extracellular matrix. Changes in the synthesis of type X collagen in response to extracellular stimuli such as vitamin D and the mineralizing agent, beta-glycerophosphate, have been determined in organ and cell culture. Since the appearance of type X precedes mineralization, a study of the factors that influence synthesis and regulate matrix calcification may elucidate the role of this protein.

316 THERMAL STABILITY OF TURKEY TENDON COLLAGEN AS A FUNCTION OF THE MINERAL CONTENT.

A.Bigi (x), G.Cojazzi (xx), N.Roveri (xx).
(x) Dipartimento di Chimica and (xx) Centro di Studio per la Fisica delle Macromolecole (CNR), Università di Bologna, Italy.

A previous study carried out using small angle X-ray diffraction technique on turkey leg tendon at different degrees of calcification allowed to establish a clear structural relationship between inorganic deposits and collagen in the range of mineralization between 20 and 70% wt (Bigi et al., Int. J.Biol. Macromol., 1988, in press).

In order to evaluate how this structural relationship affect the thermal stability of collagen, we have carried a microcalorimetric and thermogravimetric analyses on calcified turkey leg tendon at different degree of demineralization.

The results reveal a close relationship between thermal behaviour of collagen fibrils and their mineral content.

FECTS 1988

317 PROTEOGLYCAN CONTENT AND BIOMECHANICAL PROPERTIES IN HUMAN ILIAC CREST

G. Gevers*, G. Raymaekers*, D. Vanderschueren*, J. Dequeker*, R. Van Audekercke**, M. Martens**.
*Arthritis and Metabolic Bone Disease Research Unit, **Laboratory for Biomechanics, K.U.Leuven, U.Z. Pellenberg, B-3041 Pellenberg, Belgium.

Iliac crest trabecular bone specimens (n=29) from 55-75 year old women, taken at necropsy, were analyzed biochemically and biomechanically. In the EDTA extract osteocalcin, proteoglycan (as expressed by the uronic acid content) and sialoprotein (as expressed as the sialic acid content) were estimated and hydroxyproline in the residue.

Mechanical properties, elastic modulus (stiffness) and maximum compressive strength were determined on trabecular bone specimens.

Intercorrelations were calculated between biochemical and biomechanical data. Only a signficant positive correlation between elastic modulus, compressive strength and proteoglycan content was found.

Although these data are preliminary, they indicate that proteoglycans may have a biomechanical role in bone, despite the small amount present.

318 PATHOLOGICAL CELLULAR TRANSFORMATION IN HUMAN ROTATOR CUFF TENDON

R.S. Hann, J.I.L. Bayley, S.Y. Ali. Department of Experimental Pathology, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, LONDON

The mechanism by which human supraspinatus tendon undergoes pathologcial calcification is not known. We have examined several cases of calcifying tendinitis, which are clinically recognised radiologically by the formation of mineralised nodules within the supraspinatus tendon of the human rotator cuff.

By examining the mineral deposits and associated areas of tendon in the electron microscope we are able to analyse the components of crystal matrices and detect variations in the nature of the mineral within and between the samples taken.

We have also explored the current favoured hypothesis that a fibrocartilagenous transformation occurs within the tissue before mineral deposition takes place. This has been done initially by looking for histological evidence of chondrogenesis by metachromatic staining. In cryosections of unfixed tissue the immunolocalisation of cartilage specific collagen type II has been studied. Further to this the histochemical localisation of alkaline phosphatase by light and electron microscopy in this tissue provides evidence for the involvement of this enzyme in tendon calcification. Since this enzyme is also implicated in cartilage calcification this may also be indicative of tendon metaplasia.

CELLULAR AND MOLECULAR CHANGES IN CALCIFYING TENDINITIS.

Hann SR, Bayley JIL, Ali SY.

Dept. of Experimental Pathology, Institute of Orthopaedics (University College London), R.N.O.H., Brockley Hill, Stanmore, Middx. HA7 4LP.

Introduction

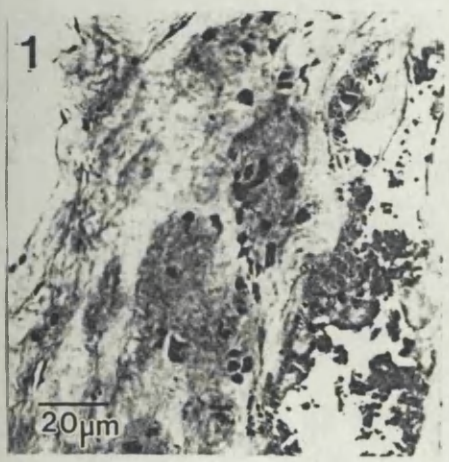
Calcifying tendinitis can be a debilitating disease and occurs where mineral becomes deposited in tendon. Those tendons situated in the rotator cuff of the human shoulder are particularly vulnerable.
Cellular, molecular and enzymic features of pathological tendon in contrast to normal human tendon have been compared to those of articular cartilage and pathological ossification. From these comparisons, evidence has been obtained to suggest that this disease does not occur via a chondrogenic modulation as suggested by Uhthoff (1975).

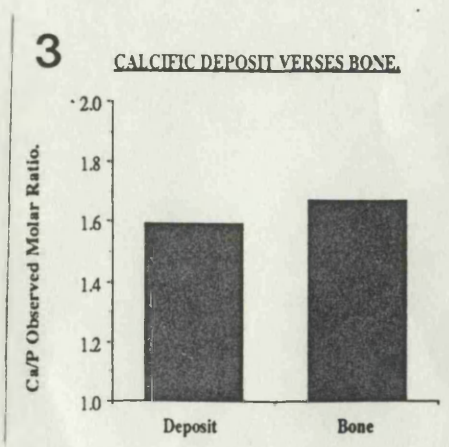
Materials and methods.

Fresh tissue samples from 11 calcifying tendinitis patients were taken from around the area of calcification and fixed or cryo-preserved in the operating theatre. Samples from a myositis ossificans patient and a sample of normal human articular cartilage were used for comparative or control purposes. Supraspinatus tendons removed whole at post mortem were used as non-pathological controls.
Each specimen was examined by light and electron microscopy. Alkaline phosphatase histochemistry was carried out on 10 of the calcifying tendinitis samples and the myositis ossificans tissue was used for a positive control. Immunohistochemical labelling studies on samples from three patients were used to investigate whether collagen type II and type VI , hyaluronic acid binding region protein and chondroitin sulphate molecules could be detected in the pathological tendon. Electron probe x-ray microanalytical studies have were carried out to compare the calcium to phosphorous ratios in focal areas of mineral found in individual patients.

Results.

Pathological specimens from patients showed variable morphological changes. Although some areas were fibrocartilaginous and despite the presence of individual rounded chondrocyte-like cells (fig: 1), the tissue was dissimilar to that exhibiting pathological endochondral ossification (fig: 2). Alkaline phosphatase is an enzyme frequently associated with calcification but, histochemically, in mineralised human tendon alkaline phosphatase activity was absent.
Immunohistochemical labelling of matrix molecules associated with the pathological tissue has primarily been observed by light microscopy. These methods could not detect the presence of collagen type II in the pathological tendon. Bone had been formed in one specimen of calcified tendon. A significant difference was observed in the Ca/P molar ratio observed in this bone when compared with the calcific deposit found in the same tissue section (fig: 3). The ratios were all closer to the expected value for hydroxyapatite rather than pyrophosphate.





Discussion.

These results show that the fibrocartilaginous region associated with the pathological calcification is morphologically dissimilar to pathological endochondral ossification and the matrix components differ to those found in human articular cartilage.

The Ca/P ratios of calcospherites formed are significantly different to those of bone when both are found in the same area of tendon.

Although it is not yet possible to provide a specific pathological mechanism for calcifying tendinitis, it appears not to be akin to endochondral ossification or primary bone formation, but may have features unique to tendon mineralisation.

Uhthoff, H.K. (1975): Calcifying Tendinitis, an Active Cell-Mediated Calcification. Virchows Arch. A Path. Anat. and Histol. 366, 51-58.