CELLULAR MECHANISMS INVOLVED IN THE BREAKDOWN OF ARTICULAR CARTILAGE

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SUMMARY

In most of the major diseases in rheumatology the chronic disabling factor is the loss of joint function through destruction of articular cartilage. The exact pathogenesis of the cartilage loss in these diseases is unknown and a clearer understanding of the precise mechanisms involved is essential for the formulation of specific therapy.

An organ culture system has been used to study the possible role of different articular cells and tissues in the destruction of cartilage matrix. This technique provides a closed, controlled environment in which to examine the potential capabilities of living tissues. Most of the work has been done on articular tissues from normal young pigs. It was impossible to dissect equivalent explants of synovium but finely divided synovial tissue explanted in measured volumes gave viable cultures.

Minced synovial tissue causes a drastic breakdown of articular cartilage when the two tissues are cultured in contact and there is loss of proteoglycan and collagen by 14 days. This effect is also seen in dead cartilage. Dead cartilage, however, is unaffected by synovial tissue if the two explants are not in contact, whereas living cartilage becomes depleted of proteoglycan and collagen with fibroblastic transformation of some of the chondrocytes. Thus, synovial tissue has a direct, presumably enzymatic effect on cartilage matrix and also an indirect destructive effect mediated through the chondrocytes. This destructive effect is not confined to synovial tissue, and it has been shown that non-articular tissue such as blood vessel also produces both a direct and an indirect destructive effect on the cartilage matrix.

That chondrocytes of isolated cartilage have the ability to destroy cartilage matrix has been confirmed by the action of retinol on isolated cartilage. After 16 days' exposure to 5 or 10 iu retinol/ml culture medium there is almost total loss of proteoglycan and extensive breakdown of collagen with fibroblastic transformation of the chondrocytes. There is a regional variation in the degree of destruction which is greatest in the deeper zone.

The extent of cartilage matrix loss depends on the net effect of the various destructive processes. This is clearly demonstrated by the action of hyperoxia (55% O_2) on cartilage and synovium. Hyperoxia increases the breakdown by synovial tissue of the collagen of cartilage matrix, stimulates the release of collagenase from the synovium but inhibits the indirect collagenolytic effect.

An attempt has been made to study the role of the mononuclear leucocytes in the destruction of cartilage since these cells are found in large numbers in the synovium in chronic inflammatory arthritis. Cells obtained from the peripheral blood of young pigs greatly enhance the destructive effect of the synovium on the cartilage; the small number of monocytes present plays an essential part in the action of the leucocytes. Stimulation with phytohaem-agglutinin did not enhance the breakdown induced by the cells from peripheral blood but increased that caused by cells derived from lymph glands.

The maintenance of cartilage matrix depends on the dynamic balance between synthesis and breakdown of the structural macro-molecules. Some earlier results suggested that the synovium might impair the synthesis of proteoglycan by the chondrocytes as well as degrading the existing matrix. This has been examined in detail

by studying the incorporation of $^{35}SO_4$ into proteoglycan. The synovium was found to inhibit the synthesis of proteoglycan by chondrocytes in the original explant, but cells that have emigrated from the explant seem to be unaffected and often form new cartilage on the cut surface of the old. The inhibitory effect of the synovium is reversible.

Some preliminary experiments have been made on human articular cartilage in culture. Pig synovial tissue, which has such a destructive effect on pig cartilage, causes little breakdown of matrix in human cartilage from the femoral head during 16 days' cultivation. On the other hand, it severely inhibits the synthesis of proteoglycan by the human chondrocytes.

These results demonstrate the complexity of the processes involved in the breakdown of cartilage. They also show that the actions and interactions of the different tissues can be dissected apart by means of the organ culture technique to provide a better insight into the mechanisms involved. This should allow a more rational approach to the study of the pathogenesis of cartilage destruction <u>in vivo</u>.

CHAPTER 1

INTRODUCTION

Arthritis is a major cause of disability. Osteoarthritis affects more than 80% of the population over the age of sixty years (1) and there are at least one million people with rheumatoid arthritis in the United Kingdom (2). In both diseases loss of cartilage is a prominent feature resulting in permanent joint deformity and loss of function. The detailed mechanism of cartilage destruction in either disease is poorly understood and there is little evidence that any of the currently available therapeutic measures alter the progress of the cartilage destruction.

In the inflammed joint there are many different processes active at any one time. Concurrent features are proliferation of the intimal cells, infiltration of lymphocytes and plasma cells, accumulation of polymorphonuclear leucocytes in the synovial cavity, hyperaemia of the synovium, pannus formation and destruction of the articular cartilage (3). In this complex situation it is difficult to sort out the factors which are important in the pathogenesis of the cartilage erosion and those which are epiphenomena. Even in the simpler situation found in osteoarthritis there are a variety of features such as fibrillation of cartilage, proliferation of chondrocytes, sclerosis of subchondral bone, osteophyte formation and subsynovial fibrosis (4). Synovial intimal cells, polymorphonuclear leucocytes and chondrocytes can all cause breakdown of the cartilage matrix but the relative importance of these cells in any one disease is not clear. To add to the problem very little is known about the mechanisms which control these destructive processes.

In the hope of understanding further the mechanisms of cartilage destruction that are active in these diseases, a study has been made

of the breakdown of normal cartilage using an *in vitro* organ culture technique. This technique can provide information about the potential capabilities of the cells and tissues. The interaction between different tissues and the factors controlling the behaviour of the tissues in culture can be studied. It is likely that articular tissues can respond in only a limited number of ways and therefore it is hoped that information on the ability of different cell types to breakdown cartilage matrix *in vitro* will provide a better understanding of the pathogenic mechanisms of cartilage breakdown *in vivo*. This information might suggest therapeutic possibilities.

Articular joints are subject to a variety of mechanical forces which undoubtably play a role in the normal function of the cartilage and probably also in some aspects of cartilage destruction (5). This study, however, has been concerned with the cellular and biochemical responses of articular tissues and not with the mechanical aspects of joint damage.

CHAPTER 2

HISTORICAL REVIEW

As the experimental work of this thesis is concerned with the breakdown of articular cartilage, the pathological and experimental data on the destruction of articular cartilage will be reviewed. This will not be confined to the active loss of matrix components but will also consider the turnover of matrix and the proliferation and transformation of the chondrocytes.

To help interpret this evidence and the subsequent experimental results the structure and function of normal articular tissue will be briefly described.

I STRUCTURE OF ARTICULAR TISSUES

(a) Structure and Metabolism of Articular Cartilage

The normal articular cartilage consists of two major components: the chondrocytes which occur throughout the cartilage, and the matrix which consists of a fibrous network of collagen and hydrated proteoglycan. There are no blood vessels, lymphatics or nerves in adult articular cartilage (6).

The chondrocytes The chondrocytes are distributed throughout the cartilage but display morphological and functional variations in different regions. Collins (7) has described four zones in adult cartilage, but in immature articular cartilage Vaughan (8) describes three ill-defined layers of cells: (i) a superficial layer (zone 1) in which the cells are flattened and small and lie with their long axes running parallel to the joint surfaces; (ii) an intermediate layer (zone 2) where the cells are somewhat larger and arranged in groups of two or four; (iii) a layer adjacent to the epiphyseal bone (zone 3) where the cells are hypertrophic and arranged roughly

roughly in columns at right angles to the surface. The fourth zone in Collins' classification is a region adjacent to the underlying bone where the matrix is calcified.

Typical chondrocytes are ovoid with a maximum diameter of 10 to 30 um (9). In section the cell has a scalloped edge with cytoplasmic processes projecting beyond the lacuna in which the cell resides. The adult chondrocyte has few mitochondria and the predominant cytoplasmic organelles are the granular endoplasmic reticulum and the Golgi complex. The cells in the superficial zone have the morphology of fibroblasts, rather than chondrocytes (10,11). Chondrocytes have normal major histocompatibility antigens on their surface (12).

The matrix The collagen and proteoglycan account for most of the organic matter in the extracellular matrix (13) and are responsible for the mechanical properties of the cartilage that allow it to withstand the forces exerted through the joint (14).

The proteoglycans of cartilage are large molecules composed of hydrophilic carbohydrate chains covalently linked to protein. As many as 50 to 100 chains may be attached to one protein molecule and between 20 and 30 of these protein molecules may be bound to a single hyaluronate molecule. The total molecular weight of this giant molecule is in the order of 50×10^6. The evidence for this has been reviewed recently (15). The majority of carbohydrate chains consist of two different sulphated glycosaminoglycans: chondroitin sulphate which has a repeating disaccharide unit of glucuronic acid and N-acetyl galactosamine, with about 25-30 units in each molecule, and keratan sulphate which has a repeating unit of N-acetyl glucosamine and galactose and about 13 units per molecule.

Although both chondroitin and keratan sulphate are attached to the same protein molecule there are variations in the relative

concentrations of these glycosaminoglycans. The assembly of glycosaminoglycans onto the protein core does not seem to be random since more keratan sulphate is attached to the end that is linked to the hyaluronic acid (16). The concentration of proteoglycan is highest in the deeper zones of the cartilage (17) where the proportion of keratan sulphate is greatest (18). The composition of the matrix also varies within the deep zones with less keratan sulphate adjacent to the chondrocytes.

It has been proposed that there are different pools of proteoglycan in the matrix with different structures (19) and rates of turnover (20). While the reasons for the regional and structural variations of the proteoglycan are unknown, it is thought that the major function of the proteoglycan is to hold water within the cartilage matrix during periods of mechanical compression and so act as a cushion protecting both the collagen fibres and the chondrocytes and also reducing the forces transmitted through to the underlying bone (21).

The collagen in the cartilage is a fibrous protein composed of three identical polypeptide chains (α chains) arranged in a triple helix. This particular form of collagen seems to be confined to cartilage and is classified as type II collagen. The polypeptide chain undergoes intracellular hydroxylation, glycosylation and disulphide bonding before assembly into the triple helix. This procollagen is secreted from the cell with extra terminal peptides which are lost in the final assembly of the collagen fibre. The final collagen fibre consists of many triple helix subunits covalently linked. Further details of the biochemistry of the structure and function of collagen are to be found in a recent review (22). Although type II collagen seems to be unique to hyaline cartilage it is not yet possible to relate the primary structural differences of the different collagens to their

functional roles (23). Not all cartilage has type II collagen since the fibrocartilage in the menisci within the knee joint has type I collagen (24).

While the structure of the matrix is known in considerable detail we have very little information about the turnover of the component molecules. A variety of enzymes have been found in articular cartilage which are capable of degrading the glycosaminoglycans, the proteoglycan core proteins and collagen (25,26,27,28,29), but the physiological role of these enzymes is unknown.

Cartilage matrix does more than just withstand mechanical loads. The proteoglycan fraction has been found to inhibit calcification (30), and the matrix contains extractable factors, which inhibit the invasion of vascular tissue (31), and also proteinase inhibitors (32,33).

Several age related changes have been described in cartilage. The proportion of keratan to chondroitin sulphate increases during growth and development. Keratan sulphate is almost absent in very young cartilage and in man reaches a plateau level during the third and fourth decades (34,35). Similarly in the foetal pig the articular cartilage has a ratio of 1:27 keratan to chondroitin sulphate which changes to 1:4 in three year old animals (36). It has been suggested that keratan sulphate is formed preferentially under anaerobic conditions and that such conditions occur when the layer of calcified cartilage appears forming a barrier between the marrow and the articular cartilage (35). Other work, however, suggests that since the distribution of collagen, chondroitin and keratan sulphate vary greatly between individuals it is likely to be genetically determined rather than related to age or sex (37). The amount of lipid in the cartilage matrix rises with

age which is found mostly in the superficial zone (38). Senescent pigment has been described predominantly in the deeper layers (39). The functional significance of these observations is unknown.

Metabolism of cartilage The route of nutrition for articular cartilage depends on the maturity of the tissue. The joint cartilage from immature animals is not only a weight-bearing surface but also part of the epiphyseal nucleus. This cartilage derives its nutrition from both the synovial fluid and the underlying marrow cavity (40). A study on lambs suggests that growing cartilage derives a significant part of its nutrition from the underlying marrow (41). Adult cartilage, however, seems to rely entirely on the synovial fluid (42,43). This view is supported by the observation that severe osteonecrosis of the femoral head does not seem to affect the metabolism of the chondrocytes in the femoral articular cartilage (44).

The diffusion of dyes into cartilage seems to diminish if the tissue has been dead for more than one hour (42) but the diffusion of sulphate is the same in living and dead cartilage (45).

Not much is known about the nutritional requirements of articular chondrocytes. They have an active glycolytic system and enzymes from the Krebs cycle have been identified (47). There is a low but detectable uptake of oxygen (46) and the oxygen tension in cartilage is much lower than in vascular tissue (48). The optimum oxygen tension *in vivo* is unknown, but high or low tensions reduce the synthetic activity of the chondrocytes *in vitro* (49,50). Chondrocytes are, however, much more resistant to hypoxia than other cells (51).

One of the major metabolic activities of the chondrocyte is the production of the extracellular macromolecules. Proteoglycan

has a half-life of 8 days (52) or 200 days (45) depending on the source of tissue. $^{35}SO_4$ is rapidly taken up into the Golgi of the chondrocyte and then extruded into the matrix bound to proteoglycan (53,54,55). The superficial cells synthesize less proteoglycan than the deeper layers (56,57) and there is evidence that some chondrocytes secrete chondroitin sulphate-rich proteoglycan while others secrete keratan sulphate-rich proteoglycan (58). There is little information on the collagen metabolism in adult articular cartilage (9); this is partly because the rate of synthesis of collagen in mature cartilage is very low (59).

(b) Structure and Metabolism of the Synovium

The morphdogy of the synovium varies widely in different joints and in different regions of the same joint. It consists of a lining layer, the intima, usually one to three cells thick resting on a vascular subintimal tissue which may be adipose or fibrous according to its site. The synovium extends over villi which project into the joint cavity. There is no basement membrane under the intima (60). In a study of the human synovial intima two distinct cell types were found: phagocytic cells resembling macrophages which were termed A cells and fibroblast-like cells that were termed B cells (61). There were also cells of an intermediate type. These observations have been confirmed in rabbits (62) and pigs (63). Both studies found the macrophage-like cell to be in the minority. In rabbits there was a change in the population of cells during *in vitro* culture, with a gradual loss of macrophage-like cells. Whether this was migration or transformation of the cells is not clear (62). In an ultrastructural study of synovial villi from the pig, explanted on a Millipore membrane and grown in organ culture, the intimal cells on the lower surface of the villus which were in contact with the

Millipore membrane resembled B type cells and the cells on the free surface appeared to be of the A type and were actively phagocytos (63). Further studies showed that cells of the A type had Fc surface receptors and resembled macrophages in culture (64). It was concluded that the intimal synoviocytes belonged to a single cell type that are part of the mononuclear phagocyte system. It is not known whether the cells are replaced by mitosis or by incorporation of new cells via the blood.

A variety of functions have been attributed to the synovial intima. The macrophage-like cells are thought to be important for the ingestion of autologous or foreign material that has gained access to the joint (60). Other functions of the intima include the synthesis and release of structural macromolecules including type I and type III collagen (24), proteoglycan (65,66), hyaluronic acid (67), and degradative enzymes such as hyaluronidase (68), other lysosomal enzymes (69) and collagenase (70). A polypeptide hormone which has been called connective tissue activating peptide has been isolated from synovial tissue (71). This hormone is found in various tissues and affects the synovial tissue by raising the metabolic activity of the cells with an increased release of hyaluronic acid.

The importance of these currently identified factors in the metabolism of normal or pathological joints is unknown. For instance, it is not clear whether the hyaluronic acid is important in the lubrication of the joint or whether the associated protein is more relevant (72). The evidence for a pathological role for the degradative enzymes will be discussed in the next Section.

II BREAKDOWN OF CARTILAGE MATRIX

It is generally thought that in rheumatoid arthritis synovial cells invade and destroy the matrix while in osteoarthritis the breakdown of cartilage is due to mechanical factors combined with enzymatic activity of the chondrocytes. The evidence for this and the breakdown of cartilage matrix in other pathological and experimental conditions will be reviewed in this Section.

(a) Rheumatoid Arthritis

Nichols and Richardson (73) were among the first people to suggest the concept of inflammed synovium eroding cartilage; they described rheumatoid arthritis as a "Proliferative arthritis . . . Where the pannus comes in contact with cartilage it usually produces a destruction and absorption of the cartilage." Over the years evidence has accumulated to support an active role for the synovial tissue.

Hammerman and co-workers showed that in culture cells from rheumatoid synovium caused the loss of proteoglycan from human cartilage whereas normal human synovial cells did not (74).

In one study of early rheumatoid arthritis the first recognisable disturbance was an acute synovitis (75). It has been suggested that this synovitis is directly correlated with the degree of cartilage breakdown (76) but other workers have not been able to corroborate this observation (77,78). The synovial changes include hyperplasia of the intimal cells, proliferation of the subsynovial tissue and infiltration of lymphocytes and plasma cells (3). An ultrastructural study of the synovium suggested that the macrophage-like A cells had many cytoplasmic granules which contained high levels of acid phosphatase (79). Elevated levels of other lysosomal enzymes have been found in rheumatoid synovium (80,81) and a recent study of

rheumatoid synovium by Poole and co-workers suggests that there is some extracellular release of the lysosomal enzyme cathepsin D (82). A dilemma that arises if lysosomal enzymes are to be implicated is that these are most active at an acid pH and the extracellular medium is neutral (83). It is possible, however, that there are local acidic micro-environments which allow the enzymes to be active. On the other hand, neutral proteinases which can degrade the matrix have been identified in rheumatoid synovium (84,85) and some of the results implicating cathepsin D might have been due to a contaminating neutral proteinase (86). Rheumatoid synovium has been shown to cause breakdown of collagen in autologous tendon (87). With the discovery of a specific enzyme for degrading collagen (88) it was not long before a similar enzyme was found in rheumatoid synovium (89,90,91) and this was postulated as being responsible for the collagen destruction (92). This enzyme is inactivated by synovial fluid owing to the presence of α_1 anti-trypsin and α_2 macroglobulin (93,94). A complex hypothesis has been put forward by Harris and co-workers (95) who suggest that collagenase from the pannus of rheumatoid arthritis is released in a latent form which is not inhibited by the proteinase inhibitors and is activated by some substance which perhaps simultaneously saturates local proteinase inhibitors (95). A less complex explanation is that the local release of collagenase at the interface of the cartilage and pannus is sufficiently high to have active enzyme. With the use of a specific antibody to synovial collagenase, Wooley and co-workers have demonstrated immunoreactive enzyme at the junction of cartilage and pannus (96). They have found no evidence for this collagenase in the chondrocytes and very little in the rest of the synovial membrane. The macrophage-like cells

from rheumatoid synovium release not only enzymes such as collagenase or lysozyme but also chemical mediators such as prostaglandins (97). The exact role of the prostaglandins, which are inflammatory mediators, is not known, but they may be involved in potentiating the disease.

A feature of rheumatoid synovitis is the large number of polymorphonuclear leucocytes present in the synovial fluid. Several workers have suggested that these cells play a role in cartilage breakdown. The presence of enzyme inhibitors in the synovial fluid, however, was thought to make the participation of these cells unlikely but ultrastructural studies have shown polymorphonuclear leucocytes adhering to the cartilage surface (98). In vitro, polymorphonuclear leucocytes can cause enzymatic degradation of proteoglycan (99,100,101). Ingestion of bacteria or aggregated IgG induces polymorphonuclear leucocytes to release a super oxide radical which is also able to degrade cartilage proteoglycan (102).

Hammerman, Barland and Janis (103) have suggested that the chondrocytes might be involved in the breakdown of the cartilage. They state that "the advancing margin of the pannus is, in reality, the retreating remains of the cartilage, in which chondrocytes, no longer trapped in their matrix lacunae, and surrounded by residual collagen fibres, have come to resemble fibroblasts." Two histological studies of early cartilage lesions in rheumatoid arthritis support this possibility (104,105). Mills studied 26 biopsies taken from the joint margin of the knee to include cartilage bone and synovium. He found no evidence of pannus but a chronic synovitis and a fibroblastic proliferation of the perichondrium with cells penetrating the articular cartilage. From the histological section it is impossible to determine whether the 'penetrating' fibroblasts

originated from the perichondrium or were transformed chondrocytes. Tateishi's report was an ultrastructural study of the junction between cartilage and synovium. No infiltrating cells were seen at the junction and Tateishi concluded that the "chondrocytes immediately adjacent to the pannus . . . divide into fibroblast like cells following alteration of the cartilage matrix." Kobayashi and Ziff describe several features of the junction between the cartilage and synovium, one of which is that phagocytic and fibroblastic cells invade the cartilage (106). The alternative explanation that the chondrocytes have transformed to fibroblast-like cells should be considered.

Chondrocytes may cause breakdown of cartilage matrix before the pannus reaches the cartilage since in early rheumatoid arthritis there is matrix depletion with no evidence of pannus formation (107). In a macroscopic study by Chaplin the cartilage at a distance from any eroded area was much softer than normal cartilage (108). These soft areas, however, were in contact with the synovial membrane covering the alar fat pad and regions which were in contact with other cartilage were unaffected. Decreased amounts of collagen, non-collagenous proteins and hexosamine have been found in apparently normal areas of cartilage from rheumatoid joints (109). Elevated levels of the degradative enzymes cathepsin D and β-acetyl glucosaminidase have also been discovered in cartilage not associated with pannus (110). Several ultrastructural studies of rheumatoid cartilage have shown changes in chondrocyte morphology in areas at a distance from the pannus (111,112) and in one recent report fragmentation of collagen and phagocytosis by the chondrocytes has been described (113).

(b) Osteoarthritis

The majority of cases of osteoarthritis are idiopathic (114) but some are a consequence of trauma, previous inflammatory arthritis, or metabolic diseases such as acromegaly (for review see Peyron, 115). This disease is best considered as the final common pathway of cartilage in response to many diverse stimuli.

The histological changes in osteoarthritis are characterised by loss of proteoglycan (116,117), fibrillation and splitting of the cartilage (118), some chondrocyte death and clusters of proliferating chondrocytes. There is progressive loss of cartilage thickness and eventually the cartilage disappears to expose underlying bone.

One of the earliest changes in the cartilage is an increase in the water content of the matrix (119). This appears before the decrease in the glycosaminoglycan content and is evidence that there is a primary alteration in the collagen meshwork allowing the hydrophilic proteoglycan to swell (120). This might be due to direct mechanical factors or to a biochemical alteration of the collagen. Osteoarthritic cartilage has been shown to contain elevated levels of proteoglycan-degrading enzymes (121, 122) and, in culture, it releases collagen-degrading enzymes (123,124). The reason for the increase in degradative enzymes is not known. Acid phosphatase and alkaline phosphatase levels are also raised in osteoarthritic cartilage and in the deeper layers the number of matrix vesicles, which are thought to contain hydroxyapatite, is increased (125,126).

It has been suggested that the alteration in chondrocyte function is a consequence of mechanical factors (5) but recently an hypothesis has been proposed suggesting the synovium as the

site of the (as yet unknown) primary lesion (127). The synovium is commonly involved in osteoarthritis with subsynovial fibrosis as a prominent feature (128,129). Inflammatory changes may occur in the synovium sometimes at an early stage of the disease but more often in the late stages, possibly due to shedding of cartilage fragments into the synovial fluid (130,131,132). The importance of the synovial tissue for normal cartilage function has been stressed by Creuss who found almost complete destruction of cartilage in slipped femoral epiphyses associated with extensive fibrotic reaction in the capsule and absence of thesynovial lining (133). He thought that the cartilage loss was secondary to the synovial disfunction. As stated in an earlier Section the adult cartilage derives all its nutrition from the synovium.

(c) Other Conditions Causing Cartilage Destruction

Although many arthritic diseases other than rheumatoid arthritis and osteoarthritis cause cartilage destruction the mechanism of matrix loss in most of them has not been studied. There is often a dearth of histological data and the changes in the cartilage are poorly understood.

Before we consider other diseases, an example of normal chondrocytes degrading the matrix may be mentioned. In the normal development of the human ulna the hyaline cartilage in the sigmoid notch undergoes destruction (134). In this area the matrix is completely resorbed by the chondrocytes leaving a mass of fibroblast-like cells in the notch.

Several diseases which are associated with crystal formation in connective tissue cause destruction of cartilage. In gout, crystals of sodium urate are deposited radially in the superficial portion of the articular cartilage (135). This sometimes leads to disintegration

of the articular cartilage by an unknown mechanism with secondary osteoarthritis, massive obliteration of the joint structure, or a chronic tophaceous synovitis with diffuse, non-specific acute and chronic inflammatory reaction and small areas of pannus. The factors which control these different patterns are unknown.

Although calcium pyrophosphate arthropathy can present with an acute synovitis similar to that of gout, the pattern of crystal deposition in the cartilage differs. The crystals appear first in the layers of the intermediate or mid zone and not the articular cartilage surface, the calcified layer or the bone (136). It was also noted that there was depletion of proteoglycan in the matrix of mildly involved areas. It is not known whether the loss of matrix is induced by the chondrocytes in the middle zone or is due to an alteration in the synthesis of proteoglycan (137).

In ochronotic arthropathy the crystals of homogentisic acid are also deposited in the deeper zones of the cartilage with no evidence of discoloration of the articular surface. This leads to focal areas of cartilage erosion exposing shaggy, fibrillated, black cartilage. Unlike osteoarthritis, however, the chondrocytes along the fissures of degrading cartilage do not proliferate (138). An ultrastructural study of the cartilage showed severe degradation of the collagen fibres (139) but it was not clear whether this was an early or a late change.

Recently it has been suggested that some of the features of osteoarthritis might be due to deposition of hydroxyapatite (140). Crystals of apatite have been identified in the deeper layers of the cartilage (141), but again it is not known whether they are a primary feature of the disease.

Chondromalacia patellae causes erosion of patellar cartilage mainly in young people. The earliest feature is softening of the cartilage with loss of proteoglycan (142), followed by fibrillation, fissuring and erosion of articular cartilage with formation of clusters of proliferating chondrocytes and sclerosis of subchondral bone (143). The histology suggests a primary role for the chondrocytes whose function might be altered by mechanical or humoral factors.

In relapsing polychondritis there is loss of proteoglycan from the cartilage matrix with preservation in the immediate perilacunar zones around the unaffected chondrocytes (144). Focal chondrolysis is seen with infiltration of mononuclear leucocytes, subsequent fibrosis, focal calcification and the formation of matrix vesicles around affected chondrocytes (145,146). Increased transformation of lymphocytes from patients with relapsing polychondritis when exposed to proteoglycan with an increase in the production of macrophage inhibition factor suggest a cell mediated response (147). From the histology it would seem that the depletion of matrix is caused by the infiltrating mononuclear cells and not primarily by the chondrocytes.

The early lesion in ankylosing spondylitis has been described as resembling polychondritis more closely than rheumatoid arthritis (148). The earliest change is a local inflammatory erosion of cartilage with infiltration of mononuclear leucocytes at the cartilage margins which is followed by fibrous and bony ankylosis (149). Later changes in the synovium are very similar to rheumatoid arthritis with villous hypertrophy, hyperplasia and infiltration of lymphocytes, plasma cells and histiocytes (150).

The study of the pathogenetic mechanisms involved in cartilage breakdown is complicated by the similarity of the histological changes

in many of the diseases. For example in systemic lupus erythematosis synovial changes indistinguishable from those of rheumatoid arthritis may occur in the absence of any erosion or other destructive effects in the cartilage. On the other hand, in a small minority of cases both synovitis and erosion of cartilage are present (151).

In summary, there appears to be an active role for the chondrocytes and synovium in the destruction of the cartilage matrix in many types of arthritis. Study of the human pathology has not yet unravelled these problems, an understanding of which is crucial to the control of the diseases.

(d) In Vivo Experiments

In an attempt to simulate rheumatoid arthritis many experimental models have been described. Most of these involve the parenteral administration of material into laboratory animals. Page-Thomas has considered these experiments in two broad groups: a direct inflammatory arthritis caused by the injection of arthritogenic substances into the joint and an experimental allergic arthritis induced by the injection of antigen into the joint in a previously sensitised animal (152).

Early work with noxious substances such as turpentine injected into the joint are difficult to interpret but the injection of certain macromolecules has produced some parallels with rheumatoid arthritis.

An example of a direct inflammatory arthritis is the intra-articular injection into rabbit knees of the mucopolysaccharide carragenin (153). Repeated injections produce a chronic joint lesion with synovial hyperplasia, lymphocytic foci, fibrin deposition and marginal erosions of cartilage. Recent studies of this model

have shown that one of the earliest features is loss of proteoglycan from the matrix with up to 60% lost after a single injection (154). Further injections cause only a small decrease in the proteoglycan but instead of the proteoglycan being replaced as occurred after a single injection the cartilage is unable to recover. This absence of recovery coincides with the appearance of erosion in the cartilage surface. Although histological features of inflammatory arthritis can be seen the very extensive loss of proteoglycan from the matrix in the absence of invading pannus suggests that either the matrix is being depleted by active enzymes from the synovial fluid or that the chondrocytes have a direct role in the response to the carragenin. It is not easy in the animal model to decide which of the mechanisms is active.

A variety of other agents injected directly into joints have been shown to induce a synovitis with variable cartilage breakdown. These include streptococcal cell walls (155), E.coli (156), immune complexes (157), purified gammaglobulin (158), poly-lysine (152), cartilage polysaccharide (159), blood (160), filipin (161,162), lymphokines (163), and lectins (164). In all these models an active role for the synovial tissue or the chondrocytes is possible. The chondrocytes have been implicated in the arthritis caused by filipin (161).

The model of direct inflammatory arthritis tends to be a self-limiting arthritis which can last up to a year but often settles much earlier (152). The experimental model of allergic arthritis, however, has a much more chronic course. The arthritis is induced by injecting an antigen into the joint of a previously sensitised animal. For the sensitising procedure Freund's complete adjuvant is usually utilised. This system was originally described by Frizdberger in 1913 (152)

but is commonly known as the Glynn Dumonde model after their description of the arthritis induced in rabbits by this technique using human fibrin as the antigen (165). This model produces synovial cell hyperplasia, perivascular accumulation of lymphocytes, lymphoid follicles, diffuse plasma cell infiltration, pannus and cartilage erosion. It has been suggested that the chronicity of the arthritis is due to deposition of immune complexes within the cartilage; there is some evidence for the existence of such complexes in the cartilage (166).

The chondrocytes might be contributing to the breakdown of the matrix. Histochemical changes are seen in cells at a distance from the invading pannus (167). Elevated levels of alkaline phosphatase, acid phosphatase and ATP ase are found in the deeper zones of cartilage from the arthritic joint, and there is diffuse loss of proteoglycan from the matrix unrelated to the invading pannus.

Recently an allergic model has been described which does not rely on any intra-articular stimulus to produce the arthritis (168). An acute arthritis was induced in one fifth of the rabbits studied, by the repeated intravenous injection of bovine serum. Histological changes of synovitis and pannus-formation were seen, and immune complexes were found in the joint tissues (169). Again the exact mechanism of matrix destruction cannot be deduced from these experiments.

The consequences of enzymatic depletion of cartilage were first shown by Thomas after the intravenous injection of crude papain into rabbits (170). This produced reversible collapse of the ears and depletion of proteoglycan in all cartilaginous tissues. Repeated intra-articular injection of papain into rabbit knees causes degradation of the cartilage especially in the weight bearing regions (171).

Excess vitamin A (retinol), which is known to stimulate the release of lysosomal enzymes (172), produces depletion of proteoglycan in the cartilage matrix of rabbit knees (173,174), and excess of intra-articular vitamin A causes osteoarthritic changes in the animals (175).

There are several other models for human osteoarthritis which cause proteoglycan depletion, chondrocyte proliferation and fibrillation of cartilage. Experimental rupture of the cruciate ligament in dogs invariably produces osteoarthritis (176). This model has been studied in detail by Muir and co-workers and the sequence of events seems to be very similar to natural osteoarthritis in dogs and humans (177) (for review see Muir, 21). Study of the early changes demonstrates that an increase in water content is one of the first detectable events, suggesting widespread damage to the collagen network which could be either mechanically or chemically induced. It is also of interest that there is microscopic evidence of osteophyte formation within 3 days of the rupture of the ligament, before there is loss of proteoglycan or of collagen (178).

Other models which mimic human osteoarthritis include joint compression (179), partial menisectomy (180,181), and intra-articular steroid injection (182). Scarification of the cartilage fails to produce osteoarthritis consistently (183,184,185).

Cartilage necrosis by cold injury is of interest because it fails to produce degenerative changes in vivo (186). Areas of articular cartilage in rabbits were subjected to local freezing. This killed the chondrocytes but did not denature the collagen. After 10 days there was loss of proteoglycan but the breakdown

of cartilage seemed to stop at that stage and even after 6 months there was no visible destruction of the collagen.

The loss of proteoglycan, therefore, does not necessarily result in the mechanical breakdown of collagen and suggests that living chondrocytes may be implicated when such destruction takes place.

Although synovium clearly has a destructive role in inflammatory arthritis normal synovium also may have a deleterious effect on articular cartilage when the tissues are in contact. This has been shown in a study of persistent joint deformities in rabbit knees (187). In longstanding and persistent joint deformity the articular cartilage is grossly abnormal: sometimes it is completely absent over the portion of the joint surface which is no longer in contact with the opposing articular surface, but which is in continuous contact with the synovial membrane. Remobilisation of the joint fails to induce recovery and the pathological changes progress to osteoarthritis. Articular cartilage may also be resorbed by normal tissue when homografts are transplanted into a joint (188,189,190) or into the muscle (188).

Finally, a study of intra-articular loose bodies suggested that there is transformation of a fractured portion of the articular surface from an angular to a rounded body. The peripheral chondrocytes lyse the surrounding matrix and revert to fibroblasts before proliferating and forming fibrocartilage (191).

In summary, in many of the animal models for human arthritis there is evidence that both the synovial cells and the chondrocytes participate in the loss of the cartilage matrix. The relative importance of these or other cell types has not been determined.

(e) In Vitro Experiments

For most of the *in vitro* experiments on the breakdown of cartilage matrix an organ culture technique has been used. The object of the technique is to keep differentiated tissues alive and functional *in vitro* and is discussed in detail in the Chapter on methods.

Excess vitamin A was found to have a drastic effect on the cartilage matrix of embryonic chick limb bone rudiments (192). After 10 days in culture the cells were not dead but the matrix was severely depleted. This was not just a toxic effect as higher doses of the vitamin were less effective in causing breakdown. Vitamin A was found to inhibit the incorporation of sulphate into cartilage matrix and also caused release of that already present (193). Further studies indicated that the vitamin greatly increased the synthesis and the extracellular release of an acid protease (194) and this enzyme release was later shown to be largely responsible for the degradation of the cartilage matrix (195). Studies on lysosomes suggested that vitamin A had a direct effect on the membrane causing release of the lysosomal enzymes without disruption of the lysosome (172).

The matrix of chick limb bone rudiments can also be degraded by culturing the rudiments in an atmosphere of 85% oxygen (196). Again this is thought to be due to increased release of lysosomal enzymes (197).

The cartilage matrix in embryonic chick limb bones is affected by antibodies raised against tissue antigens (for review see Fell, 198). In the earliest experiment rabbit antiserum to minced foetal mouse tissue caused resorption of bone and loss of metachromasia

from the terminal cartilage of the foetal mouse bone (199). This work was repeated with chick limb bone rudiments and an antiserum to chick erythrocytes. The antiserum caused extensive breakdown of the cartilage matrix and some resorption of bone. The effects were complement dependent. The osteoblasts and chondrocytes became fibroblast-like (200). There was a change in the lysosomal physiology leading to extrusion of lysosomal enzymes (201). Antibodies of the IgG or IgM classes raised against chick erythrocytes were effective but an antiserum against serum protein was ineffective (202). Although the result is very similar to the effect of vitamin A the antiserum exerts its effect through the cell surface and not directly on the lysosomal membranes (203). The exact mechanism by which the antibodies act is not known.

The addition of sucrose to cultures causes loss of proteoglycan from the articular cartilage of chick limb bone rudiments with an increased production and release of lysosomal enzymes. The sucrose is taken up by the articular chondrocytes and perichondral tissue and accumulates in the lysosomes (204). When transferred to normal medium the sucrose is lost from the cells and the synthesis and release of acid protease returns to normal (205).

There are at least two major disadvantages in the use of limb bone rudiments. First, the embryonic epiphyseal chondrocytes will not necessarily reflect the behaviour of adult articular chondrocytes and second, the cartilage is surrounded by a layer of fibrous perichondral tissue which might influence the behaviour of the chondrocytes. Much of the earlier work has, therefore, been repeated with post-foetal articular cartilage. A study of excess vitamin A on pig articular cartilage suggested that isolated cartilage was only

slightly affected by the vitamin and that the presence of other tissues such as marrow or joint capsule were required to produce severe depletion (206). With this associated tissue, there was extensive breakdown of proteoglycan and collagen with transformation of the chondrocytes to fibroblast-like cells. There seemed to be little recovery after removal of the excess vitamin. With slightly different culture conditions, however, the vitamin was found to have a direct effect on cartilage with loss of proteoglycan (207).

The effect of rabbit antiserum to pig erythrocytes on the pig articular tissues has been extensively studied by Fell and co-workers (for review see Fell, 198). The antiserum had no deleterious effect on isolated hyaline cartilage but in the presence of joint capsule or marrow there was extensive breakdown of matrix. This effect is complement dependent. It was found that IgG was unable to penetrate normal hyaline cartilage beyond the superficial fibrous layer (208). If, however, the proteoglycan was removed from the matrix then the antiserum entered the cartilage and caused necrosis of the chondrocytes. The depletion of proteoglycan could be caused either by pretreatment of the cartilage with exogenous enzymes, such as trypsin (209), or by the enzymatic breakdown induced by invading marrow (207) and joint capsule (210).

The destructive effect of the antiserum is reversible if proteoglycan only has been lost, but if the breakdown has progressed as far as extensive loss of collagen then the process seems to be irreversible. This was found with both subchondral marrow (211) and capsular tissue (212). Interestingly, the antiserum reacted strongly with the chondrocytes but not with the fibroblast-like cells (211).

III CONTROL OF SYNTHESIS OF CARTILAGE PROTEOGLYCAN

The proteoglycan is an essential component of the cartilage matrix and extensive loss produces severe alterations in the physical properties of the cartilage (213). As described earlier loss of the proteoglycan is an early feature of rheumatoid arthritis, osteoarthritis, and in many of the experimental models of arthritis. The factors that control the synthesis of proteoglycan are central to any study of cartilage changes in arthritis. In adult articular cartilage the proteoglycan has a high rate of turnover, unlike the collagen which is very low, and hence inhibition of synthesis of the proteoglycan will produce depletion in the matrix.

A characteristic feature of cartilage proteoglycan is the amount of sulphate that it contains. The incorporation of radioactive sulphate ($^{35}SO_4$), therefore, can be used as a method for assessing the active synthesis of sulphated proteoglycans (214). $^{35}SO_4$ is incorporated into growing cartilage; it is first taken up by the chondrocytes and then released into the matrix bound to the proteoglycan (215).

Adult cartilage also incorporates $^{35}SO_4$ (216,217) and some authors claim that in osteoarthritis, despite the relative depletion of proteoglycan, the rate of synthesis is increased (216,218,219,220, 221). A similar study on human femoral head cartilage, however, failed to show any increase in the uptake of $^{35}SO_4$ in the osteo-arthritis cartilage (222).

Rheumatoid arthritic cartilage in culture incorporates more $^{35}SO_4$ if the cartilage comes from an eroded region than if it is taken from a non-eroded region in the same joint (223). Since other studies suggest that even the macroscopically intact areas

are often affected in rheumatoid arthritis it is difficult to interpret this observation. Rheumatoid synovial tissue has been shown to inhibit the synthesis of sulphated proteoglycan in rabbit articular cartilage (224) and bovine nasal cartilage (225). In the first study the used medium from cultures of rheumatoid tissue inhibited proteoglycan synthesis but that from normal synovial tissue did not. The effect was prevented by indomethacin and it was suggested that the active factor might be a prostaglandin.

A similar inhibition of synthesis has been found in the carragenin-induced arthritis in rabbits (226) and in antigen induced arthritis (227). A single injection of carragenin caused a 50% decrease in the rate of proteoglycan synthesis in the articular cartilage.

Experiments have shown that a variety of other factors decrease the synthesis of proteoglycan by chondrocytes. These include cortisol (228,229), a serum factor which might be a glucocorticoid (230), oestradiol (231), a pituitary factor (232), hyaluronic acid (233), lymphokines (234), hyperoxia (235), prostaglandins (236), diphosphonates (237), aspirin (238), and immobilisation of the joint (239,240,241).

Opposing this heterogenous group are factors which stimulate the synthesis of proteoglycan in cartilage. These include growth hormone *in vivo* (242) but not *in vitro*, somatomedins (243), insulin (243), non-suppressible insulin-like activity (244), thyroxine *in vivo* (245) but not *in vitro* (243), intracellular cyclic AMP (246) and the interruption of the nerves to the joint (247). Enzymatic depletion of the cartilage stimulates the synthesis of proteoglycan to replenish the matrix (248,209,212).

The relative importance of this bewildering array of potential controlling factors is unknown. Some factors such as growth hormone and thyroxine do not seem to have a direct effect on the synthesis of proteoglycan by post foetal chondrocytes but are mediated through other hormones. Much of the experimental work has been on foetal or epiphyseal cartilage or in immature laboratory animals and it cannot be assumed that these data will apply to adult articular cartilage. For instance, thyroxine stimulates chondrogenesis in embryonic chick chondrocytes (249) but has no detectable effect on rabbit articular chondrocytes (250). The response to somatomedin by rabbit articular chondrocytes is different from rabbit epiphyseal chondrocytes (251).

It would appear that inhibition of synthesis of proteoglycan does not necessarily mean increased destruction of cartilage. Thus Rosner and co-workers showed that in osteoarthritis induced by partial menisectomy in the rabbit, oestradiol suppressed the synthesis of proteoglycan in both the experimental and control joints but the severity of the osteoarthritis was not affected by the hormone (252). It is possible that the steroid is having other effects which might counter the diminished synthesis of proteoglycan, such as reduction of the release of catabolic enzymes.

IV PROLIFERATION AND TRANSFORMATION OF ARTICULAR CHONDROCYTES

The growth of articular cartilage is by an interstitial process rather than by appositional growth (253). A study of rats which had been exposed briefly to ^{3}H-thymidine while growing showed the persistence of the label in the adult chondrocytes which would not

be expected if the cells were dividing frequently (254). Blackwood (255) showed that ^{3}H-thymidine was taken up in the intermediate zone and that the label progressed down to the hypertrophic zone. He could find no evidence of migration to the articular surface. A study by Mankin (256,257) in rabbits suggested that there are three different patterns of cell division depending on the age of the animal. In the newborn the mitotic figures were evenly distributed throughout the articular cartilage. In two month old animals the mitoses were in two separate zones: one 5-12 cells deep lying parallel to the articular surface and about 5 cells in thickness and another 10-15 cells in thickness lying 5-15 cells above the calcifying zone. He postulated that the superficial zone was supplying the new cells for the articular surface and that the deeper zone was the chondroblastic phase of endochondral ossification of the epiphyseal nucleus. In older animals (four months) mitoses were only found in the basal layer above the calcified cartilage. No mitosis was seen in mature animals. Although cell division has not been described in articular cartilage from normal adults, it is impossible to say that the chondrocytes never divide.

Structural damage to adult articular cartilage is poorly repaired by chondrocytes. During the process of cartilage loss the chondrocytes either die or cease to resemble chondrocytes. It is important to know whether or not the chondrocytes could proliferate and retain the ability to produce normal extracellular matrix.

Chondrocytes have at least three potential ways of responding to a situation. They may be unable to replicate in which case the cartilage will have only a limited capacity for repair. They may proliferate and maintain their chondrogenic form; this will mean that the cartilage has the potential to repair substantial defects.

Lastly, they may dedifferentiate and cease to express the features of a chondrocyte; in this situation the chondrocytes might augment any breakdown in the cartilage matrix. There is evidence that chondrocytes can both proliferate as chondrocytes and dedifferentiate to fibroblast-like cells under suitable conditions.

Clusters of chondrocytes surrounded by proteoglycan, have been recognised for a long time in osteoarthritic cartilage. It was suggested that these clusters merely represented original surviving chondrocytes brought together by depletion of the intercellular matrix, but it is now thought that the clusters are due to proliferation of the chondrocytes. Increased DNA synthesis has been shown in osteoarthritic cartilage (258) and in this disease "the chondrocytes . . . seem to revert to a chondroblastic state and are capable of making new cells and matrix at a much more rapid rate than is normally seen." (259). An ultrastructural study of osteoarthritic cartilage showed that the organellar pattern of the cells was consistent with an immature chondrocyte (260). In chondromalacia patellae the histological changes are very similar to osteoarthritis and the chondrocytes in the deeper layer are hypertrophic and show mitotic activity (261).

Adult articular chondrocytes can undergo dramatic proliferation in acromegaly. In this condition the cartilage is thickened with clusters of large active basal chondrocytes. Ulceration of the cartilage also occurs "because of unequal proliferation and matrix formation in the mid zone, creating stress in the interterritorial areas and ultimately fissuring" (262). The exact mechanism is not known but probably acts through somatomedin (263).

Proliferation of articular chondrocytes has been produced experimentally <u>in vivo</u> after scarification of the cartilage in the

rabbit (264), partial menisectomy of the rabbit knee (265), papain-induced arthritis in rabbits (266), in the repair of surgical defects in the dog (267), and in experimental haemarthrosis in the dog (268). In the last example pannus was seen in the haemarthrotic joint and the cartilage underlying areas of synovial proliferation had increased numbers of chondrocytes.

As to the question of whether chondrocytes can dedifferentiate there have been few studies in human disease. One investigation of the ultrastructure of chondrocytes in chondromalacia patellae showed that the superficial cells resembled fibroblasts (269). A fibroblastic transformation has been suggested in rheumatoid arthritis; from an ultrastructural examination of the junction between synovium and cartilage it was suggested that "chondrocytes immediately adjacent to the pannus presumably divide into fibroblast like cells following alteration of cartilage matrix." (105).

There is ample evidence that, in culture, the chondrocytes can dedifferentiate (270,200). Some factors which control the chondrocyte are known. A fraction of high molecular weight from embryo extract enhances dedifferentiation whereas one of low molecular weight allows dedifferentiated cells to revert to a chondrogenic phenotype (271). Addition of vitamin C to monolayer cultures is essential for the maintenance of typically chondrified masses of cells (272). Chondrocytes grown in monolayer cultures tend to dedifferentiate with failure to synthesise matrix, but if the cells are aggregated and grown in organ culture the cells retain their capacity to produce matrix (273). A similar result was obtained if the chondrocytes were maintained in spinner cultures where they formed large quantities of proteoglycan which was not seen in monolayer cultures (274). Not only can dedifferentiated chondrocytes

return to a chondrogenic form but it has been reported that fibrocytes can transform to typical rounded cartilage cells by mechanical forces (275).

Further evidence of the transformation of chondrocytes comes from examination of the type of collagen synthesised. The collagen in normal hyaline cartilage is exclusively of type II and synthesis of this type of collagen is indicative of the normal phenotypic expression of articular chondrocytes. Where the chondrocytes appear to have dedifferentiated type I is synthesised and sometimes type III. Chondrocytes in culture readily switch to producing type I collagen (276,277) and in organ culture a switch to type I and III has been noted (278,279). These forms of collagen have been found in articular cartilage from human osteoarthritis (280,281) but other workers have been unable to confirm this observation (282,21). In rheumatoid arthritis, with synovial pannus so intimately related to the cartilage it is impossible to tell whether type I and III collagens are produced by the synovial cells or by transformed chondrocytes.

There is evidence, therefore, that under suitable conditions, adult articular chondrocytes can proliferate and remain in a chondrogenic form and under other conditions dedifferentiate to a fibroblast-like cell. Obviously a clearer understanding of the factors that determine this phenotypic expression are essential to our control of cartilage function.

CHAPTER 3

MATERIALS AND METHODS

In nearly all the experiments reported in this thesis the articular tissues from young pigs were used; the only exception is some work on human cartilage described in Chapter . The pig was chosen as a source of material partly because it was readily obtainable, but mainly because pigs suffer from a variety of arthritic diseases which are similar to the human conditions (for review see Roberts and Doyle, 283). Osteoarthritis is a common affliction in pigs and the early changes in cartilage are biochemically and histologically similar to those in human disease (284). Pigs also suffer from inflammatory arthritis caused by infectious agents such as erysipelothrix insidiosa, mycoplasma hyorhinis and clostridium perfringens, all of which produce erosion of cartilage with synovial proliferation and pannus formation (285, 286,287). In the erysipelothrix infection a chronic form develops where no infectious agent can be found and the histological changes in the joint are identical with those in rheumatoid arthritis.

It was felt, therefore, that an understanding of the mechanisms of breakdown in the pig cartilage might help unravel the process in human arthritis.

I MATERIALS

(a) Culture Medium

The tissues were grown in a chemically defined medium with the addition of 15% serum. Two chemically defined media were used. Some of the early experiments were made with the chemically defined medium BGJ_5 (288) supplemented with 5 mg % sodium acetate and 15 mg

% ascorbic acid (289). Similar results were obtained with Dulbecco's modification of Eagle's medium (290) supplemented with 12 mg % ascorbic acid and this medium was used in most of the work. Both media have a bicarbonate buffer.

In experiments with pig leucocytes 15% normal pig serum (NPS) was added to the chemically defined medium. The serum which was obtained from the local slaughterhouse, was heat inactivated (56° for 30 mins) and filtered (0.22 μm Millipore filter) before use. In all other experiments 15% heat inactivated normal rabbit serum (NRS) was used. This was obtained monthly from a pool of normal rabbits kept in the Department.

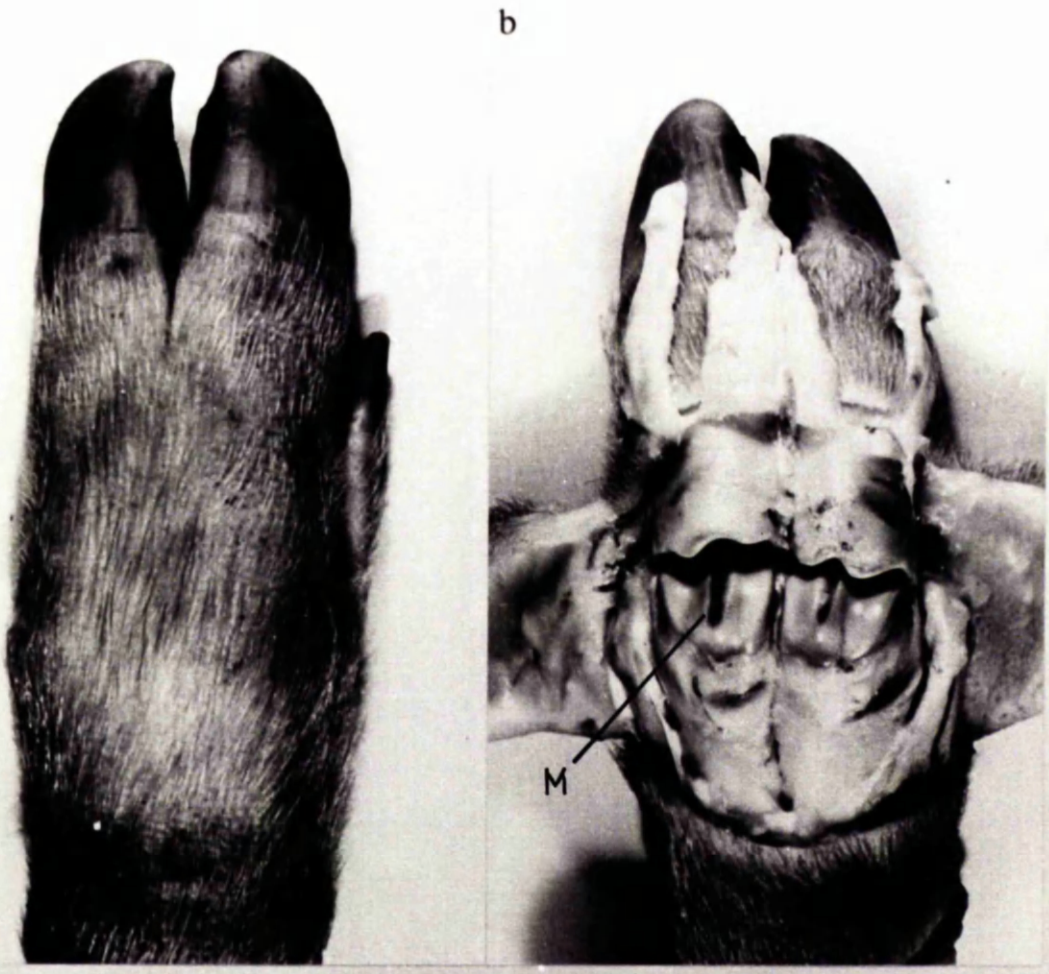
During preparation the tissues were placed in holding medium consisting of either BGJ_5 or DMEM with only 50 mg % sodium bicarbonate and a ten-fold increase in antibiotics. The reduced bicarbonate concentration gave a pH of 7.4 at atmospheric concentrations of carbon dioxide.

(b) Tissues

Pig tissues The fore trotters of young pigs were used as a source of normal articular tissue for most of the experiments. These animals, which were 6 to 9 months old and weighed between 100 and 150 kg, were being routinely killed for human consumption. The trotters were removed before the carcasses were scalded. They were then transferred to the laboratory, scrubbed in warm tap water, and either dissected immediately or stored at + 4° for up to 2 hours before dissection. Prior to dissection the trotters were immersed in 70% ethanol for 5 minutes.

The third and fourth metacarpo-phalangeal joints were opened by a dorsal approach (Fig. 1). 70% ethanol and a pot of boiling

Fig. 1



a) Pig trotter after washing and soaking in 70% ethanol.
b) Dissected trotter: Dorsal aspect of the joint capsule with the underlying synovium has been removed from the 3rd and 4th metacarpo-phalangeal joints. A strip of cartilage has been removed from each joint; a dark strip (M) is the underlying marrow which has not been included in the explant.

Fig. 2

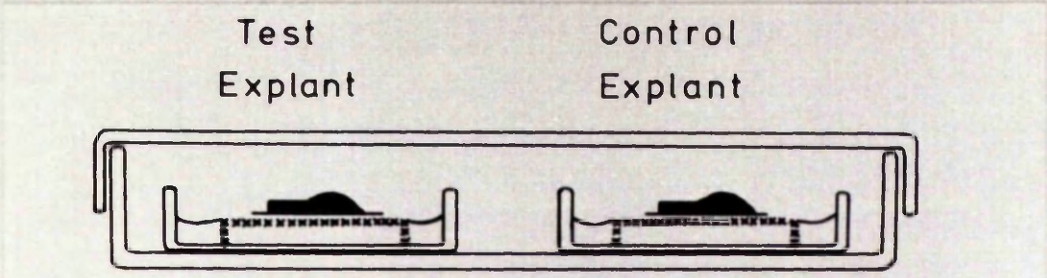


Diagram of culture system. Test and control explant are resting on a stainless steel grid in the culture vessel. Paired explants incubated in the same Petri dish.

water were used to keep the instruments sterile during the dissection. The overlying skin was reflected and the extensor tendons removed. This exposed the joint capsules of the metacarpo-phalangeal joints.

Synovium After the metacarpo-phalangeal joints had been exposed, as much as possible of the joint capsule was dissected off with its underlying synovial tissue. These sheets of tissue were placed in holding medium on a sterile Maximow slide in a Petri dish. The dishes were collected and stored for up to 1 hour at room temperature. The synovial tissue was dissected from the sheets by cutting off the villi and some subsynovial tissue with fine curved irridectomy scissors. It was impossible to cut equivalent explants from intact synovial membrane; therefore the synovial tissue was further cut up with scissors into a fine mince. The mince from several joints was pooled, centrifuged at 2,000 g for 10 minutes, resuspended in holding medium and centrifuged again. This final tissue was stirred and volumes of 0.025 ml were used as explants of synovial tissue. They were a chaotic mass of synovial intima, subsynovial fibrous tissue and blood vessels.

Cartilage The young pigs from which the material was obtained were still growing actively, so that the structure of the articular cartilage differs in some respects from that of the adult. It is differentiated into three zones which merge with one another:
(1) a thin layer of fibro-cartilage at the articular surface;
(2) a middle zone of rounded cells either isolated or in small groups;
(3) a zone in which the cells are arranged in columns in which the upper cells are proliferating and the deeper cells have become hypertrophic. The matrix of the hypertrophic region is calcified and is being invaded by finger-like processes of marrow; endochondral bone

is being deposited on the surface of the eroded cartilage. The 'tidemark' which defines the boundary between the calcified and uncalcified matrix in the adult cannot be distinguished, probably because the matrix is removed by the invading marrow soon after calcification.

A strip of cartilage about one centimetre long was cut from the metacarpal of the third and fourth metacarpo-phalangeal joints (Fig. 1). Care was taken to avoid the underlying marrow but to cut as deeply as possible. By this procedure all three zones of chondrocytes were present in most of the cartilage strips. Each strip of cartilage was trimmed at both ends and divided into two equal portions. Because the pigs were rather heterogenous and the thickness of the cartilage varied widely in different animals the two fragments from each strip served as paired test and control explants.

Blood vessels In some experiments explants of finely divided blood vessels were used. The vessels were dissected from the subcutaneous tissue on the dorsal aspect of the trotters. They were collected in holding medium, minced and treated in the same way as the synovium.

Fibrous tissue Pieces of fibrous tissue from the insertion of the extensor tendons in the trotters were removed and cut up and treated in the same way as the synovial tissue.

Mononuclear leucocytes (1) Peripheral blood leucocytes Peripheral blood was removed aseptically from the jugular vein of normal pigs. Heparin was added as an anti-coagulant. The blood was layered onto Ficoll/Hypaque with specific gravity of 1.075 (291) and centrifuged

at 900 g for 20 minutes. The mononuclear cells at the plasma-Ficoll interface were removed, washed in 5 volumes of phosphate-buffered saline (PBS) and centrifuged at 200 g for 10 minutes. The cells were resuspended in PBS, centrifuged and resuspended in a known volume of holding medium. They were counted on a Neubauer slide with phase contrast light, centrifuged and resuspended in a suitable volume of medium to give a working concentration of 3×10^6 cells per 0.05 ml medium.

(2) Lymph gland leucocytes Leucocytes from mesenteric lymph glands of 6-8 week old normal pigs were dissected out, injected with 1-2 ml of PBS and then cut open. The released cells from several lymph glands were collected and pooled. They were then centrifuged at 200 g for 10 minutes, resuspended in PBS, centrifuged, and resuspended in holding medium. The cells were counted and the volume adjusted to a cell concentration of 3×10^6 cells per 0.05 ml medium.

Human articular cartilage Femoral heads were obtained from surgical joint replacements for fractured neck of femur. The cartilage was old (age range 68-87) and there were often localised areas of degenerative arthritis. Samples were taken from macroscopically intact areas of cartilage, which were later checked by histological examination. Strips of cartilage were removed with a scalpel, care being taken to avoid the underlying calcified tissue. Eight strips of cartilage were removed from each joint which provided eight pairs of explants for culture; each strip was divided into three equal portions; two for culture and one fixed immediately for histology.

II METHODS

(a) Tissue Culture Techniques

Organ culture vessels The organ culture system is a modification of the watch glass method (292) and the system devised by Trowell for liquid media (293). The technique now used was described by Fainstat (294). The culture system consists of two glass culture vessels, each with a table of stainless steel gauze, enclosed in a Petri dish carpeted with filter paper soaked in isotonic saline to act as a moist chamber (for review see Fell, 295). This was further modified by the addition of a Millipore membrane (pore size 8 um) on the table to act as a substrate for the explanted tissue (210). The height of the table is adjusted so that 1.5 ml of culture medium just wets the surface of the grid. Explants of cartilage are placed either on the grid or on the Millipore membrane and, depending on the experiment, 0.025 ml of synovium, blood vessel, or fibrous tissue are spread on the Millipore (Fig. 2). A layer of medium drawn up by surface tension covers the explants. The Petri dishes are stacked in a modified Fildes Macintosh jar, gassed with the appropriate mixture (usually 5% CO_2, 20% O_2, 75% N_2) and incubated at 38.5° for the pig cartilage explants (pig body temperature is 39.3°), or 37.5° for the human cartilage explants.

The cultures are examined under the dissecting microscope every 2 days and the culture medium changed at that time. The used culture medium from 4-6 culture vessels is pooled and stored at -20° for analysis. At the end of the culture period the tissues are either stored at -20° for chemical analysis or fixed for histological examination.

Bottle cultures For some experiments synovium was cultured in 150 ml tissue culture bottles. The minced synovial villi from 3 or 4 joints were added to 50 ml of serum-free culture medium. The bottles were gassed with 5% CO_2 and incubated at 38.5°. The medium was decanted every 48 hours and replaced with fresh. The used medium from the 6-8 day cultures was diluted with an equal volume of fresh medium. This used medium was then ready for further organ culture experiments. Control medium was gassed and incubated at 38.5° for 48 hours, then diluted with an equal volume of fresh medium.

Leucocyte cultures (1) In organ culture vessels 3×10^6 leucocytes were added to the organ culture dish in 1.5 ml medium containing 15% normal pig serum (NPS). Affronted explants of cartilage and synovium were cultured on the grid. The vessels were enclosed in a Petri dish and incubated at 38.5° for 8 days. The medium was changed every 48 hours and inevitably many of the leucocytes were lost. At the end of the culture period the explants were removed for histological examination and the used medium collected for proteoglycan analysis. The floor of the culture dish was examined for the presence of cells by fixing with methanol for 5 minutes and staining with dilute Toluidine blue or Carazzi's haematoxylin.
(2) In tissue culture bottles In some experiments affronted explants of cartilage and synovium were cultured in the used medium from leucocyte cultures. The medium was prepared by culturing 30×10^6 leucocytes in flat bottomed 150 ml glass tissue culture bottles containing 30 ml medium with 15% NPS; the medium was gassed with 5% CO_2 and incubated at 38.5° for 48 hours. At the end of the culture period the cells had formed a layer on the bottom of the

bottle. The supernatant was carefully removed and stored at -20° until required for the organ culture experiments.

Killing of tissues When required the tissues were killed by freezing the explant to -15° and then thawing. This was repeated to ensure that the cells were dead.

Radiolabelling In some experiments the explants were exposed to ^{35}S sulphate ($Na^{35}SO_4$; Radiochemicals, Amersham). The sulphate was added to the culture medium at either 1 μCi/ml or 10 μCi/ml and the tissues cultured for 48 hours or 2 hours respectively. At the end of this time the explants were rinsed in isotonic phosphate buffered sulphate and then stored at -20° or fixed for autoradiography and histological examination.

Exposure of leucocytes to phytohaemagglutinin (PHA) The leucocytes were exposed to PHA in two ways: (a) by adding 0.25 ml PHA/10 ml medium (Crude, Wellcome) to the medium in the organ culture dish for the first 48 hours, or (b) by pre-incubating the cells with 0.25 ml PHA/10 ml medium for 2 hours before culture. The cells were pre-incubated with PHA in holding medium containing 15% NPS and after the 2 hours they were washed x 2 in holding medium.

Exposure of leucocytes to carbonyl iron The suspension of mononuclear leucocytes was depleted of monocytes by treating the cells with carbonyl iron. 150 mg carbonyl iron was added to 10 ml cell suspension in a Universal container. The bottle was rotated for 30 minutes at 37°. Holding medium containing 15% NPS was used for the incubation. The iron was removed by rotating the bottle through a horse-shoe magnet. The supernatant fluid was removed, centrifuged at 200 g and the cells washed x 2 with holding medium.

(b) Histological Techniques

Fixation Explants not required for autoradiography were fixed in Zenker's solution (296) containing 4% acetic acid for 1 hour and then Zenker's solution for a further 2 hours. The tissue was then washed for 5 hours with tap water and stored overnight in 70% ethanol. It was stained in bulk with eosin (0.05% in 95% ethanol) to facilitate orientation of the block during section cutting, dehydrated with ethanol, cleared in cedarwood oil and embedded in paraffin wax (296).

Zenker's fluid was found to be unsuitable for samples required for autoradiography and these were fixed in acetic acid and ethanol (1:3) for 30 minutes and then formol saline (215). The samples did not require washing in tap water and were dehydrated, cleared in cedarwood oil and embedded in paraffin wax.

Section cutting The wax blocks were cut on a Cambridge Rocker microtome and serial sections of 6 μm were obtained. Three histological slides were prepared from the middle third of each explant and at least ten serial sections were mounted on each slide.

Staining Toluidine blue One slide of each explant was stained with 0.5% toluidine blue in 5% ethanol. This cationic dye has metachromatic properties and stains cartilage proteoglycan purple. Cellular basophilic material stains blue.

Celestine blue, Carazzi's haematoxylin and van Gieson's stain (abbreviated to van Gieson) This sequence of dyes stains collagen red and basophilic material brown. It gives good visualisation of the cells as well as identifying the collagen. In some samples of cartilage the area around the chondrocytes in the deeper zone stained very poorly with van Gieson. These areas also stained intensely with toluidine blue.

It was not clear whether the collagen content around the deep chondrocytes was less than in the other zones or if the proteoglycan masked the staining by the van Gieson. This was examined by taking several pairs of cartilage explants and digesting one half with trypsin (0.25% for 1 hour at 35°), which removes the proteoglycan but not the collagen (209). Both halves were fixed and sections stained with toluidine blue and van Gieson. In the trypsinised half the metachromatic staining was lost and the van Gieson stained uniformly over the whole matrix. This result suggested that the proteoglycan masks the staining of collagen by van Gieson. In the analysis of the organ culture experiments, collagen degradation, as judged by the van Gieson staining, was only considered if there was also loss of proteoglycan.

Carazzi's haematoxylin and chromotrope This stain was used occasionally because it gave good cellular detail.

Autoradiography When autoradiographs were required sections of paired explants were mounted on the same histological slide. This was to minimise errors due to variation in the thickness of emulsion on different slides. A liquid nuclear emulsion was used (K5, Ilford). This was warmed to 40°, diluted by one third with distilled water and the slides for histology were dipped into the emulsion. They were dried flat with the aid of an electric fan and exposed for one week at 4°.

The film was developed with Phen X (Ilford) for 10 minutes and fixed with Hypam (Ilford) for 5 minutes. The autoradiographs were counterstained with either toluidine blue or Carazzi's haematoxylin or left unstained.

(c) Biochemical Techniques

Alcian blue assay for proteoglycan Alcian blue (G. Gurr and Co.) is a cationic dye that will bind to glycosaminoglycans (297). Whiteman (298) developed a quantitative technique for measuring glycosaminoglycans in fluids which was modified by Dingle and co-workers (207). Insoluble alcian blue-glycosaminoglycan complexes are formed. The precipitated material is collected, washed and redissolved in 2% sodium dodecyl sulphate. The intensity of colour of the resulting blue solution is read on a spectrophotometer at 720 nm and is proportional to the concentration of glycosaminoglycan present. Chondroitin sulphate (Shark's fin; Koch Light) was used as a standard which gave a linear curve up to 100 µg/ml and the proteoglycan expressed as mg chondroitin sulphate.

It was found that medium from cultures of synovium interfered with the alcian blue assay. This interference was overcome by pretreating the used culture medium with 1% trypsin (Crude, Difco) for 10 minutes at room temperature. This interaction of synovial medium with glycosaminoglycans is considered in detail in the Appendix.

Hydroxyproline assay Hydroxyproline is assayed as a measure of collagen since this amino acid occurs predominantly in collagen. It is measured after hydrolysis with 6 M hydrochloric acid for 16 hours at 106°. The hydroxyproline is oxidised with chloramine T (BDH) and then exposed to paraaminobenzaldehyde to give a coloured product (299, 300). Purified hydroxyproline (BDH) was used as a standard and results are expressed as ug hydroxyproline.

Collagenolytic activity Collagenolytic activity was measured by the breakdown of reconstituted collagen fibrils (301,70). Purified rat skin collagen labelled *in vivo* with ^{14}C-glycine was used as substrate (gift from Dr. J.T. Dingle), clostridial collagenase as a standard (Worthington) and trypsin (Difco) as a control. Release of ^{14}C-glycine from the fibrils after 16 hours' incubation at 35° was counted on a Tricarb β counter. The results were expressed as units of bacterial collagenase. The samples were pretreated with potassium thiocyanate (final concentration of 3 M) to dissociate enzyme inhibitor complexes (302). Release of ^{14}C-glycine was linear to 0.5 units of standard collagenase.

$^{35}SO_4$ incorporation Tissues for assay of the incorporation of $^{35}SO_4$ were weighed wet and then treated with papain (10 µl x 2 recrystallised; Sigma) in 2 ml phosphate buffer at 65° for 3-4 hours until digested (207). 50 µl of 10% cetyl pyridinium chloride was added to 0.5 ml of the digest to precipitate polyanions (303). After 30 minutes the samples were centrifuged at 10,000 g for 5 minutes, 250 µl formic acid was added to the pellet and heated to 70° for 10 minutes. 100 µl aliquots of the supernatant were similarly treated with formic acid. 100 µl samples of the formic acid solution were added to 2.6 ml of scintillation fluid (150 ml 2 ethoxyethanol, 175 ml toluene and 2.6 g PPO) and counted on a Tricarb β counter. The counts from the pellet give an indication of the incorporation of $^{35}SO_4$ into polyanions and were expressed as total counts per minute per explant, counts per minute per mg wet weight, or counts per minute per mg protein (assayed by Lowry, 304).

CHAPTER 4

EFFECT OF SYNOVIAL TISSUE ON THE BREAKDOWN OF ARTICULAR CARTILAGE

I OBJECT OF EXPERIMENTS

The first group of experiments was made to investigate the ability of synovial tissue to degrade cartilage.

II RESULTS

(a) Isolated Explants of Synovium and Cartilage

Synovium Sections of living synovial mince fixed immediately after explantation on the Millipore (day 0) showed fairly dense fibrous tissue, the tips of villi, and scraps of the synovial intima, adipose tissue and small blood vessels jumbled together in a chaotic mass.

During cultivation the particles of the mince fused to form a solid lump of connective tissue which became increasingly flattened. When histologically examined after 14 days' growth (21 specimens) the explants were more cellular with less collagen. Usually there was a fairly profuse outgrowth into the underlying Millipore. Biochemical analysis of the used medium showed that a small quantity of proteoglycan had been released (Fig. 3a).

Cartilage Fragments of living cartilage placed on the Millipore with the articular surface upwards underwent little change during 14 days' cultivation (Table 1, Fig. 4). A narrow zone of matrix immediately beneath the cut surfaces often became depleted of metachromatic material and the general staining of the matrix throughout the

	Explants	Number of Explants	Loss of Proteoglycan (Toluidine blue)						Loss of Collagen (van Gieson)					
			++ ++	+ ++	++	+	±	0	++ ++	+ ++	++	+	±	0
I	Isolated cartilage													
	Living	37				1	20	16					1	36
	Dead	24				4	7	13						24
II	Cartilage in contact with living synovium													
	Living	36	21	11	4				4	11	16	4		
	Dead	15	14	1						1	3	3	1	7
III	Cartilage in contact with dead synovium													
	Living	7					5	2						7
	Dead	11				1	3	7						11
IV	Cartilage separated from living synovium													
	Living	39	4	7	22	4	2			2	18	14	3	2
	Dead	16					6	10						16

TABLE 1

EFFECT OF SYNOVIUM ON BREAKDOWN OF LIVING AND DEAD CARTILAGE: ANALYSIS OF HISTOLOGY

LEGEND TO TABLE 1

The degree of depletion of the proteoglycan and collagen of the matrix, as shown by their staining reactions, is expressed by the + signs.

Proteoglycan
- $\overset{++}{++}$ = total or nearly complete absence of metachromasia;
- $\pm$ = slight effect;
- 0 = no effect.

Collagen
- $\overset{++}{++}$ = complete or nearly complete disappearance of all intercellular material;
- $\pm$ = slight degradation;
- 0 = no effect.

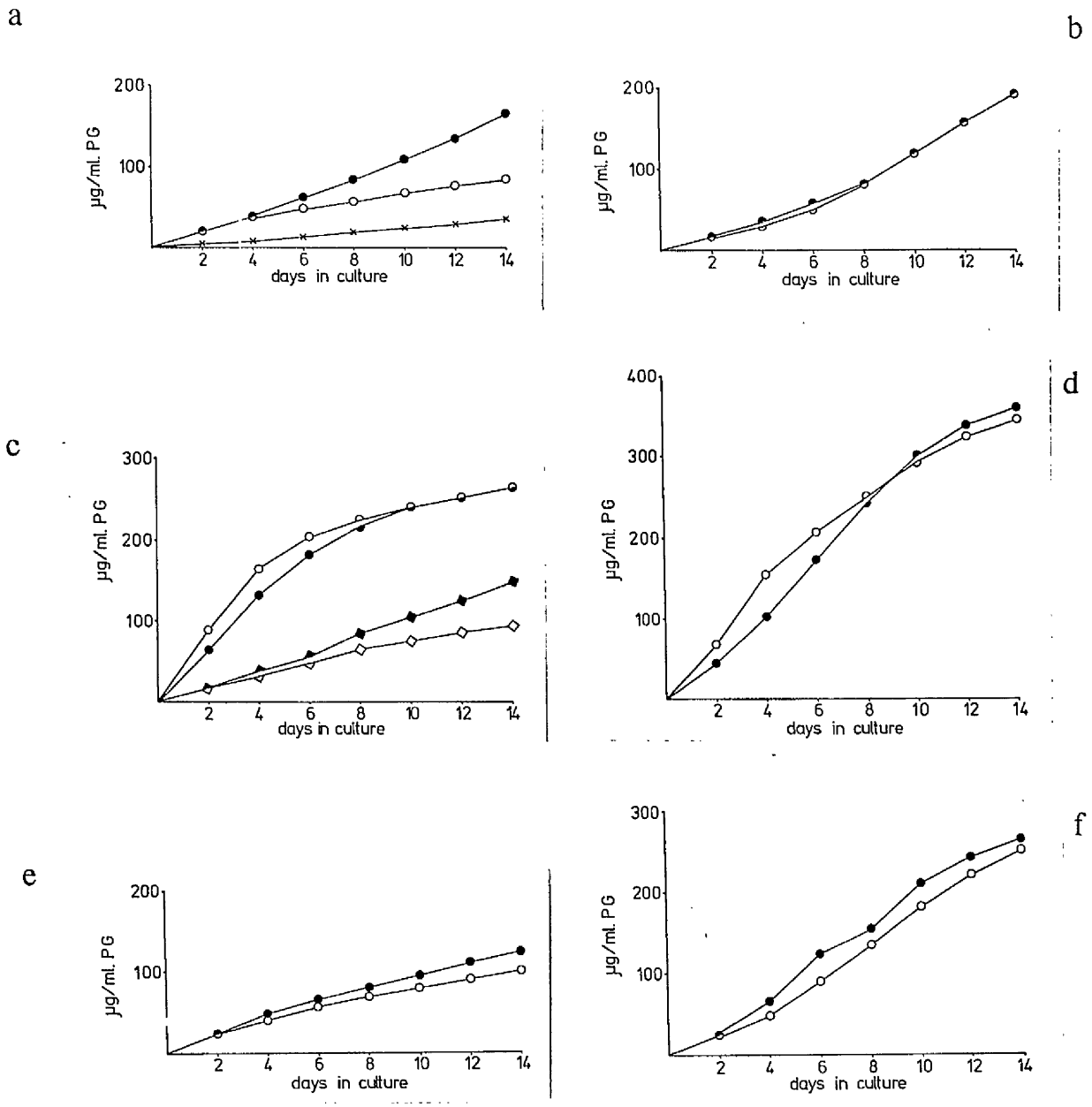
Intermediate changes in both components are represented by:

$\overset{+}{++}$ $++$ and $+$.

The number of explants in each group is shown.

In the Legends to the photographs, each specimen illustrated is graded according to the above scale.

Fig. 3



Cumulative release of proteoglycan (PG) from cartilage and synovium in organ culture. Each group is the mean of 4 or 6 explants. Paired explants are indicated by open and closed circles or diamonds.

a) Isolated explants of -- ×—× living synovium, ●—● living cartilage, ○—○ dead cartilage.

b) Comparison of paired explants of living cartilage separated from living synovium.

c) ●—● living cartilage on living synovium, ○—○ dead cartilage on living synovium, ◆—◆ living cartilage on dead synovium, ◇—◇ dead cartilage on dead synovium.

d) ●—● living cartilage overlapped by living synovium,
○—○ dead cartilage overlapped by living synovium.

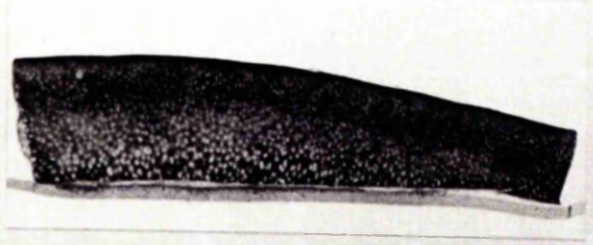
e) ●—● dead cartilage separated from living synovium,
○—○ isolated dead cartilage.

f) ●—● living cartilage overlapped by living synovium,
○—○ living cartilage separated from living synovium.

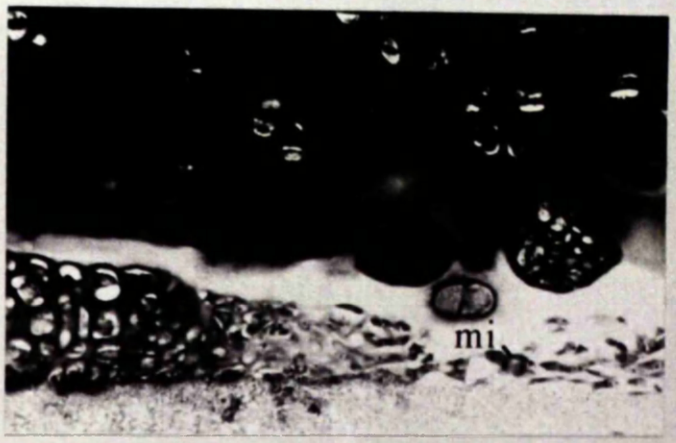
Fig. 4

a

b



c



d

a) Isolated cartilage after 14 days' cultivation; matrix intensely metachromatic. (grade of depletion: 0 ; see table 1.).Tol. blue X 15.

b) Same explant as in (a). (0; table 1.). van Gieson. X 15.

c) Similar explant showing cells emerging from the columns and the formation of new cartilage beneath the cut surface; mi: mitosis (0; table 1.). Tol. blue. X 190.

d) Same explant as in (c). (0 ; table 1.). van Gieson. X 190.

explant was usually slightly less intense than in controls fixed at day 0, especially when the articular cartilage happened to be very thin (as stated above it varied widely in thickness in different trotters). Chondrocytes emigrated from the columns of proliferative cells in the lower part of the explant; some invaded the Millipore and others often formed a layer of fibroblast-like cells, in which nodules of new cartilage were developing (Fig. 4c,d). In only one of the 37 explants in this group was loss of collagen detected; this appeared as a tiny subarticular patch in which the matrix stained pale pink with van Gieson's stain.

The only visible effect of incubating dead cartilage in culture for 14 days (Table 1) was some reduction in the intensity of metachromasia throughout the matrix.

Proteoglycan was released into the medium by both living and dead cartilage (Fig. 3a); more was released by the living tissue, probably because, unlike the dead explant, it was actively synthesising proteoglycan during cultivation (see Chapter 9). There was no release of hydroxyproline into the medium.

(b) The Comparability of Paired Cartilage Explants

In each experiment the effect of two sets of conditions was compared. The variability of the cartilage in different animals made it necessary to use paired explants for this purpose. The reliability of this procedure was tested in a control experiment; cartilage explants were prepared and the members of each pair were placed in separate culture dishes which also contained synovial mince. At 2-day intervals the used medium from each set of dishes was pooled and assayed for proteoglycan; there was

a good correspondence between the amounts released by paired explants (Fig. 3b).

(c) Living Synovium in Contact with Living Cartilage

Two types of experiment were made in which cartilage was placed, articular surface upwards, in direct contact with living synovium. In one set of experiments the cartilage explant was laid on top of the synovial mince; in the other set it was placed on the Millipore and the mince was then deposited so as to overlap one end of the cartilage fragment. Both types of experiment gave essentially the same result, and are therefore included in the same section of Table 1.

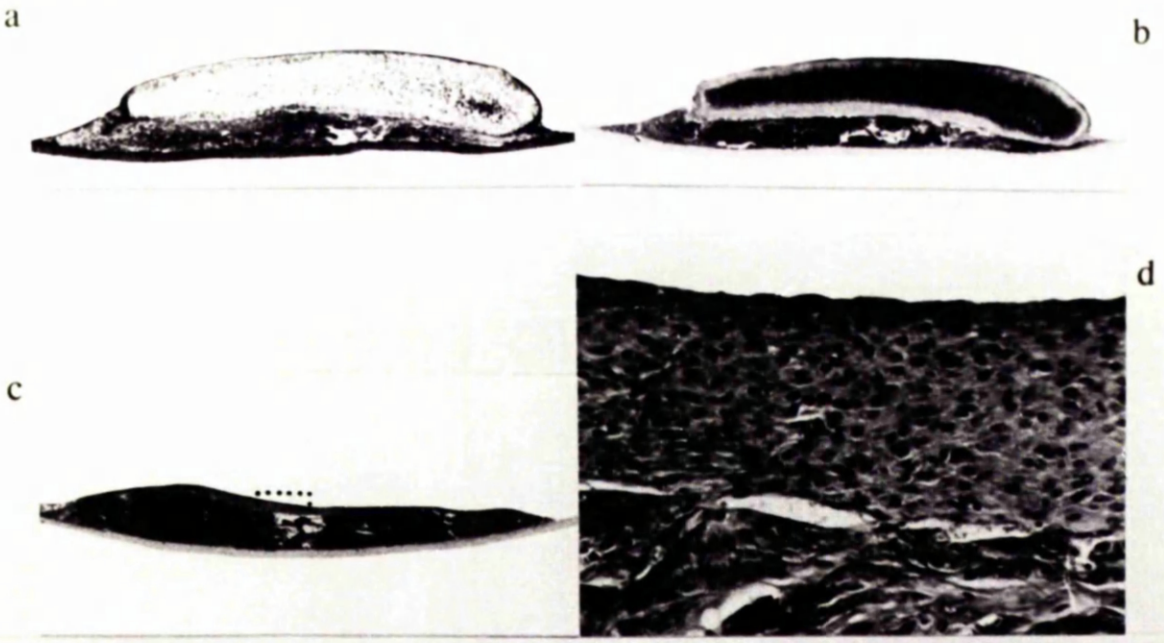
There was no obvious difference between the synovial mince grown in isolation and that cultivated in contact with cartilage.

Cartilage on the synovium Contact with the synovium had a disastrous effect on the matrix of living cartilage (Table 1). First the proteoglycan (Fig. 5a) and then the collagen (Fig. 5b) broke down; finally the matrix disappeared leaving a compact mass of viable, often actively dividing cells (Fig. 5c,d). Breakdown proceeded at different rates in different explants (Fig. 5b,c). Degradation of the collagen was accompanied by conspicuous changes in the appearance of the chondrocytes; these cytological effects will be described in the next Section.

In one experiment the cartilage was laid on a very small deposit of mince, but even this small quantity had a severe effect on the matrix of the overlying explant.

Cartilage overlapped by synovium Most of the experiments on cartilage in contact with synovial mince were made with this system. Depletion of proteoglycan began on opposite sides of the explant in the region

Fig. 5



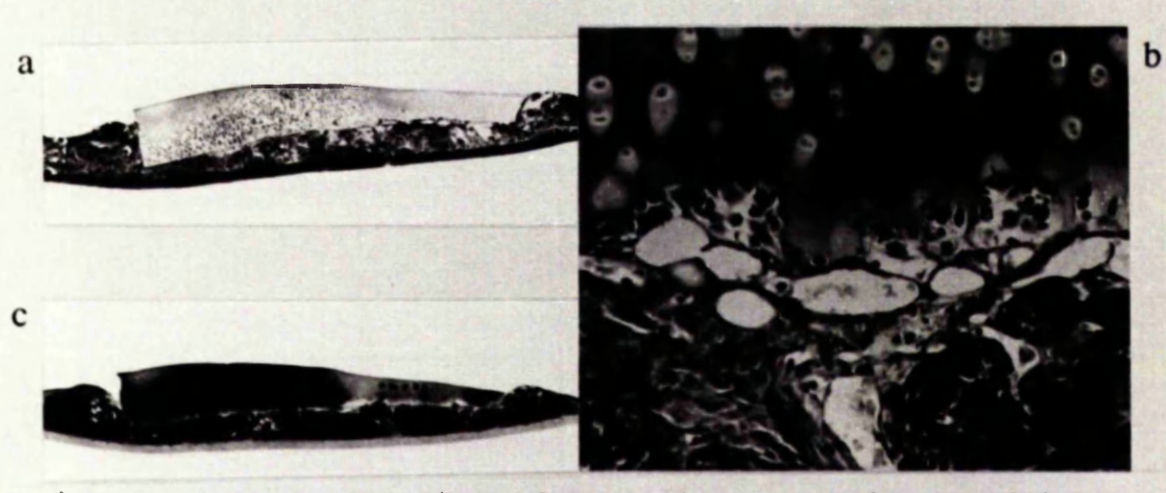
a) Cartilage explanted on synovial mince, and cultivated for 14 days'. Apart from a small patch at one end the matrix has lost its metachromasia (++++ ; table I.). Tol. blue. X 10.

b) Same explant as in (a), section stained for collagen; note peripheral zone of depletion (++ ; table I.). van Gieson. X 10.

c) Similar explant; breakdown of martix complete (++++ ; table I.). van Gieson. X 10.

d) Marked area in (c) under higher magnification. Note vaible fibroblast-like chondrocytes devoid of matrix. X 190.

Fig. 6



a) Dead cartilage after 14 days' incubation on synovium. Complete loss of metachromasia (++++ ; table I.). Tol. blue. X 10.

b) Synoviocytes invading and eroding the dead cartilage . van Gieson. X 190.

c) Same section as in (b) showing partial depletion of collagen (++; table I.). X 10.

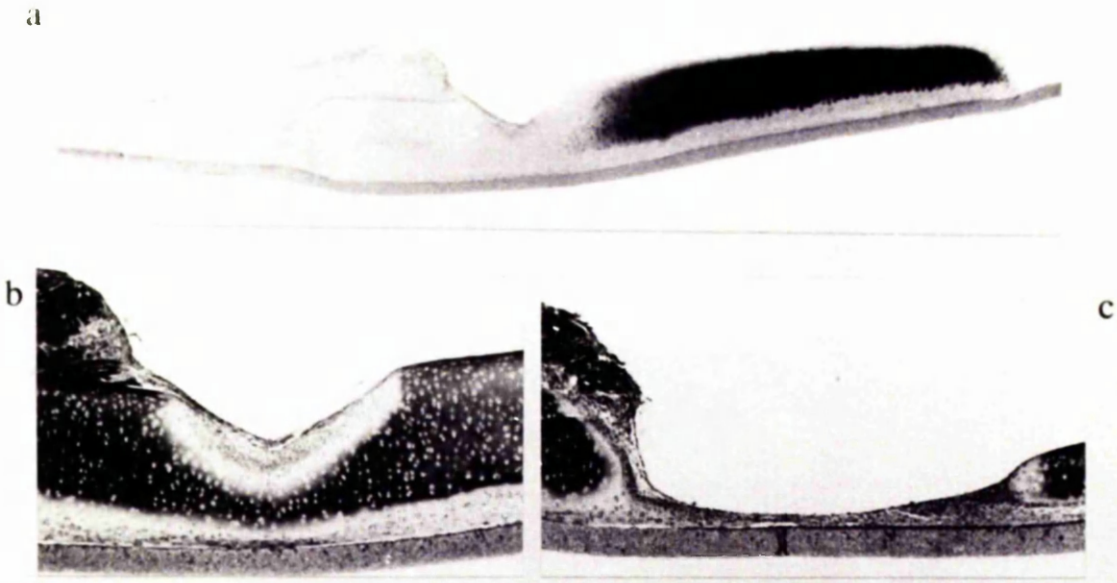
of the synovial overlap and spread to the free end of the fragment (Fig. 7a). On the upper surface breakdown of collagen often began under the leading edge of the synovial mince (Fig. 7b) where a depression was formed; on the under surface a tract of degraded matrix appeared next to the Millipore and extended both upwards towards the area of breakdown beneath the leading edge of the synovium and lengthwise towards the free end. The cartilage lying under the main mass of the synovium often disintegrated less rapidly than that beneath the leading edge (Fig. 7c).

In this type of culture, the various stages in the dissolution of the cartilage were often clearly seen as a gradient of effect in the same explant. The cells most affected were those of the cell columns in the growing zone. As the proteoglycan disappeared, the cells of the columns became irregular in shape, very basophilic and often underwent mitosis (Fig. 8a,b). The first sign that the degradation of collagen had begun was the appearance of a colourless halo (Fig. 8c) around individual cells and groups of cells in preparations stained with van Gieson's stain. These halos enlarged and fused with one another; meanwhile the chondrocytes gradually assumed a fibroblastic shape, the matrix became increasingly sparce and cavities formed around the cells. Eventually the matrix disappeared completely (Fig. 7c).

(d) Living Synovium in Contact with Dead Cartilage

From the experiments described it was not clear which cells were responsible for the destruction of the cartilage matrix. The experiments were repeated, therefore, with dead (frozen/thawed) cartilage to abolish any effect from living chondrocytes.

Fig. 7

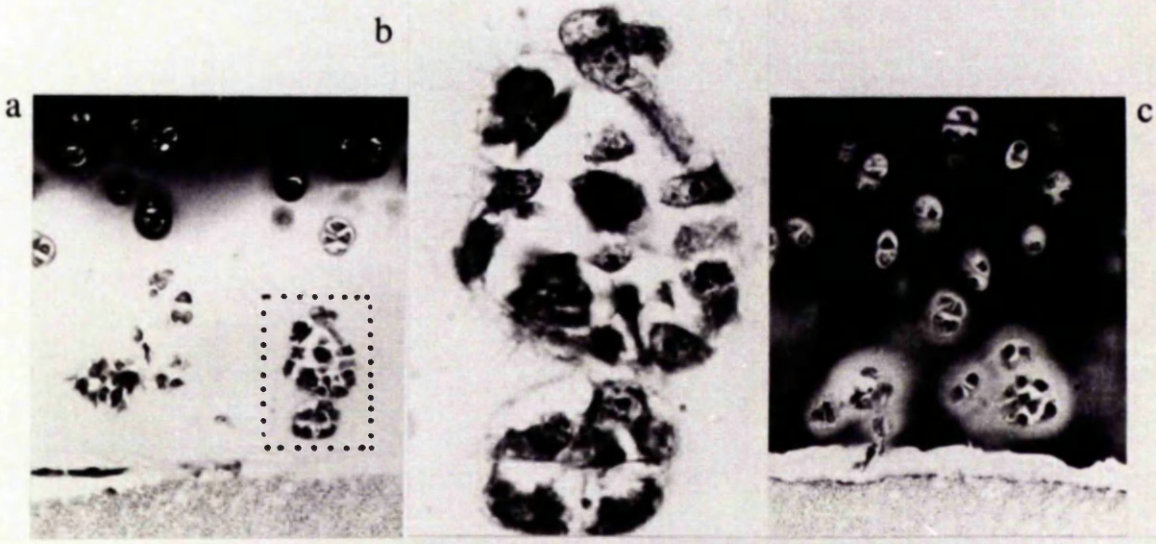


a) Cartilage explant partially overlapped by synovial mince; 14 days' cultivation. Note complete loss of metachromasia from the proximal third of the cartilage and throughout the region next next to the millipore (+++ ; table I.). Tol. blue. X 17.

b) Same explant as in (a), section showing the dissolution of collagen under the leading edge of the synovium and also beneath the cut surface next to the millipore (++; table I.).van Gieson. X 30.

c) Similar explant after 14 days' cultivation ; a more advanced stage than in (b) (+++; table I.). van Gieson. X 30.

Fig. 8



a) Cartilage with overlapping synovium, 10 days in culture; early fibroblastic transformation of the chondrocytes in the columns; loss of metachromasia in this region. Tol.blue. X 190.

b) Same section; marked area in (a) under higher magnification. The cells are strongly basophilic, irregular in shape and two are in mitosis; in the upper group the intercellular partitions have disappeared. X 750.

c) Same explant as in (a) showing halo of degraded collagen around the groups of transforming chondrocytes. van Gieson. X 190.

Cartilage on the synovium When dead cartilage was placed on living mince (Table 1) its metachromatic material was lost exactly as in the living cartilage (Fig. 6a); this was confirmed by estimations of the proteoglycan released into the medium (Fig. 3c). Synovial cells invaded the dead tissue and excavated small peripheral cavities which sometimes contained mutinucleated chondroclasts (Fig. 6b). In some explants there was evidence of a general degradation of the collagen in certain areas (Fig. 6c); these regions had shrunk, the matrix stained feebly or not at all with van Gieson's stain but it did not disintegrate as in living cartilage.

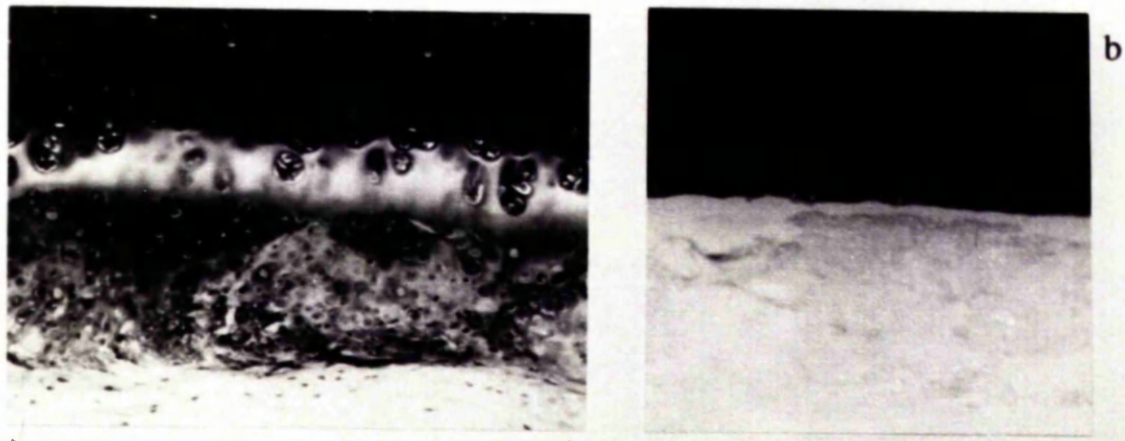
Cartilage overlapped by synovium When overlapped by synovium and cultured for 14 days, the cartilage displayed the same changes as those already described for dead cartilage placed on top of living synovium, i.e. loss of proteoglycan (Fig. 3d), areas of partially degraded collagen, and peripheral erosion of the matrix by invading synovial cells.

(e) Dead Synovium in Contact with Living or Dead Cartilage

For comparison with the results described experiments were made in which living or dead cartilage was explanted in contact with dead synovial mince killed by freezing and thawing (Table 1).

During 14 days' cultivation, the matrix of both living and dead cartilage remained strongly metachromatic (Fig. 9a,b) and much less proteoglycan was shed into the medium than when the explants were associated with living synovium (Fig. 3c). The living cartilage released more proteoglycan into the medium than the dead, probably because it continued to synthesize proteoglycan during cultivation.

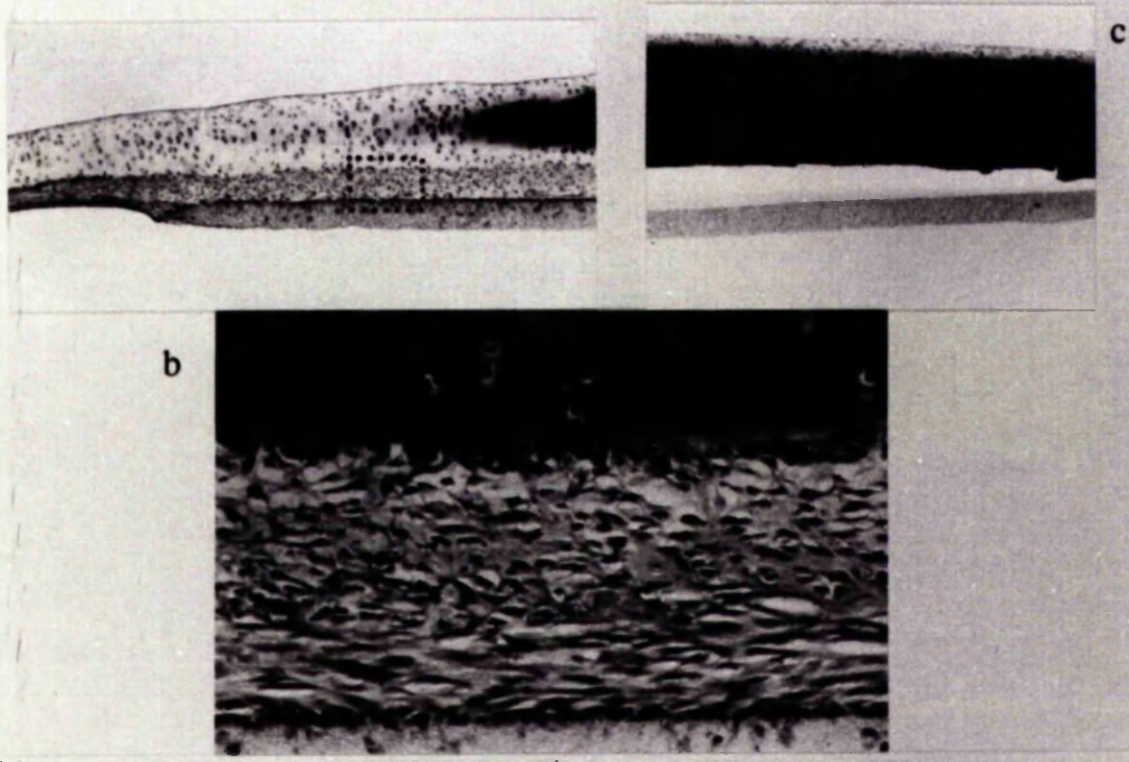
Fig. 9



a) Living cartilage cultivated for 14 days on dead synovium. Matrix strongly metachromatic except near the cut surface; outwandering chondrocytes have repopulated the synovium, multiplied and formed chondroid tissue in the dead mince. Tol.blue. X 75.

b) Paired explant, killed and incubated on dead synovium for 14 days; intense metachromasia; no viable cells in the mince. Tol blue. X 75.

Fig. 10



a) Living cartilage cultivated for 14 days on the same millipore as synovium, but with the two tissues widely separated. Note severe loss of metachromasia, and breakdown of the matrix above the millipore. (+++; table I.). Tol. blue. X 30.

b) Same explant as in (a), section shows disintegration of the collagen and fibroblastic transformation of the chondrocytes. van Gieson. X 190.

c) Paired cartilage, killed and incubated for 14 days at a distance from a living synovial explant on the same millipore (0; table I.). Tol. blue. X 30.

Chondrocytes wandered out from the columns of proliferative cells in the living cartilage and repopulated the underlying dead mince. In one experiment these cells gave rise to masses of new cartilage and chondroid tissue in the substance of the synovial explant (Fig. 9a).

(f) Living Synovium Separated From Living Cartilage

Experiments were made to see if direct contact between the cartilage and synovium was essential for the breakdown of cartilage matrix. The cartilage and synovial mince were either explanted on the same Millipore but well separated or explanted on different Millipores in the same culture vessel; the results were the same. After 14 days' cultivation loss of metachromasia was extensive in most of the explants (Fig. 10a) and the collagen was severely degraded, with associated fibroblastic transformation of the chondrocytes (Fig. 10b, Table 1). The amount of proteoglycan shed into the medium was approximately the same as from paired explants grown with overlapping synovium (Fig. 3f).

(g) Living Synovium Separated From Dead Cartilage

Having shown that a humoral factor is contributing to the cartilage breakdown, the experiments in the previous Section were repeated with dead cartilage to eliminate any action by the chondrocytes. Serum is known to contain enzyme inhibitors which might affect the of synovium on cartilage when the tissues are not in contact.

Histologically no effect could be detected after 14 days in culture and the explants were indistinguishable from dead cartilage 'cultivated' in the absence of synovium (Fig. 10c, Table 1). In experiments with paired explants, analysis of the used culture media showed that the amount of proteoglycan released by dead cartilage placed at a distance from the synovium was the same as that liberated

by isolated dead cartilage (Fig. 3e) when the small amount released by the synovium (Fig. 3a) was taken into account.

(h) Cartilage and Synovium in Different Culture Vessels

To confirm that the effect of the synovium on the cartilage was mediated through the culture fluid, experiments were performed in which the synovium was cultured in tissue culture bottles. The culture fluid from 6 to 8 day synovial cultures was collected, diluted with an equal volume of fresh uncultured medium and then used in the organ cultures of isolated cartilage. After 14 days in culture, there was extensive loss of proteoglycan and collagen with fibroblastic transformation of the chondrocytes in the zone above the Millipore membrane (Fig. 11a,b). There was no effect in control medium which had been incubated at 38.5° for 48 hours before use in the organ cultures (Fig. 11c). Breakdown was not seen in dead cartilage explanted in used synovial medium (Table 2).

(i) The Effect of Reverse Orientation on the Response of Living Cartilage to Synovial Tissue

The results described so far, refer to cartilage orientated with the articular surface upwards, but the effect of turning the cartilage upside down was also investigated.

The response of isolated cartilage cultivated in this way for 14 days (4 explants) was not significantly different from that of explants with the articular surface upwards.

Cartilage laid with the articular surface downwards on synovial mince (6 explants) behaved in the same way as cartilage with the articular surface upwards, and in some explants the matrix completely disappeared.

	Number of Explants	Loss of CPG						Loss of Collagen					
		++ ++	+ ++	++	+	±	0	++ ++	+ ++	++	+	±	0
SYNOVIAL MEDIUM													
Living Cartilage	20			12	6	1	1			1	11	6	2
Dead Cartilage	12						12						12
CONTROL MEDIUM													
Living Cartilage	20					1	19						20
Dead Cartilage	12						12						12

Grading of depletion as in Table 1.

TABLE 2

EFFECT OF SYNOVIAL CULTURE MEDIUM ON ISOLATED CARTILAGE - ANALYSIS OF HISTOLOGY

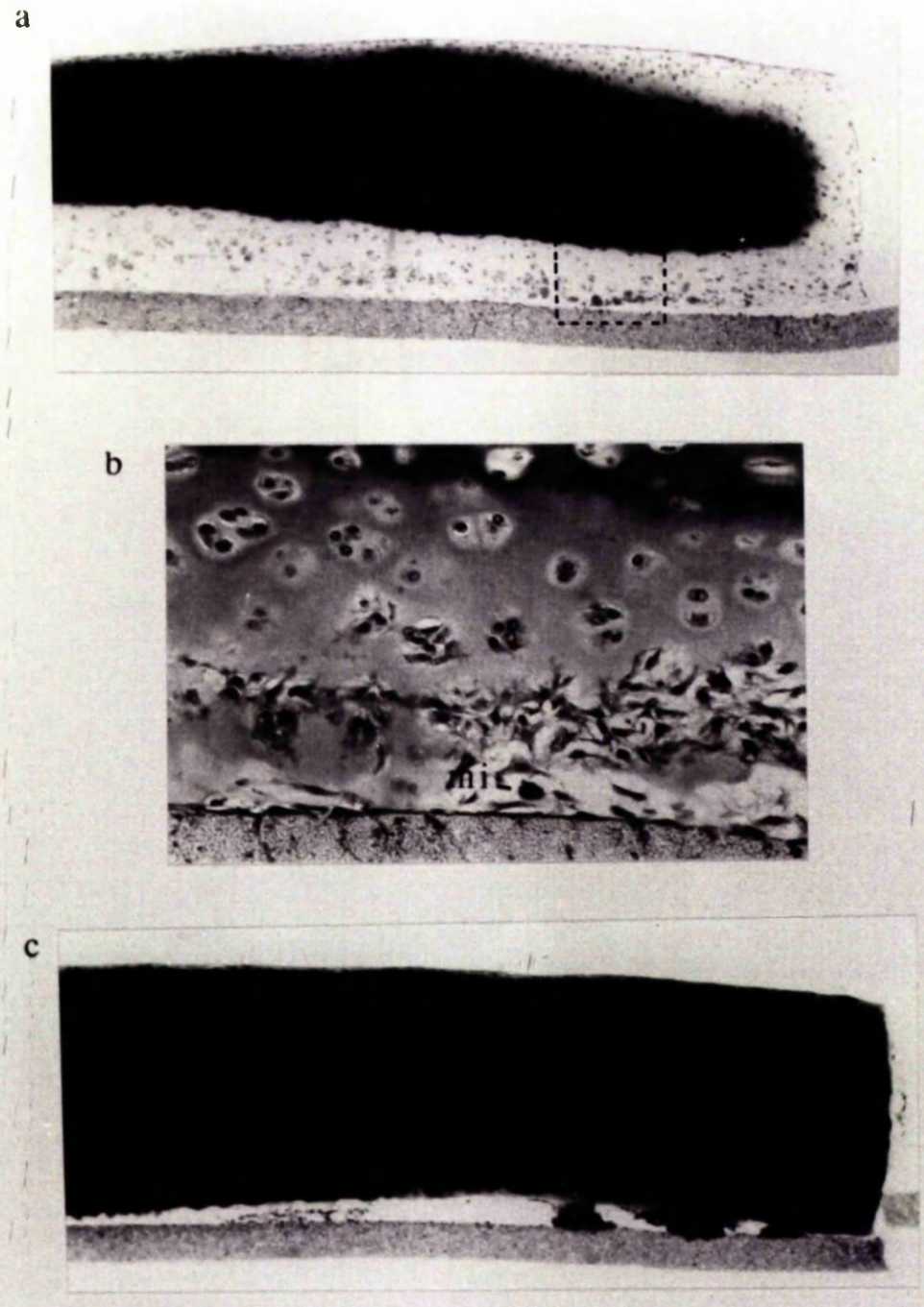
Orientation	Number of Explants	Loss of Proteoglycan (Toluidine blue)						Loss of Collagen (van Gieson)					
		++	+ ++	++	+	±	0	++	+ ++	++	+	±	0
Cut surface of cartilage on the Millipore	8		1	7						5	3		
Articular surface of cartilage on the Millipore	7			2	5							7	

Grading of depletion as in Table 1

TABLE 3

EFFECT OF ORIENTATION ON THE BREAKDOWN OF MATRIX IN PAIRED CARTILAGE EXPLANTS SEPARATED FROM LIVING SYNOVIUM - ANALYSIS OF HISTOLOGY

Fig.11



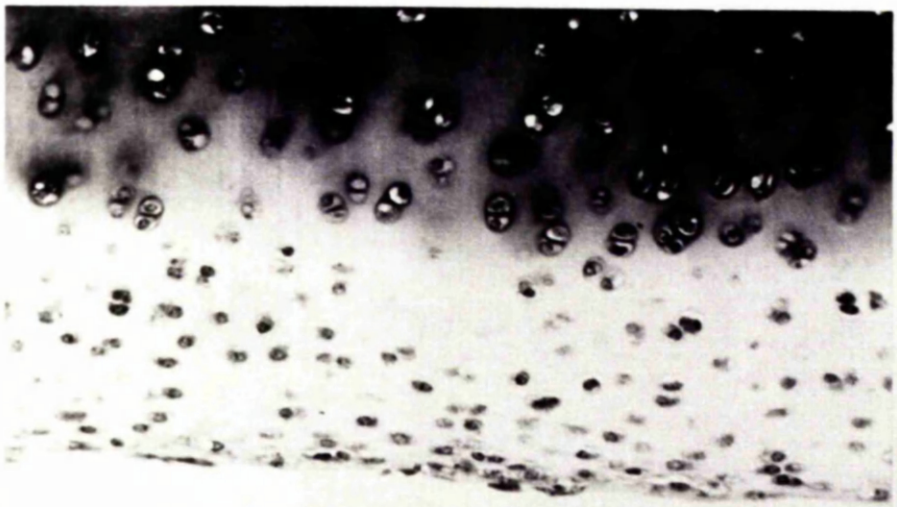
a) Isolated cartilage explant maintained for 14 days in the used medium from 6 - 8 day synovial cultures diluted with an equal volume of fresh medium. (++). Tol. blue. x 40.

b) High power view of area marked in (a) showing breakdown of collagen and fibroblastic transformation of the chondrocytes. mi = mitosis. van Gieson. x 188.

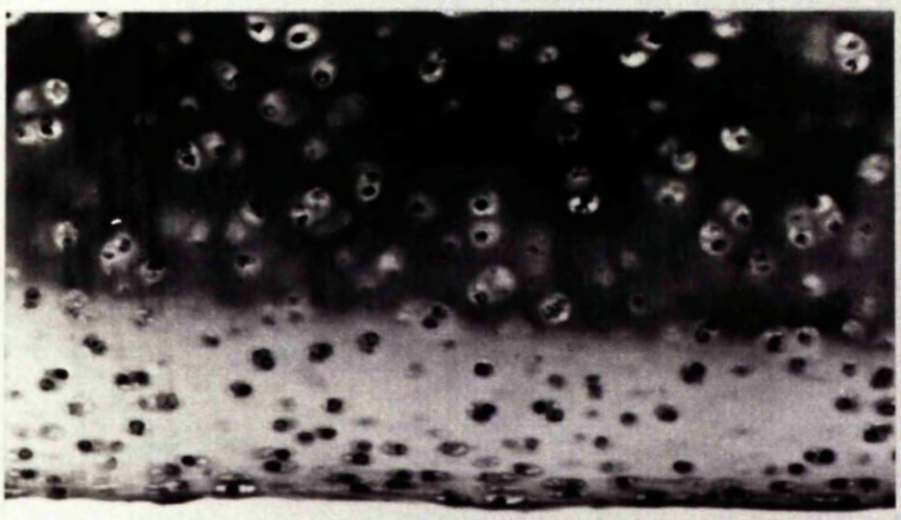
c) Paired cartilage explant maintained for 14 days in medium that had been incubated at 38.5° for 48 hours and diluted with an equal volume of fresh medium. (0). Tol. blue. x 40.

Fig. 12

a



b



a) Living cartilage cultivated for 14 days at a distance from living synovium; articular surface on millipore. Note loss of metachromasia. Tol.blue. X 200.

b) Same explant as in (a), showing some loss of collagen. van Gieson. X 200 .

When grown at a distance from synovium the cartilage showed similar changes to those seen in paired explants with the articular surface upwards (Table 3, Fig. 12a,b). In particular, the breakdown of collagen and fibroblastic transformation of chondrocytes, however, was less advanced. The histological appearance suggested that the fibrous cartilage at the articular surface was less susceptible to the influence of the medium, as 'conditioned' by the synovium than the columns of cells in the proliferative zone.

III SUMMARY

1. Pig synovium can be maintained in a viable condition for 14 days in organ culture.
2. Pig articular cartilage remains in a viable condition during 14 days in organ culture with nodules of new chondroid tissue forming at the cut surfaces and release of proteoglycan into the medium.
3. Living cartilage loses both proteoglycan and collagen when maintained in contact with living synovium.
4. Dead cartilage loses both proteoglycan and collagen when maintained in contact with living synovium.
5. Dead synovium has no destructive effect on cartilage.
6. Living cartilage loses both proteoglycan and collagen when maintained at a distance from synovial tissue, or in used synovial medium.
7. Dead cartilage is unaffected by synovial tissue at a distance or by used synovial medium.
8. Living synovium induces fibroblastic transformation of articular chondrocytes, either when the tissues are in contact or separated.

IV CONCLUSION

Synovial mince in culture has a direct, presumably enzymatic, effect on cartilage matrix with loss of both proteoglycan and collagen and an indirect effect, mediated through living chondrocytes, which also causes loss of proteoglycan and collagen.

CHAPTER 5

EFFECT OF NON ARTICULAR TISSUES ON THE BREAKDOWN OF CARTILAGE

As described in the Section on materials, the synovial tissue, which had such a disastrous effect on the cartilage matrix, was composed of intimal cells, subsynovial fibrous tissue and blood vessels. It was not clear whether the effect was due to the intimal cells, one of the other cell types or several different types of cell.

I OBJECT OF EXPERIMENTS

Experiments were made to study the response of articular cartilage to explants of blood vessel or fibrous tissue taken from the subcutaneous layers of the fore-foot of the pig.

II RESULTS

(a) Blood Vessel in Contact with Living Cartilage

Explants (0.025 ml) of minced blood vessel were cultivated for 14 days in contact with cartilage and compared with explants of paired isolated cartilage maintained for the same period (Table 4). Histological examination of the explants associated with blood vessels showed extensive and often complete loss of proteoglycan (Fig. 13a). Collagen breakdown was present in all the explants (Fig. 13b) and affected both the articular surface at the leading edge of the minced blood vessel and also the matrix above the Millipore. There was proliferation of the chondrocytes in this region with fibroblastic transformation. The isolated cartilage lost little proteoglycan or collagen (Table 4, Fig. 13c), and released much less proteoglycan into the medium (Fig. 14a) than the cartilage in contact with blood vessel.

Tissue with cartilage	Number of explants	++ ++	+ ++	++	+	±	0
-	12				1	1	10
Blood vessel	16	6	7	3			
Fibrous tissue	12	3	4	5			
Synovial tissue	16	2	7	7			

(a) Loss of Proteoglycan

Tissue with cartilage	Number of explants	++ ++	+ ++	++	+	±	0
-	12					1	11
Blood vessel	16		2	8	6		
Fibrous tissue	12		2	6	4		
Synovial tissue	16		3	4	9		

(b) Loss of Collagen

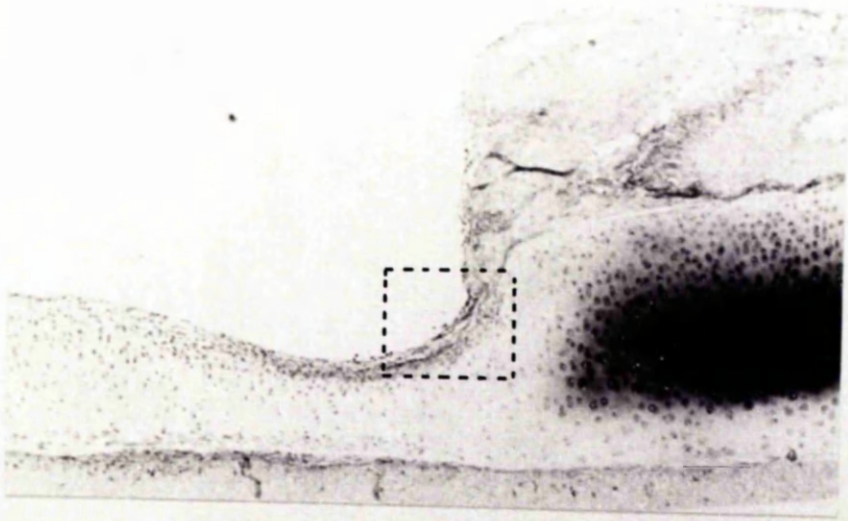
Degree of depletion as in Table 1

TABLE 4

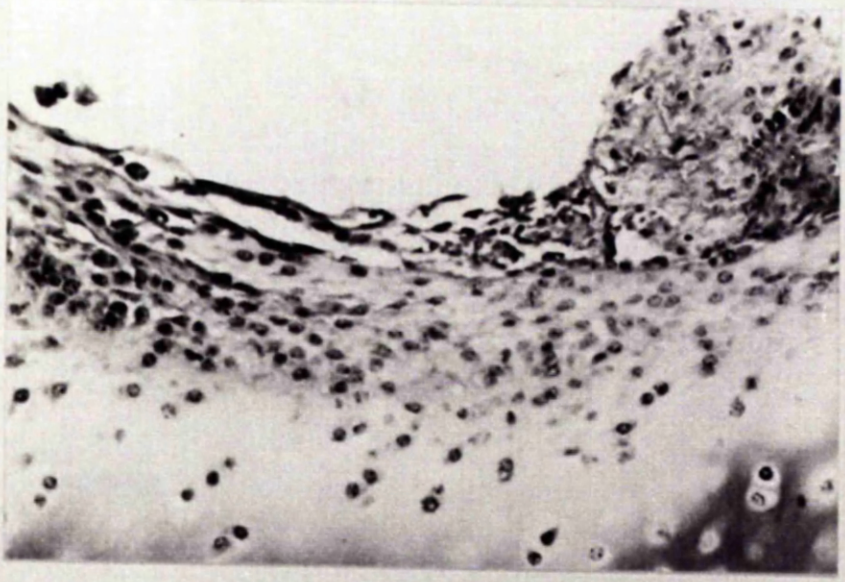
EFFECT OF NON ARTICULAR TISSUES ON LIVING CARTILAGE IN CONTACT AFTER 14 DAYS IN CULTURE - ANALYSIS OF HISTOLOGY

Fig. 13

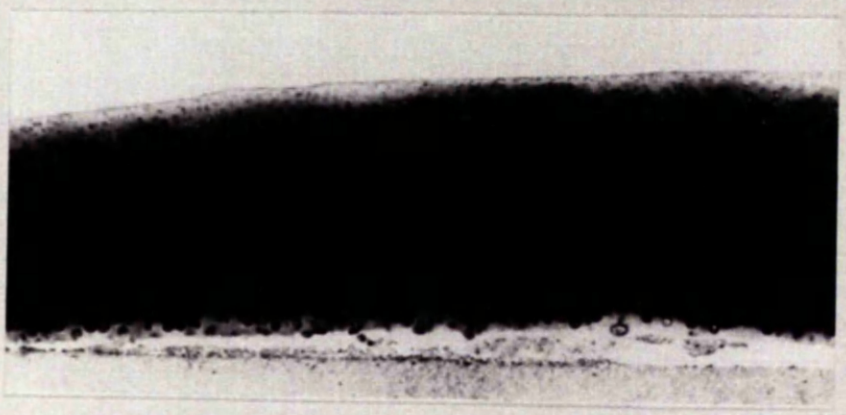
a.



b.



c.



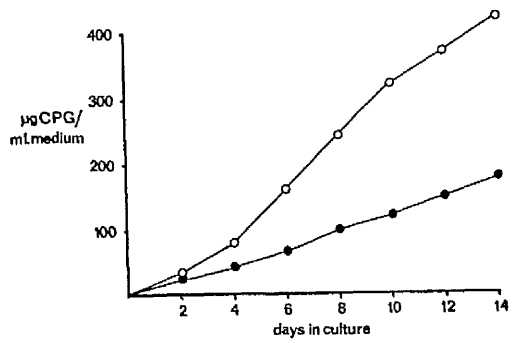
a) Minced blood vessel overlying living cartilage after 14 days in culture. Tol.blue. X 40 .

b) High power of cartilage at leading edge of blood vessel tissue. Same explant as in (a). van Gieson. X 200 .

c) Paired isolated living cartilage after 14 days in culture. Tol. blue. X 40 .

Fig. 14

a



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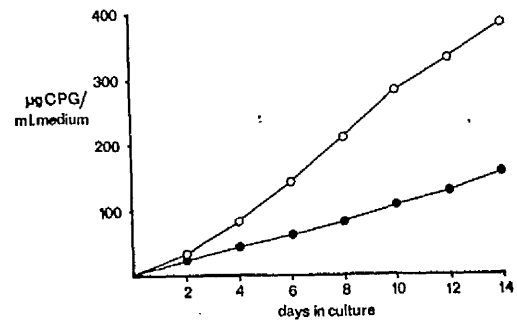


Cumulative release of proteoglycan after 14 days in culture.

a) O——O living cartilage in contact with living blood vessel.

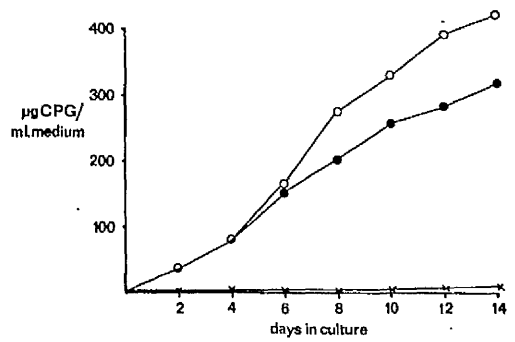
●——● paired isolated living cartilage.

b) O——O living cartilage in contact with living fibrous tissue.

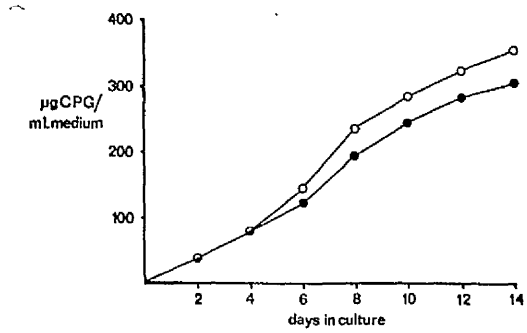
●——● paired isolated living cartilage.

Fig. 16

a



b



Cumulative release of proteoglycan after 14 days in culture.

a) O——O living cartilage in contact with living blood vessel.

●——● paired living cartilage in contact with living synovial tissue. ×——× isolated living blood vessel.

b) O——O living cartilage in contact with living fibrous tissue.

●——● paired living cartilage in contact with living synovial tissue.

(b) Fibrous Tissue in Contact with Living Cartilage

The experiment was repeated with minced fibrous tissue instead of blood vessel. After 14 days the matrix of the cartilage in contact with the fibrous tissue had lost most of its metachromatic staining and there was loss of collagen in all the explants (Table 4). The pattern of breakdown was the same as in the cultures with blood vessel (Fig. 14b, Fig. 15a,b,c) with little breakdown in the isolated cartilage.

(c) Comparison of Blood Vessel or Fibrous Tissue with Synovial Tissue

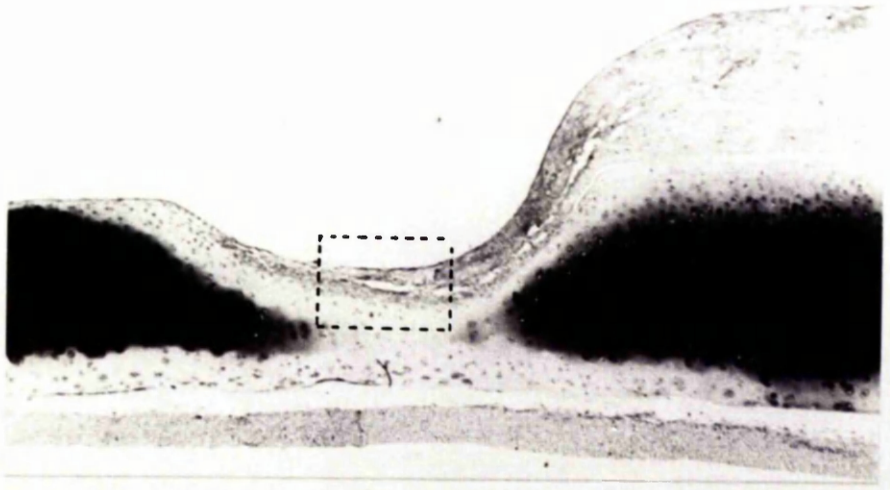
Paired cartilage explants were cultured with either blood vessel compared against synovial tissue or fibrous tissue compared against synovial tissue. In all four groups there was severe depletion of proteoglycan and to a lesser extent of collagen (Table 4). The histological appearance of the paired cartilage explants was very similar with loss of matrix and fibroblastic transformation of the chondrocytes, but the explants associated with blood vessel released more proteoglycan into the medium than their pairs with synovial tissue (Fig. 16a); this was not due to polyanionic material from the blood vessel since cultures of isolated blood vessel released only small amounts. There was a similar but less marked difference between cartilage with fibrous tissue and that with synovium (Fig. 16b).

(d) Blood Vessel in Contact with Dead Cartilage

The above experiments demonstrate that non articular tissues can have a destructive action on cartilage matrix. However, it was not clear from the results whether the process was similar to the action of synovial tissue with a direct effect and an indirect effect

Fig. 15

a.



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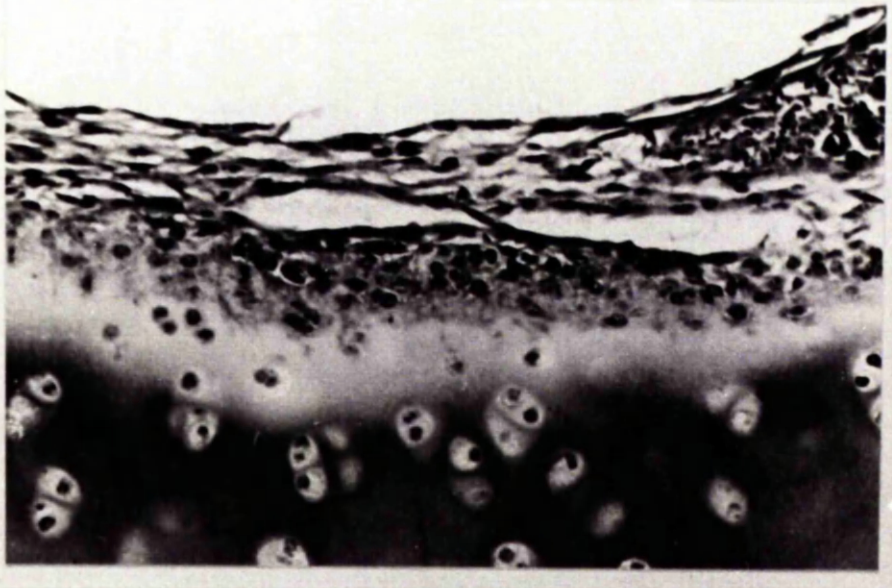
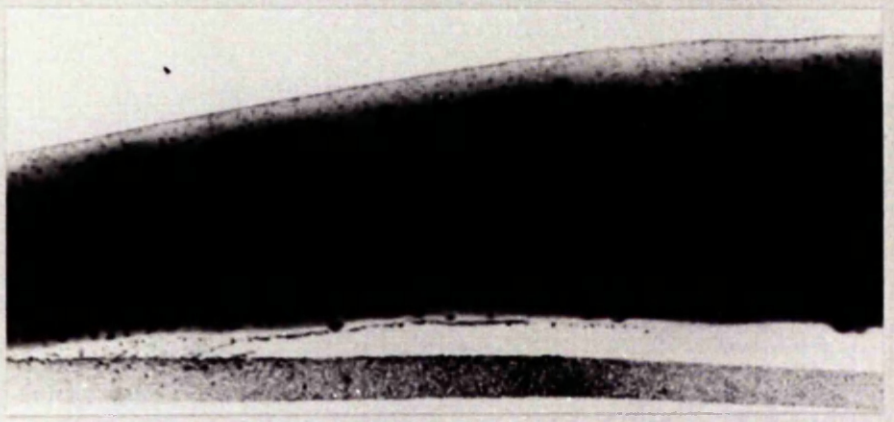


c



a) Minced fibrous tissue overlying living cartilage after 14 days in culture. Tol. blue. X 40 .

b) High power of cartilage at leading edge of fibrous tissue. Same explant as in (a). van Gieson. X 200 .

c) Paired isolated living cartilage after 14 days in culture. Tol. blue. X 40 .

mediated through the chondrocytes. This aspect was studied in more detail with the minced subcutaneous blood vessel.

Two types of experiments were made with blood vessel in contact with dead cartilage: in one the dead explant with blood vessel was paired with isolated dead cartilage and in the other with living cartilage in contact with blood vessel. In both types of experiment the dead cartilage associated with blood vessel lost most of its proteoglycan (Table 5, Fig. 17a); there was some visible loss of collagen in 2 out of the 8 explants (Fig. 17b). The release of proteoglycan was slightly less than from living cartilage with blood vessel (Fig. 18a) but much more than from isolated dead cartilage (Fig. 18b). The isolated dead cartilage showed no histological loss of proteoglycan or collagen (Table 5, Fig. 17c).

(e) Blood vessel Separated from Living Cartilage

Explants of living cartilage cultivated in the same dish as blood vessel but not in contact underwent some breakdown after 14 days (Table 5). All showed loss of proteoglycan and collagen in the zone above the Millipore membrane with fibroblastic transformation of some of the chondrocytes (Fig. 19). These changes were less advanced than those in living cartilage in contact with blood vessel. The release of proteoglycan was similar to that from isolated cartilage until about 6 or 8 days after which it was much greater (Fig. 20b).

(f) Blood Vessel Separated from Dead Cartilage

In this group there was no histological evidence of breakdown of matrix after 14 days' cultivation (Table 5). There was some release of proteoglycan into the medium (Fig. 20a) but no more

Condition of cartilage	Number of explants	++ ++	+ ++	++	+	±	0
Isolated, dead	8						8
In contact, dead	8	4	1	3			
Separated, dead	8						8
Separated, living	8	2	1	4	1		

(a) Loss of Proteoglycan (Toluidine blue)

Condition of cartilage	Number of explants	++ ++	+ ++	++	+	±	0
Isolated, dead	8						8
In contact, dead	8				1	1	6
Separated, dead	8						8
Separated, living	8			1	4	3	

(b) Loss of Collagen (Van Gieson)

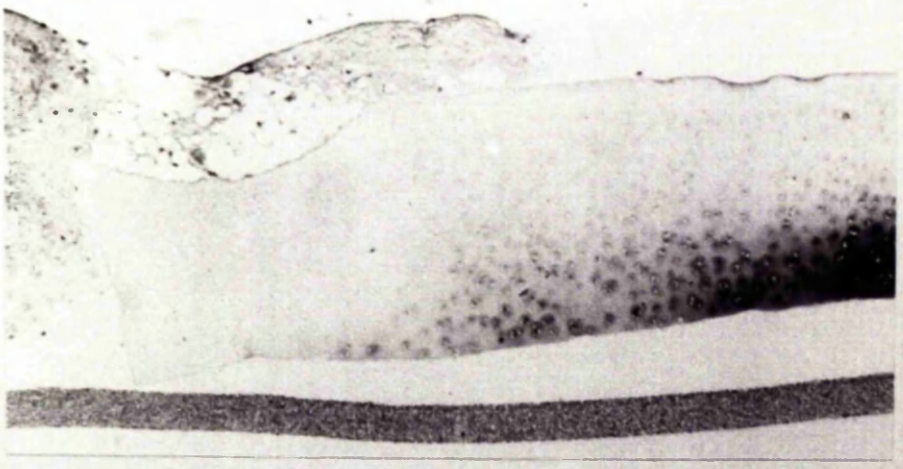
Grading of depletion as in Table 1

TABLE 5

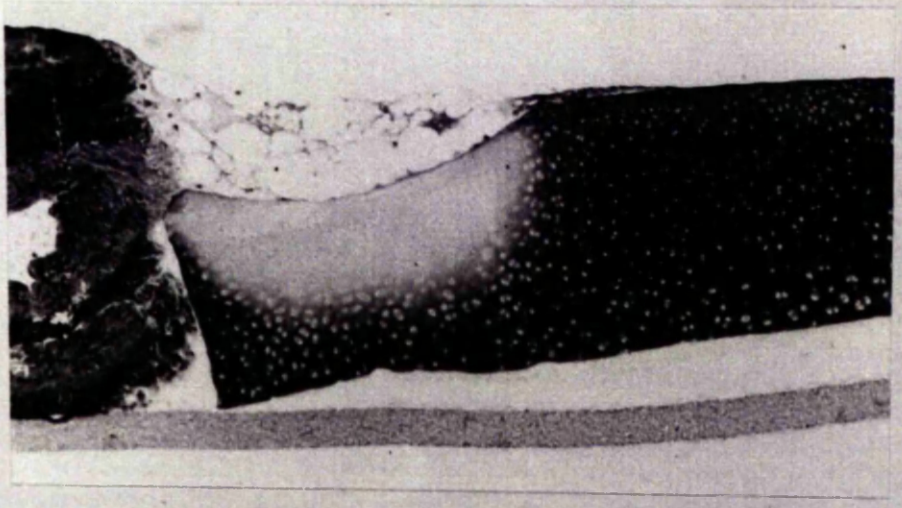
EFFECT OF MINCED BLOOD VESSEL ON CARTILAGE IN CULTURE FOR 14 DAYS - ANALYSIS OF HISTOLOGY

Fig. 17

a



b



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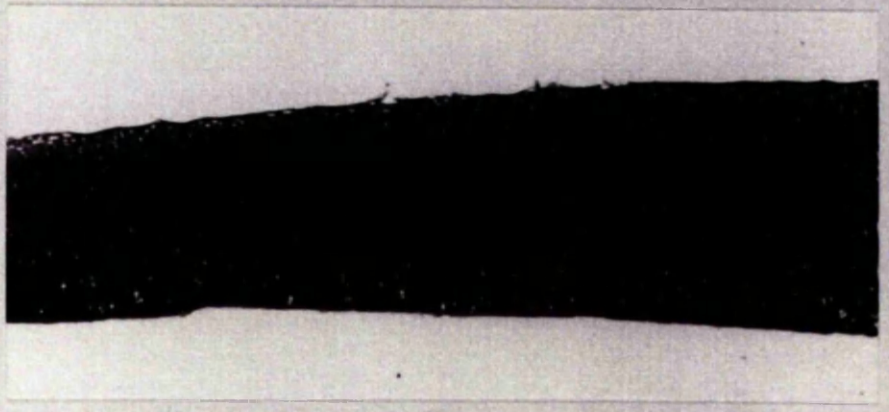
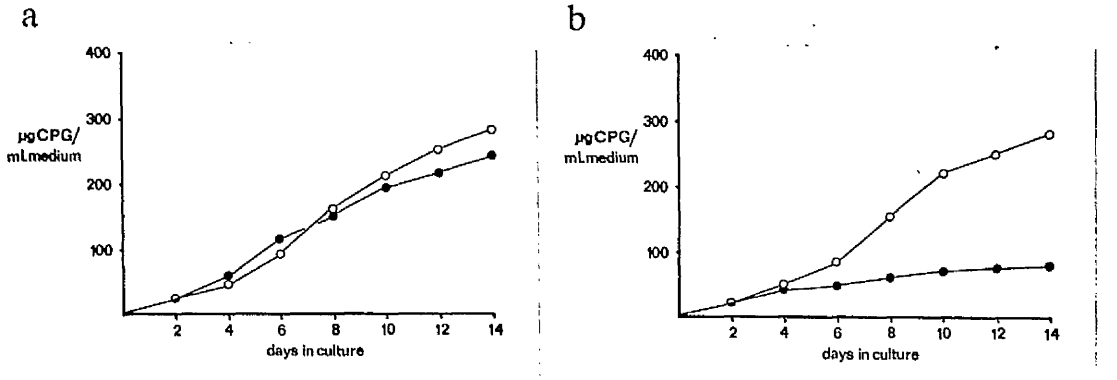


a) Minced blood vessel overlying dead cartilage after 14 days in culture. Tol. blue. X 40 .

b) Same explant as in (a) showing loss of collagen. van Gieson. X 40 .

c) Paired isolated dead cartilage after 14 days in culture. Tol. blue. X 40 .

Fig. 18



Cumulative release of proteoglycan after 14 days in culture.

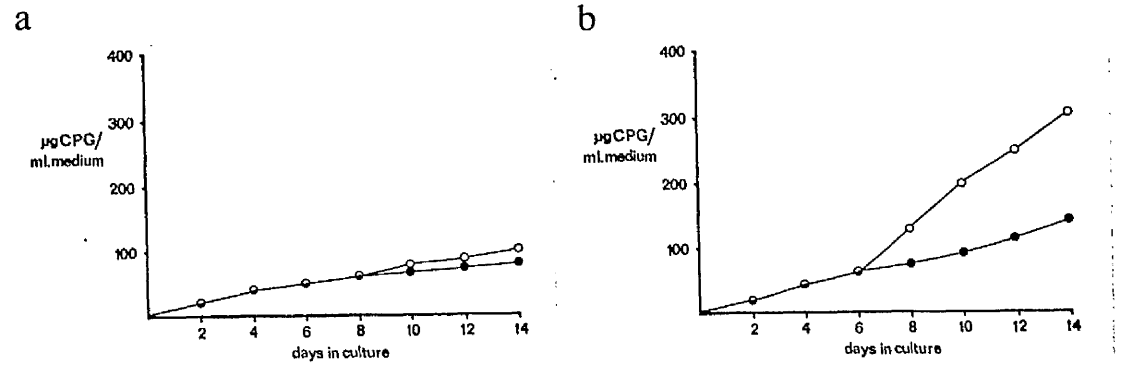
a) O——O living cartilage in contact with living blood vessel.

●——● paired dead cartilage in contact with living blood vessel.

b) O——O dead cartilage in contact with living blood vessel.

●——● isolated dead cartilage.

Fig. 20



Cumulative release of proteoglycan after 14 days in culture.

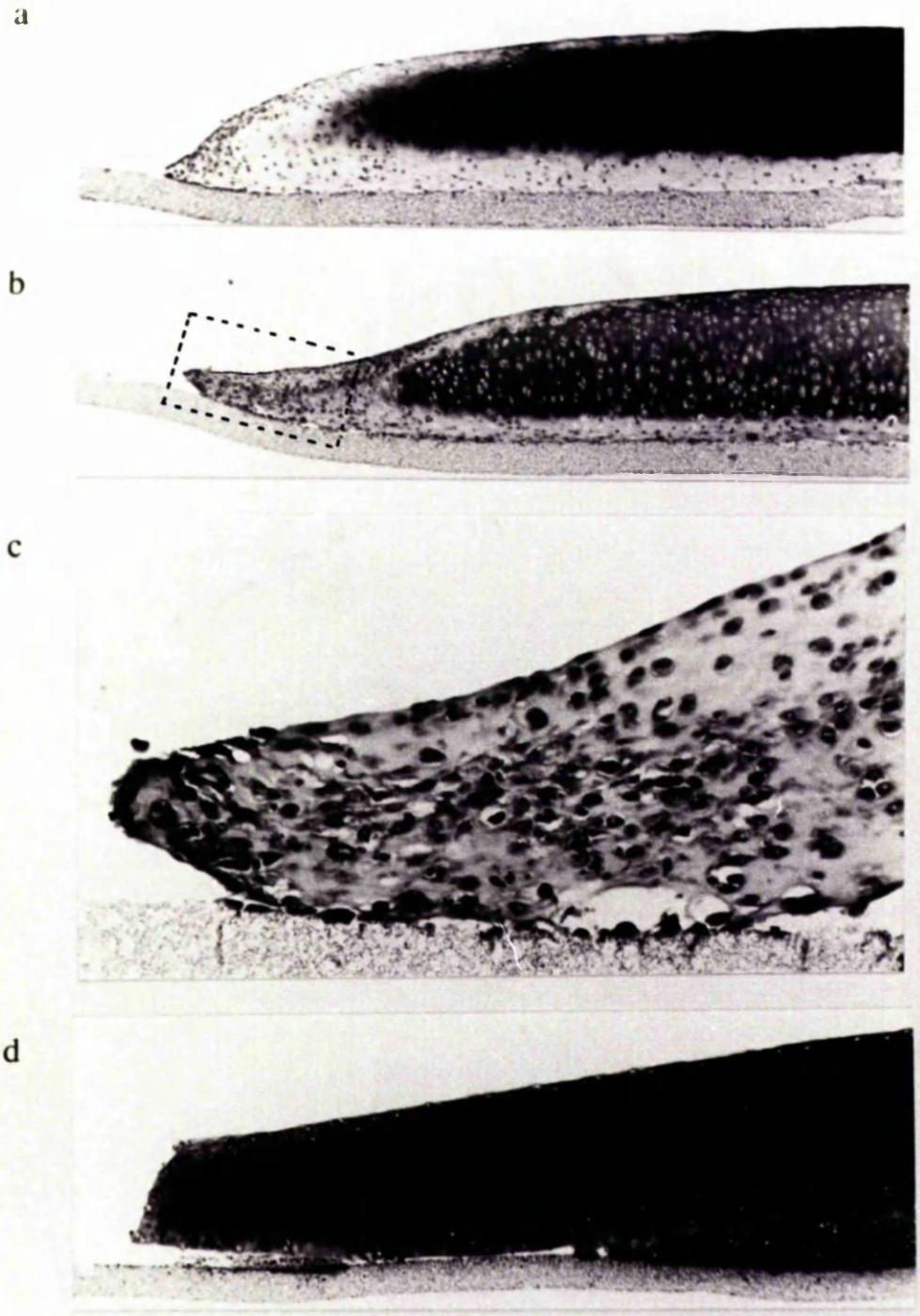
a) O——O dead cartilage at a distance from living blood vessel.

●——● paired isolated dead cartilage.

b) O——O living cartilage at a distance from living blood vessel.

●——● paired isolated living cartilage.

Fig. 19



a) Living cartilage maintained at a distance from blood vessel for 14 day in culture. Tol.blue. X 40 .

b) Same explant as in (a) stained for collagen. van Gieson. X 40.

c) High power of (b). van Gieson. X 200 .

d) Paired isolated living cartilage after 14 days in culture. Tol.blue. X 40 .

than that released by paired explants of isolated dead cartilage when the small amount released by the blood vessel was taken into account (Fig. 16a).

III SUMMARY

1. Minced blood vessel causes loss of proteoglycan and collagen from living and dead cartilage when in contact.
2. Minced blood vessel causes loss of proteoglycan and collagen from living but not dead cartilage when separated.
3. Minced blood vessel produces the same amount of cartilage breakdown as an equivalent volume of minced synovial tissue.
4. Minced blood vessel induces fibroblastic transformation of articular chondrocytes.
5. Minced fibrous tissue causes loss of proteoglycan and collagen from living cartilage when in contact.
6. Minced fibrous tissue causes the same amount of cartilage breakdown as an equivalent volume of minced synovial tissue.

IV CONCLUSION

The results demonstrate that non-synovial tissue can have a direct and an indirect destructive action on articular cartilage matrix.

CHAPTER 6

EFFECT OF RETINOL ON THE BREAKDOWN OF CARTILAGE

The results reported in Chapters 4 and 5 demonstrate that the chondrocytes can have a role in the breakdown of both proteoglycan and collagen. It was not clear whether the chondrocytes were releasing the enzymes that were degrading the matrix or merely activating synovial enzymes. That the chondrocytes can cause the loss of proteoglycan has been shown in an earlier study of the effect of retinol (vitamin A) on articular cartilage in culture (207); after 16 days an almost complete disappearance of proteoglycan was noted but loss of collagen was very slight.

I OBJECT OF EXPERIMENTS

The collagenolytic potential of the chondrocyte in response to retinol has been re-investigated. The retinol (5 or 10 iu per ml of medium - Roche Products) was in ethanolic solution and the same quantity of ethanol was added to the control medium.

II RESULTS

(a) Full Thickness Explants of Cartilage

As in previous work (207) in sections stained with toluidine blue, metachromasia disappeared from the matrix of cartilage explants during cultivation in medium containing 10 iu retinol/ml. This loss spread inwards from the surface of the fragment (Fig. 21a) and often was nearly complete by the 16th day (Table 6).

Breakdown of collagen (Table 6) first appeared (Fig. 21b) as a row of small round cavities a short distance above the cut surface;

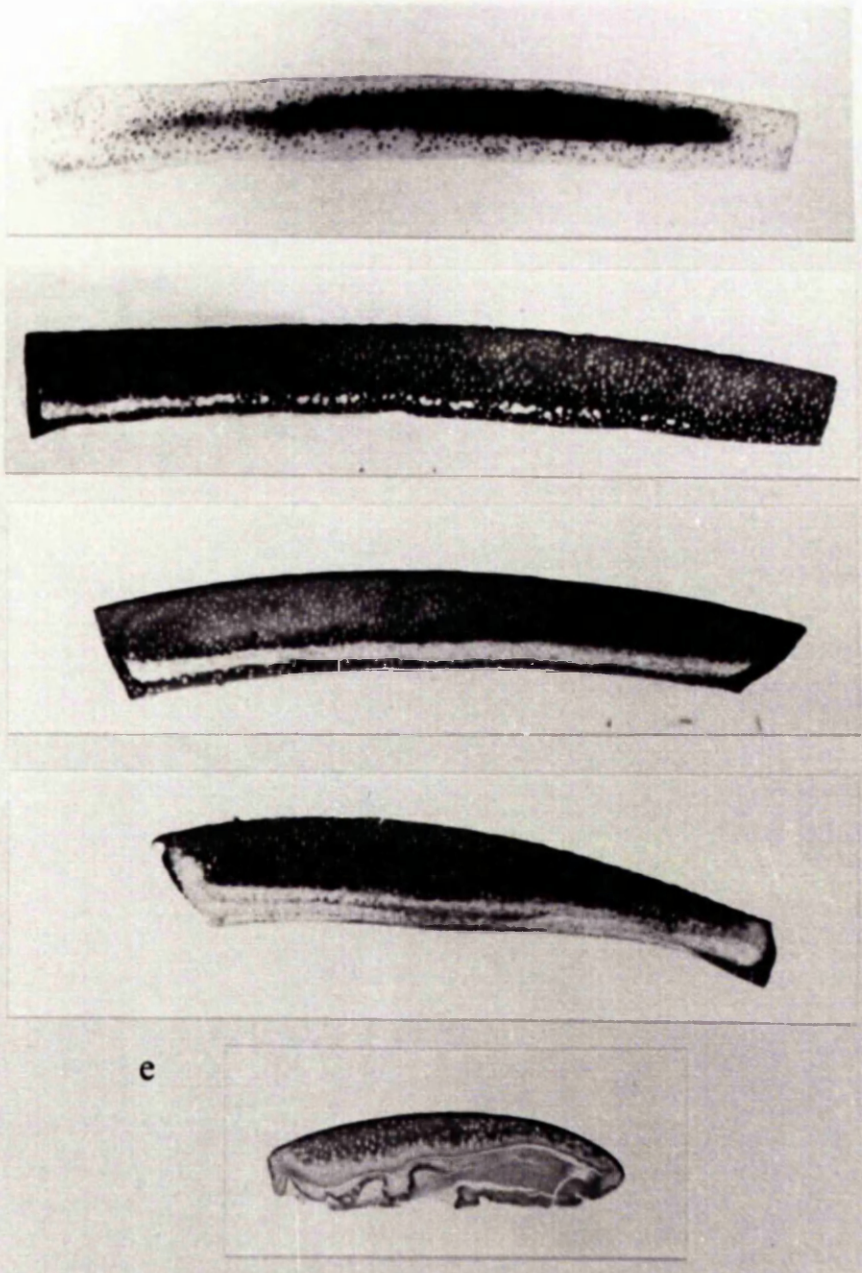
Concentration of retinol	Number of explants	Loss of Proteoglycan (Toluidine blue)						Loss of Collagen (van Gieson)					
		++ ++	+ ++	++	+	±	0	++ ++	+ ++	++	+	±	0
10 iu/ml	24	6	10	8				1	8	4	5		
5 iu/ml	24	4	9	8	3			1	4	10	7		
0	48			2	15	8	23			9	6	3	

Depletion of matrix graded as in Table 1.

TABLE 6

EFFECT OF RETINOL ON ARTICULAR CARTILAGE AFTER 16 DAYS IN CULTURE - ANALYSIS OF HISTOLOGY

Fig. 21



Explants of isolated cartilage cultivated for 16 days in medium containing 10i.u. retinol/ ml.

a) Loss of metachromatic material (+++, Table 6). Tol. blue X 32.

b) Same explant as in (a) ; first stage in the breakdown of collagen (±). van Gieson. X 32.

c)Later stage in the breakdown of collagen; tract of degraded matrix with intact collagen above and necrotic cartilage below (+). van Gieson. X 32 .

d) Further stage in the destruction of matrix (++). van Gieson. X 32.

e) Little intact matrix left (+++). van Gieson. X 32.

below this level the cartilage was largely necrotic. Some of the cells in the holes were degenerate but others, some of which underwent mitosis, were flattened against the wall of the cavity. The holes enlarged, coalesced and became filled with liberated chondrocytes which continued to multiply (Figs. 21c, 22a). These proliferating cells invaded and eventually destroyed the underlying necrotic cartilage (Figs. 21d, 22b). In one explant the lower half had completely disintegrated leaving a compact mass of viable, proliferating cells (Fig. 21e).

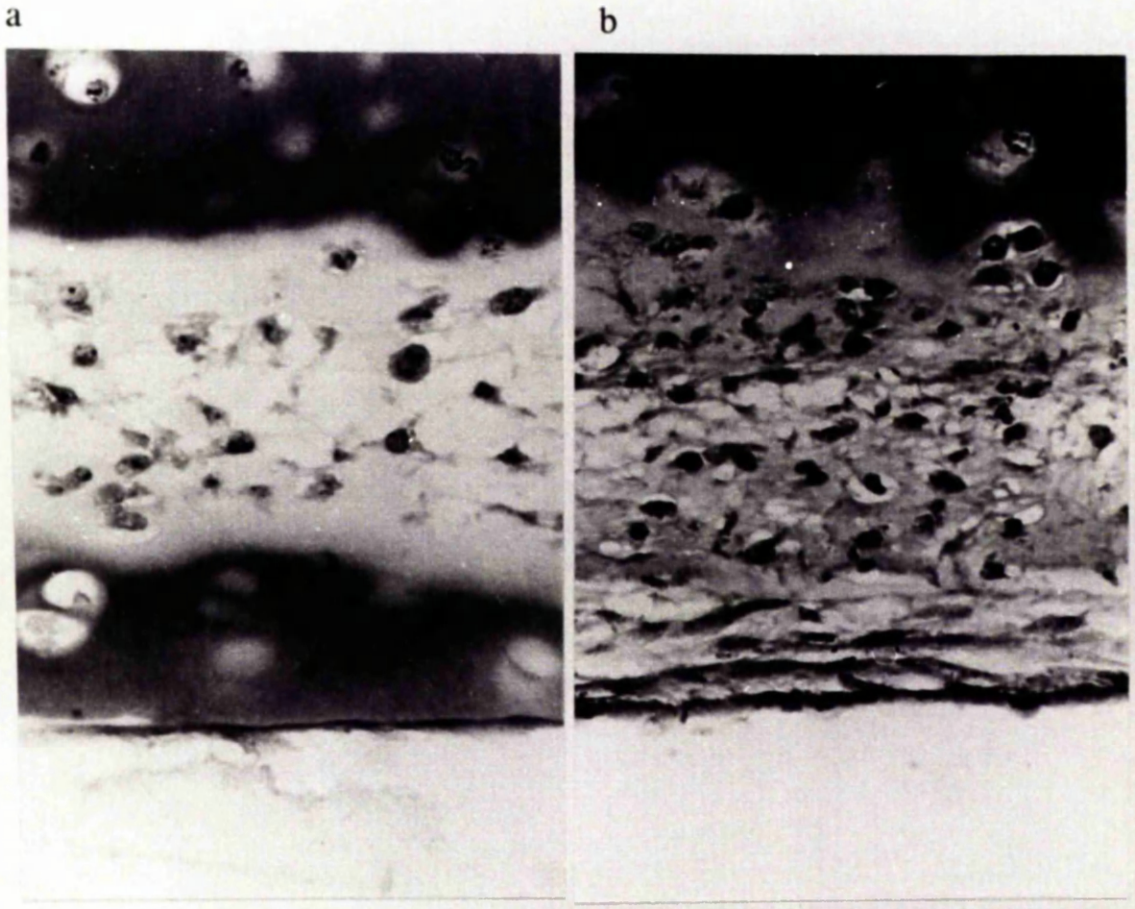
In three experiments (Table 6) one set of explants was treated with 5 iu retinol/ml and another with 10 iu retinol/ml. In the first experiment the lower dose caused much more breakdown of the matrix, but in the other two experiments the effects of the two concentrations were essentially the same. The explants in control medium showed little breakdown of matrix (Table 6) and there was usually an outgrowth of proliferating, often chondrifying cells from the cut surface.

The used culture medium was analysed for proteoglycan and hydroxyproline (Fig. 23). Proteoglycan was released from the beginning of the culture period and by 16 days the retinol group had liberated about 70% more than the controls (Fig. 23a). The hydroxyproline, however, only began to appear in the medium at 8 to 10 days and by 16 days the retinol group had released on average four times the amount present in the control culture medium (Fig. 23b).

(b) Partial Thickness Explants of Cartilage

The histological appearance of the retinol-treated cartilage suggested that the severity of the collagen breakdown might be

Fig. 22



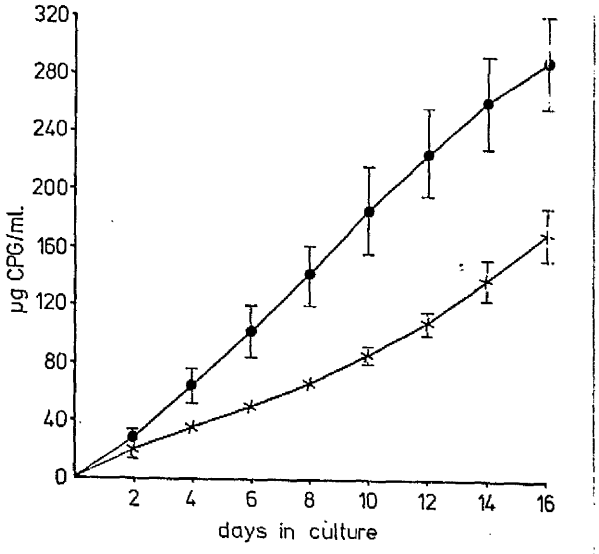
Two explants showing details of the breakdown of collagen in retinol treated cartilage.

a) Same specimen as in fig. 21c. Note: fibroblastic transformation of the chondrocytes, one of which is in mitosis. van Gieson. X 400.

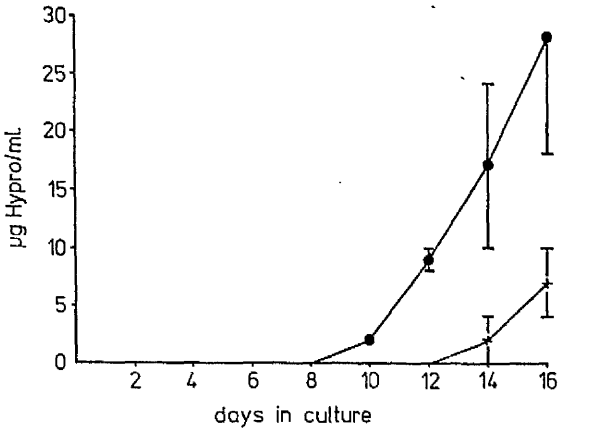
b) Same specimen as in fig. 21d. showing fibroblast-like chondrocytes invading the dead layer. van Gieson. X 400.

Fig.23

A



B



Graphs showing the mean cumulative release of proteoglycan (A) and hydroxyproline (B) from 12 pairs of explants from 3 experiments (four pairs in each experiment); one pair of each was cultivated in retinol-containing medium (10 i.u. retinol/ml) ●—● , and the other in control medium ×—× . Bars represent the standard error of the mean.

correlated with the amount of zone 3 included in the original explants. Two series of experiments were made to test this hypothesis.

In two experiments (Table 7) two explants, one thick and one thin, were cut from each condylar ridge; it was assumed that only the thicker fragment would be likely to contain zone 3. Some of these unequal pairs were grown in retinol-containing medium (10 iu retinol/ml) and the rest in control medium. Apart from the two thinnest of the explants treated with retinol which probably consisted mainly of zone 1 and showed a severe breakdown of collagen ($\overset{++}{(++}$, Table 7), the data recorded in Table 7 indicated that in both the experimental and control cultures, the degradation of collagen was greater in the thicker fragments.

The experiments were unsatisfactory, however, owing to the very wide variation in the thickness of the articular cartilage in different trotters. This meant that zone 3 might have been present in a thin explant or absent from a thick one depending on the initial thickness of the articular cartilage in individual animals. Once cartilage has begun to disintegrate, it is hard to tell whether zone 3 was originally present.

To obtain more precise information, strips of cartilage of different thicknesses were sliced from the articular condyles and each strip was then divided into two approximately equivalent parts; one part was fixed as a zero control (Figs. 24a,c) and the other cultivated for 16 days in either retinol-containing or control medium (Figs. 24b,d). It will be seen from Table 8a that in the presence of retinol, the loss of collagen was much greater when zone 3 was included in the explant (Fig. 24) although loss of collagen could be seen in explants of all thicknesses.

	Concentration of retinol	Number of explants	Loss of Collagen					
			++ ++	+ ++	++	+	±	0
Thick explants	10 iu/ml	16		1	6	7	2	0
Thin explants	10 iu/ml	16	2		2	3	6	3
Thick explants	-	16				4	3	9
Thin explants	-	16					1	15

Loss of collagen graded as in Table 1

TABLE 7

EFFECT OF RETINOL ON THICK OR THIN EXPLANTS

OF ARTICULAR CARTILAGE - ANALYSIS OF HISTOLOGY

Zones in explant	Concentration of retinol	Number of explants	Loss of Collagen					
			++ ++	+ ++	++	+	±	0
1 + 2	10 iu/ml	23		1	1	6	8	7
1 + 2 + 3	10 iu/ml	24		4	11	8	1	
1 + 2	-	25					6	19
1 + 2 + 3	-	23				1	8	14

(a) Loss of Collagen

Zones in explant	Concentration of retinol	Number of explants	Loss of Collagen					
			++ ++	+ ++	++	+	±	0
1 + 2	10 iu/ml	23	17	5	1			
1 + 2 + 3	10 iu/ml	24	14	8	2			
1 + 2	-	25	1		2	8	7	7
1 + 2 + 3	-	23			2	6	7	8

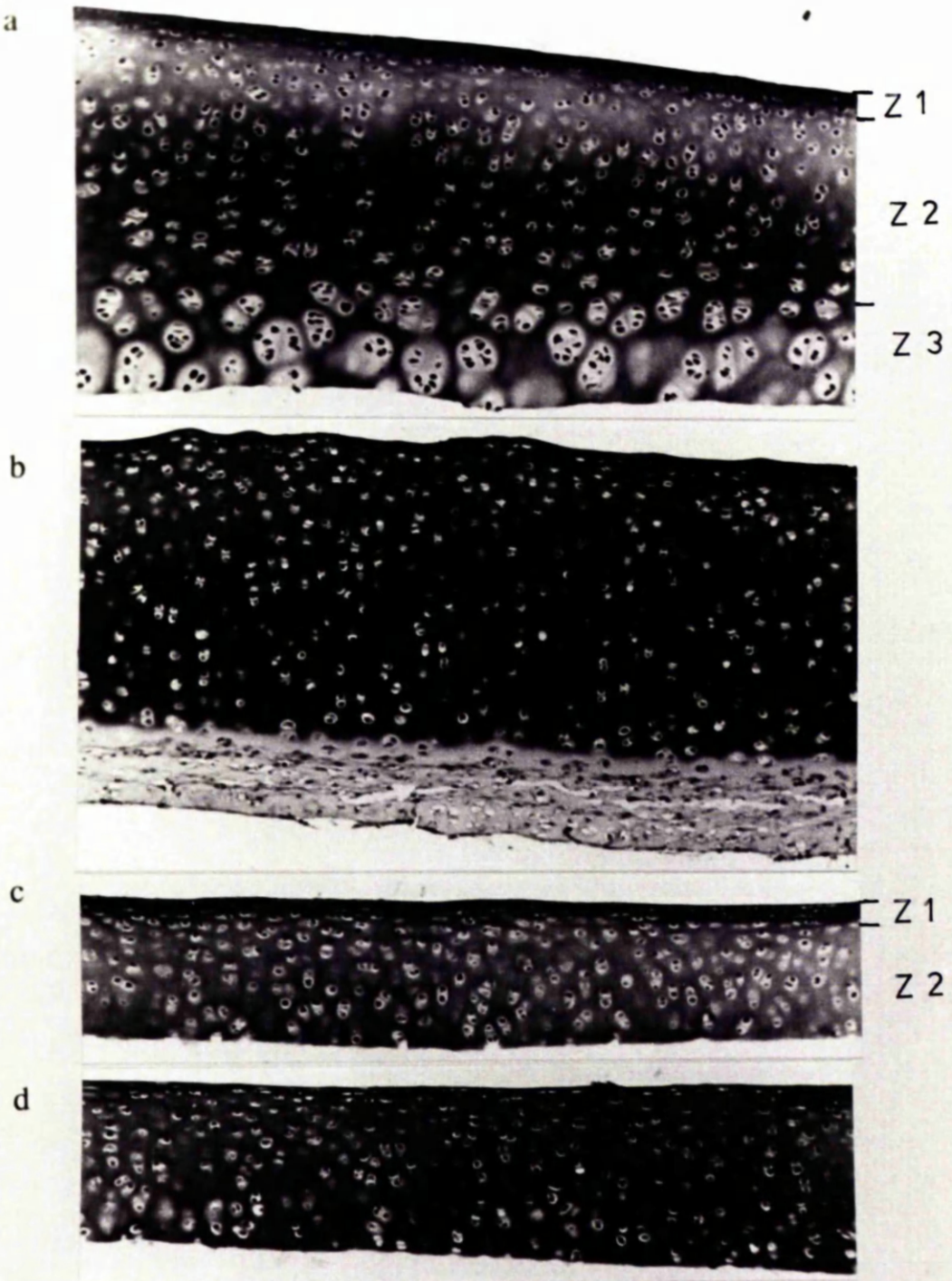
(b) Loss of Proteoglycan

Loss of proteoglycan graded as in Table 1

TABLE 8

EFFECT OF RETINOL ON BREAKDOWN OF MATRIX FROM DIFFERENT ZONES WITHIN ARTICULAR CATILAGE - ANALYSIS OF HISTOLOGY

Fig. 24



Explants illustrating the influence of zone 3 on the breakdown of collagen during 16 days' cultivation in the presence of retinol (10 i.u./ml). van Gieson. X 100.

a,b) Two fragments containing zone 3 (Z3) cut from the same strip of cartilage. (a) Fixed immediately. The colourless areas around the groups of chondrocytes in zone 3 (Z3) do not indicate absence of collagen (see p.). (b) Fixed after 16 days' cultivation in the presence of retinol; the collagen is severely degraded in zone 3.

c,d) A pair of fragments containing zone 1 and the upper part of zone 2 only, both taken from the same strip of cartilage. (c) Fixed immediately. (d) Fixed after 16 days' cultivation in medium containing retinol; the collagen is intact.

In the controls, the only explant showing significant degradation of collagen was one of those that contained all three zones. Depletion of proteoglycan (Table 8b) in both the retinol-treated and control specimens was unaffected by the presence of zone 3.

(c) Outgrowth in the Culture Vessels

In control and occasionally in retinol-containing medium, chondrocytes emerged from the cut surface of the cartilage and proliferated to form a layer of new tissue beneath the explant. Examination of the culture vessels at the end of the experiment showed that in many of the control cultures cells derived from the cartilage had populated both the supporting grid and the floor of the vessel. Forty-eight pairs of used culture vessels were studied in each of which two cartilage explants had been maintained for 16 days; one of each pair had contained control medium and the other medium to which 10 iu retinol/ml had been added.

Both on the grid and on the floor of the vessels outgrowth was more frequent and more abundant in the controls than in the retinol-treated cultures (Table 9). In control medium some of the cells which fell through the grid onto the floor aggregated to form tiny nodules inside which metachromatic material could sometimes be demonstrated by staining with dilute toluidine blue. Other cells adhered to the glass and gave rise to actively proliferating colonies of different sizes; some colonies consisted of rounded or polyhedral cells with large rounded nuclei and very basophilic cytoplasm; others were composed of expanded cells resembling fibroblasts but more commonly both forms were present along with many intermediate cells.

Only a small proportion of the cultures exposed to retinol produced colonies on the floor (Table 9); the cells were always of the fibroblastic form and no metachromasia was observed.

Concentration of retinol	Number of culture vessels	Outgrowth on floor of culture vessel		
		Slight	Scattered colonies	Profuse
10 iu/ml	48	42	5	1
-	48	3	32	13

TABLE 9

EFFECT OF RETINOL ON THE MIGRATION AND PROLIFERATION OF CELLS FROM ARTICULAR CARTILAGE

III SUMMARY

1. 5-10 iu per ml retinol stimulates chondrocytes from all zones of articular cartilage to degrade the surrounding proteoglycan and collagen during 16 days in culture.
2. Under the influence of retinol some of the chondrocytes proliferate and undergo fibroblastic transformation.
3. Breakdown of collagen is greatest in zone 3 of the cartilage studied which is composed of proliferating and hypertrophic chondrocytes.
4. The colonisation of the floor of the culture vessel by chondrocytes and phenotypic expression of these cells is inhibited by retinol.

IV CONCLUSION

A simple agent, such as retinol, is able to stimulate articular chondrocytes, degrading proteoglycan and collagen with fibroblastic transformation of the cells.

CHAPTER 7

EFFECT OF HYPEROXIA ON THE BREAKDOWN OF CARTILAGE BY SYNOVIUM

Hyperoxia has been shown to increase the lysosomal enzyme activity of Hela and chick embryonic cells in cell culture (197). A similar effect was found by Sledge and Dingle in embryonic limb bone rudiments maintained in organ culture in 85% oxygen; after 8 days there was loss of metachromatic staining in the cartilage matrix associated with increased cathepsin D activity in the medium (196). As the limb bone rudiments consist of chondrocytes and fibrous perichondral tissue, it is not clear whether the chondrocytes are causing the depletion. Loss of proteoglycan from isolated articular cartilage in organ culture has been reported in rabbit knee cartilage cultivated in 90% oxygen (49) and in human cartilage from the femoral head in 95% oxygen (57). At this level of oxygen, however, three-quarters of the chondrocytes in the human cartilage were dead after 8 days.

I OBJECT OF EXPERIMENTS

It was decided to study the effect of hyperoxia on isolated articular cartilage, synovial tissue and on the interaction between the two during cultivation in organ culture.

II RESULTS

(a) Isolated Articular Cartilage

Explants of cartilage were cultured in 20%, 55% or 95% O_2. After 10 days, histological examination showed no sign of depletion of

either proteoglycan or collagen in the two hyperoxic groups as compared with their paired controls in 20% O_2 (Table 10, Fig. 25). In some of the explants exposed to 95% O_2, pyknotic nuclei were present among the superficial cells in immediate contact with the atmosphere, but otherwise the cells of the hyperoxic cartilage appeared viable.

The cumulative release of proteoglycan into the medium was slightly lower in the hyperoxic group, but this was not statistically significant, and there was no release of hydroxyproline. Collagenolytic activity was not detected (Table 11).

(b) Isolated Synovium

The synovial explants that were maintained in 55% O_2 appeared macroscopically smaller than the controls in 20% O_2, but no microscopic difference could be seen. In both groups the cells were viable with many invading the Millipore substrate and mitotic figures were common.

Analysis of the used medium (Table 11) showed significantly more proteoglycan and hydroxyproline in the hyperoxic than in the control cultures. Latent collagenolytic activity was present in the medium of both hyperoxic and control cultures; it was 36% higher in the hyperoxic group.

(c) Cartilage in Contact with Synovium

After 10 days' cultivation in contact with synovial tissue metachromasia was often completely absent in both the hyperoxic (55% O_2) and the control explants (Table 12, Fig. 26a,b), but breakdown of collagen was greater in the hyperoxic cartilage (Table 12). In sections stained with van Gieson's stain it was

Number of explants	% oxygen	Loss of Proteoglycan (Toluidine blue)					
		++ ++	+ ++	++	+	±	0
12	20				2	4	6
12	55					3	9
9	95					3	6

Degree of depletion as graded in Table 1

TABLE 10

EFFECT OF OXYGEN ON ISOLATED CARTILAGE IN ORGAN CULTURE FOR 10 DAYS - ANALYSIS OF HISTOLOGY

	Cumulative release of proteoglycan (µg/ml)			Cumulative release of hydroxyproline (µg/ml)			Cumulative release of collagenolytic activity (units/ml)		
	20% oxygen	55% oxygen*	p value**	20% oxygen	55% oxygen	p value	20% oxygen	55% oxygen	p value
Isolated cartilage	86 (13)+	−14% (12)	n.s.	0	0		0	0	
Isolated synovium	23 (8)	+50% (34)	<0.05	50 (3)	+45% (13)	<0.005	3.17 (3.5)	+36% (19)	<0.01
Cartilage + synovium in contact	208 (102)	+5% (7)	n.s.	56 (16)	+62% (24)	<0.001	4.0 (1.9)	+58% (38)	<0.005
Cartilage + synovium separated	180 (25)	−20% (19)	n.s.	86 (24)	+24% (12)	<0.02	1.33 (0.75)	+51% (22)	<0.01

* Results in 55% oxygen expressed as a percentage of their paired controls in 20% oxygen

** Paired explants analysed by paired 't' test

\+ Standard deviation

TABLE 11

EFFECT OF OXYGEN ON BREAKDOWN OF CARTILAGE BY SYNOVIUM AFTER 10 DAYS IN CULTURE

ANALYSIS OF CULTURE MEDIUM. EACH VALUE IS THE MEAN FROM AT LEAST FIVE EXPERIMENTS

Number of explants	% oxygen	Loss of Proteoglycan (Toluidine blue) ++ ++	+ ++	++	+	±	0	Loss of Collagen (van Gieson) ++ ++	+ ++	++	+	±	0
13	20	6	5	1	1					1	1	10	1
15	55	8	3	4				4	4	6	1		
		not significant						*p <0.001					

* Paired explants analysed by paired 't' test

The degree of depletion is as described in Table 1

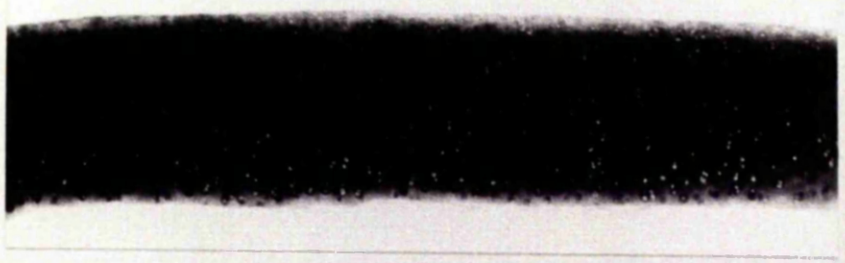
TABLE 12

EFFECT OF OXYGEN ON CARTILAGE IN CONTACT WITH SYNOVIUM IN CULTURE FOR 10 DAYS - ANALYSIS OF HISTOLOGY

Fig. 25

a

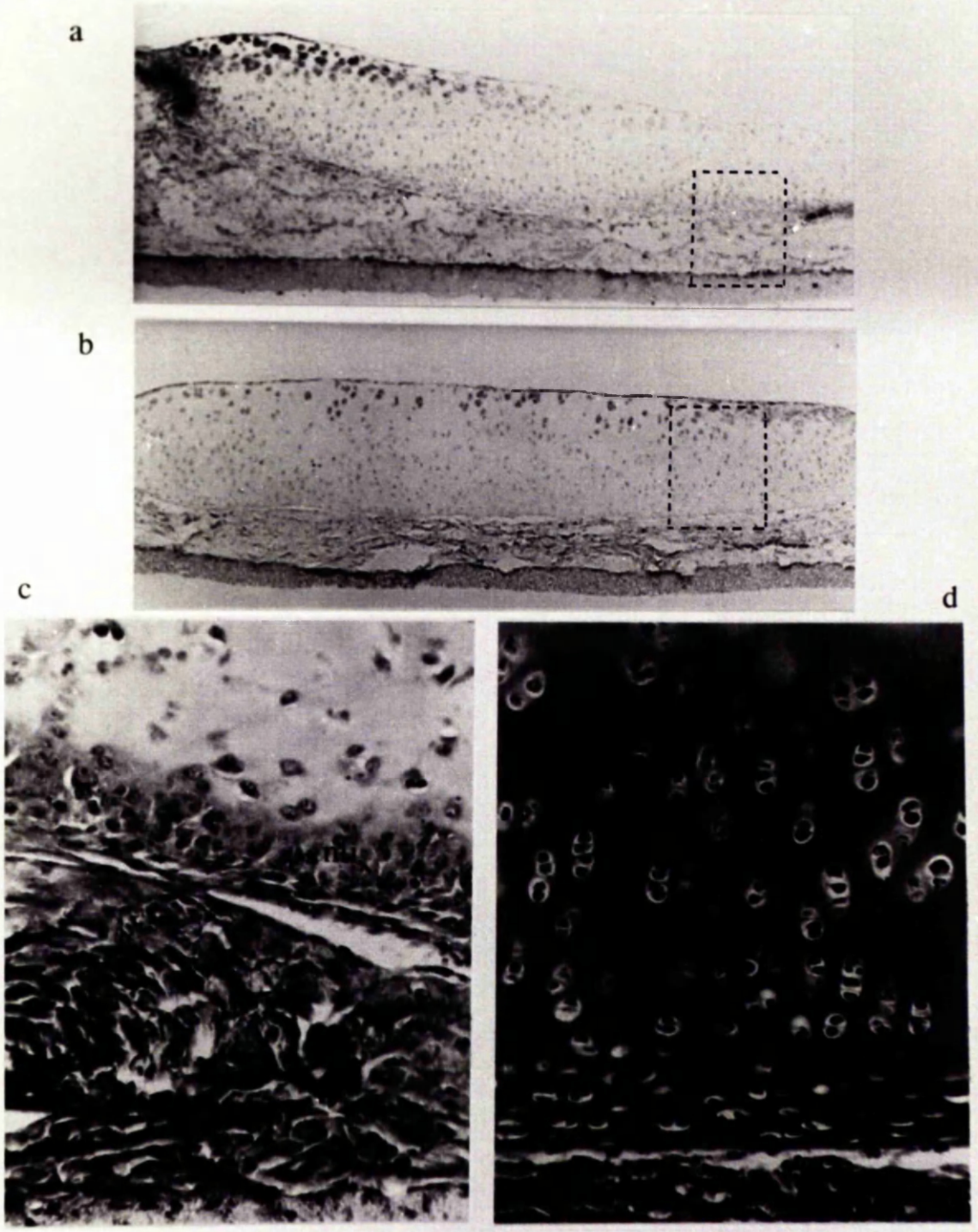
b



a) Isolated living cartilage ; 10 days culture in 55% O_2.
No loss of metachromasia. (0; table 10). Tol. blue. X 40 .

b) Paired living cartilage explant ; 10 days culture in 20% O_2.
No loss of metachromasia. (0; table 10). Tol. blue. X 40 .

Fig. 26



a) Living cartilage on top of living synovial mince; 10 days culture in 55% O_2. Complete loss of metachromasia.(++++; table 12). Tol. blue. X 40 .

b) Paired living cartilage on living synovium; 10 days culture in 20% O_2. Complete loss of metachromasia. (++++; table 12). Tol. blue. X 40 .

c) Same explant as in (a) under higher magnification showing loss of collagen from the cartilage with viable chondrocytes; mi = mitosis. van Gieson. X 250.

d) Same explant as in (b) under higher magnification. No loss of collagen. van Gieson. X 250 .

seen that the degradation of the collagen began at the margin of the articular zone, the surface of which was in contact with the synovial tissue. There was considerable variation in the degree of breakdown in different pairs of explants and in some the matrix had almost disappeared in the specimens cultured in high oxygen (Fig. 26c,d).

The biochemical findings confirmed the histological observations. There was little difference either in the amount of proteoglycan liberated by the two groups after 10 days or in the pattern of its release (Fig. 27). In the hyperoxic cultures, however, the collagenolytic activity of the used medium was about 50% higher than that of the controls and about 60% more hydroxyproline was released (Table 11).

(d) Cartilage Separated from Synovium

From the above results it was not clear whether the hyperoxia accelerated the breakdown of cartilage in contact with synovium by enhancing the direct enzymatic action of the synovium on the cartilage matrix, by increasing its indirect effect mediated through the chondrocytes or by stimulating both processes. The experiments were therefore repeated with the cartilage and synovium grown on the same Millipore but at a distance from each other; this system would exclude the direct action of the synovium, for which contact between the two tissues is required.

Histological examination of the cartilage explants after 10 days' incubation under these conditions, showed that far from increasing the indirect action of the synovium, the hyperoxia actually diminished the loss of both metachromatic material and collagen from the matrix (Table 13, Fig. 28). The cartilage was placed on the Millipore with

Number of explants	% oxygen	Loss of Proteoglycan (Toluidine blue)						Loss of Collagen (van Gieson)					
		++ ++	+ ++	++	+	±	0	++ ++	+ ++	++	+	±	0
16	20		2	8	5	1				2	10	4	
15	55			2	7	5	1					4	5	6
			*p <0.005						p <0.001				

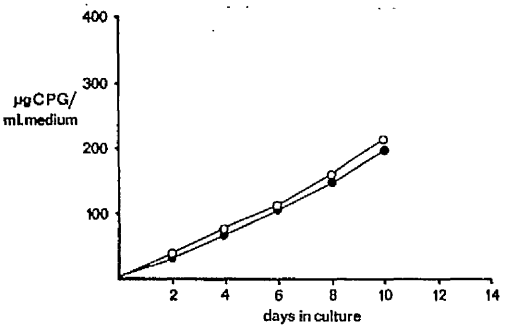
* Paired explants analysed by paired 't' test

The degree of depletion is as described in Table 1

TABLE 13

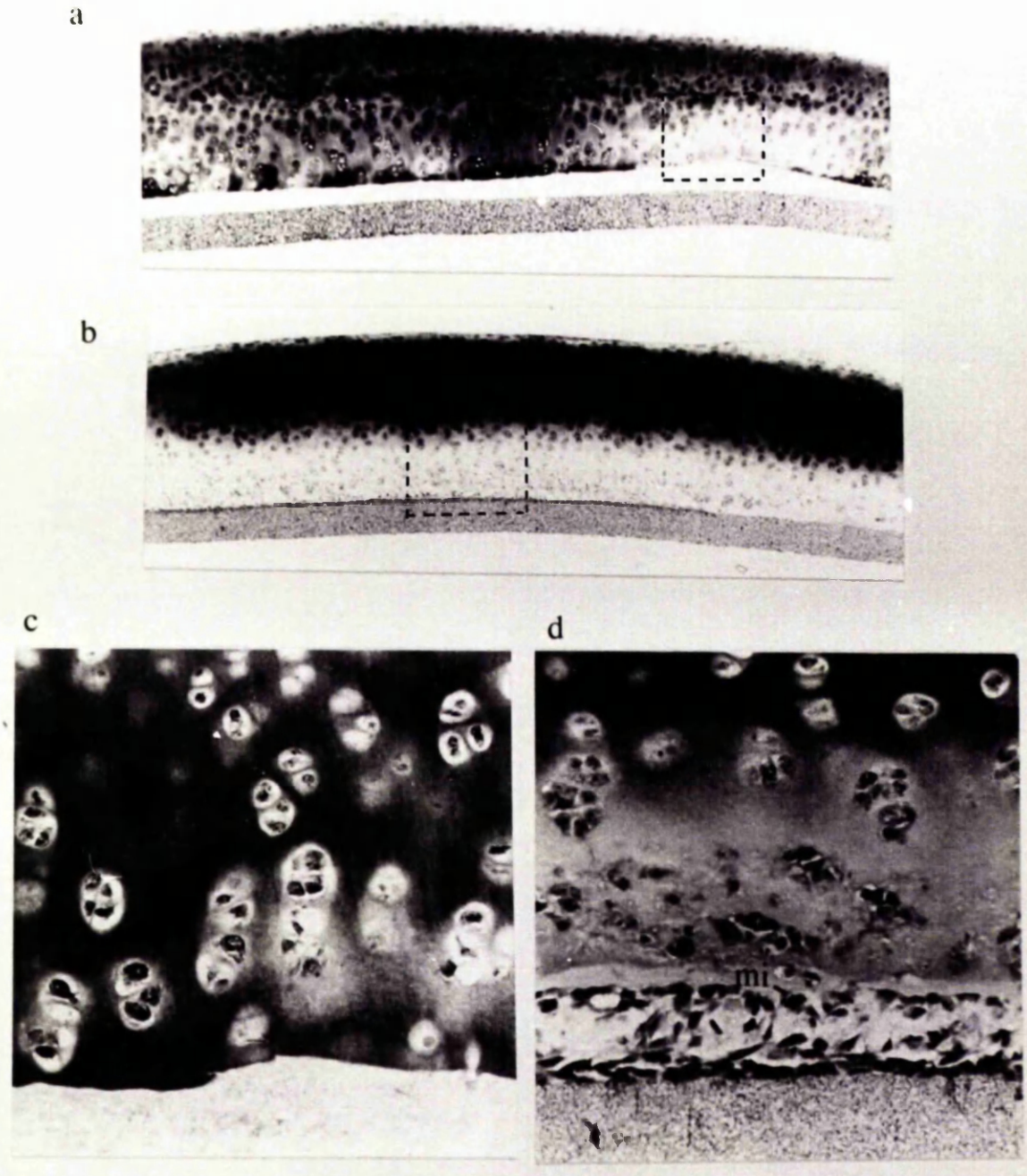
EFFECT OF OXYGEN ON CARTILAGE SEPARATED FROM SYNOVIUM IN CULTURE FOR 10 DAYS - ANALYSIS OF HISTOLOGY

Fig. 27



Cumulative release of proteoglycan (PG) after 10 days in culture of paired explants of living cartilage in contact with living synovium: O——O explants maintained in 55% O_2 .
●——● explants maintained in 20% O_2 . Curves represent the mean values from four paired explants.

Fig. 28



a) Living cartilage separated from living synovialtissue; 10 days culture in 55% O_2. Patchy loss of metachromasia above millipore (+ ; table 13). Tol. blue. X 40 .

b) Paired living cartilage separated from living synovium ; 10 days culture in 20% O_2. Extensive loss of metachromasia (++; table 13). Tol. blue. X 40 .

c) Same explant as in (a) under higher magnification. Minimal depletion of collagen. van Gieson. X 250.

d) Same explant as in (b) under higher magnification. Loss of collagen with proliferation of the chondrocytes; mi = mitosis. van Gieson. X 250.

the articular surface upwards; in the controls (20% O_2) the area immediately above the Millipore, which included the cells of the columnar zone, showed depletion of the matrix and the transformation of the chondrocytes into basophilic cells of irregular shape, often in active mitosis (Fig. 28d). These changes, though not entirely suppressed, were less obvious in the cultures exposed to 55% O_2 (Fig. 28c).

Biochemical assay of the used medium showed that the hyperoxic tissues released less proteoglycan into the medium than the controls in 20% O_2 (Table 11). Virtually all of this material is derived from the cartilage, as the synovium liberates very little (see p. 65). The hydroxyproline in the used medium, which reflects the breakdown of collagen, comes from both the cartilage and the synovium and in these experiments it was only 24% above that in the used control medium, in contrast to a difference of 60% when the two tissues were in contact. The latent collagenolytic activity, which is produced entirely by the synovial explant, was about 50% higher in the hyperoxic group.

These results imply that the increased breakdown of cartilage matrix, induced by hyperoxia (55% O_2) when the cartilage is in contact with the synovial tissue, is due to an enhancement of the direct enzymatic action of the synovium on the matrix. The release of proteoglycan is unaffected and the greater destruction of the matrix is due to an increase in the release of collagenolytic activity from the synovium. This increased breakdown takes place despite inhibition of the collagenolytic action of the chondrocytes.

III SUMMARY

1. Hyperoxia (55% and 95%) does not induce breakdown of isolated articular cartilage from the pig.
2. Hyperoxia (55%) increases the release of hydroxyproline, proteoglycan and collagenolytic activity from isolated synovial tissue.
3. Hyperoxia (55%) enhances the breakdown of cartilage by synovial mince when the tissues are in contact.
4. Hyperoxia (55%) inhibits the destructive effect of the synovium on the cartilage that is mediated through the chondrocytes.

IV CONCLUSION

Hyperoxia does not induce the breakdown of isolated pig articular cartilage, but it has a dual effect on the breakdown induced by synovial tissue by stimulating the direct effect while inhibiting the indirect effect.

CHAPTER 8

EFFECT OF MONONUCLEAR LEUCOCYTES ON THE BREAKDOWN OF CARTILAGE IN CONTACT WITH SYNOVIUM

A prominent feature of the synovium in erosive joint disease is the presence of large numbers of chronic inflammatory cells (3). It is not known what role, if any, they play in the pathogenesis of the cartilage erosion.

An experimental erosive arthritis can be produced in rabbits by the intra-articular injection of factors from activated lymphocytes (305) and phytohaemagglutinin (PHA) has a similar effect (164). PHA predominantly stimulates T lymphocytes (306) which then release lymphokines. These factors from stimulated lymphocytes have been shown to enhance the release of collagenase (307) and lysosomal enzymes (308) from macrophages. They increase the secretion of collagenase by rheumatoid synovial cells (309) and they induce the breakdown of cartilage proteoglycan by a monocyte-dependent mechanism (310). It has been suggested that the lymphocytes enhance the destruction of cartilage by attracting monocytes into the rheumatoid synovium (311) but others have suggested that a marked lymphocytic infiltration is associated with less joint damage (76).

I OBJECT OF EXPERIMENTS

The experiments described in this Chapter were made to study the influence of mononuclear leucocytes on the breakdown of articular cartilage when in contact with synovium. In these experiments the culture period was reduced to 8 days, normal heat-inactivated pig serum was added to the medium instead of rabbit serum, and PHA was used to stimulate the lymphocytes.

II RESULTS

(a) Mononuclear Leucocytes from Peripheral Blood

Leucocytes added to the organ culture When added to the medium in the culture dish blood leucocytes (3×10^6 cells) enhanced the loss of proteoglycan from affronted explants of cartilage and synovium. After 8 days there was almost total loss of metachromatic staining from the cartilage with lymphocytes, in contrast to control cultures, devoid of leucocytes in which there was only partial depletion of the matrix (Table 14, Fig. 29). Analysis of the used medium indicated more release from the explants cultured with leucocytes than from their paired controls (Fig. 30a).

Monocyte-depleted leucocytes added to the organ culture Blood mononuclear leucocytes are a mixture of monocytes and lymphocytes. The experiments were repeated with cell preparations from blood that had been treated with carbonyl iron and then exposed to a magnetic field to reduce the number of monocytes. The effectiveness of this procedure was assessed at the end of the culture period by fixing and staining the cells on the floor of the culture dish. It was fairly easy to see whether or not the macrophage-like cells were present. In two experiments carbonyl iron treatment failed to reduce the population of macrophages visible on the floor; these experiments were discarded. In other experiments, however, carbonyl iron treatment greatly reduced the number of macrophages; in these cultures there was no enhancement of the cartilage breakdown (Table 14, Fig. 31) and the explants were not significantly different from the control devoid of leucocytes. Analysis of the medium from paired explants showed that the release of proteoglycan was less in the monocyte-depleted group (Fig. 30b).

3×10^6 leucocytes added to cultures	Number of explants	Loss of Proteoglycan (toluidine blue) ++ ++	+ ++	++	+	±	0
Control	32		2	18	12		
Blood leucocytes	28	12	11	3	2		
Lymph gland leucocytes	12			2	5	5	
Monocyte depleted blood leucocytes	20	2	5	6	6	1	
Blood leucocytes 2 hr pre-incubation with PHA	8	4	2	2			
Lymph gland leucocytes 2 hr pre-incubation with PHA	16			4	10	2	

Blood leucocytes vs Control $p < 0.0001$ (student's 't' test)

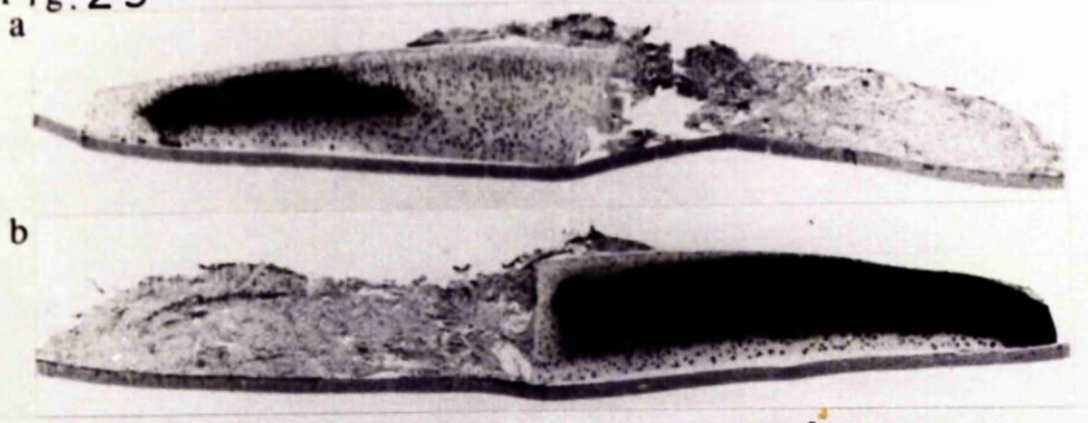
Blood leucocytes vs Monocyte depleted blood leucocytes $p < 0.001$

Degree of depletion as in Table 1

TABLE 14

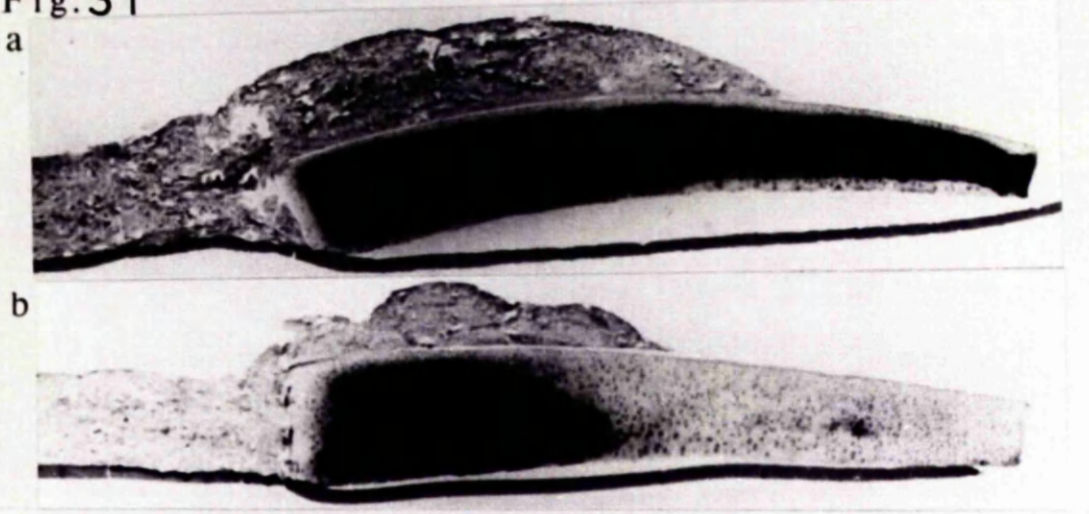
EFFECT OF BLOOD AND LYMPH GLAND MONONUCLEAR LEUCOCYTES ON THE LOSS OF PROTEOGLYCAN FROM CULTURES OF CARTILAGE IN CONTACT WITH SYNOVIUM FOR 8 DAYS IN CULTURE. ANALYSIS OF HISTOLOGY

Fig. 29



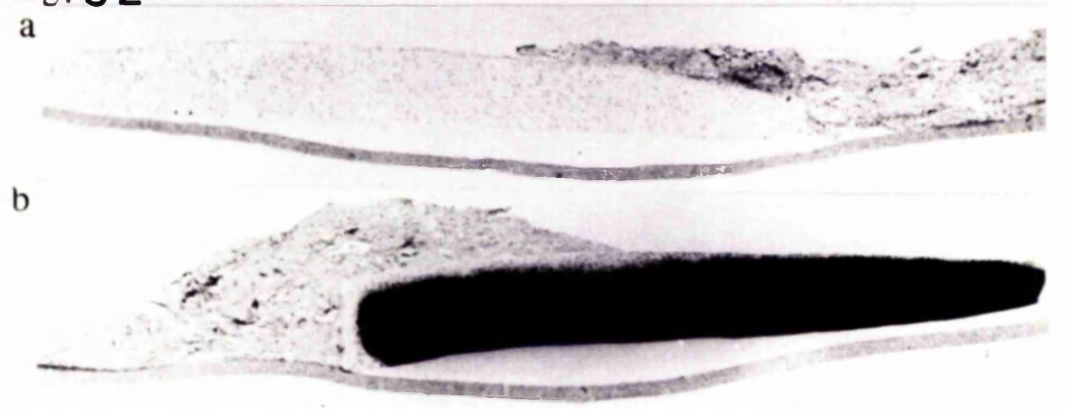
a) Cartilage and synovium cultured in contact for 8 days in the presence of 3 x 10^6 blood mononuclear leucocytes. Note extensive loss of metachromasia. (+++). Tol. blue. x 15 .
b) Paired explant of cartilage with synovium after 8 days in control medium. (+). Tol. blue. x 15 .

Fig. 31



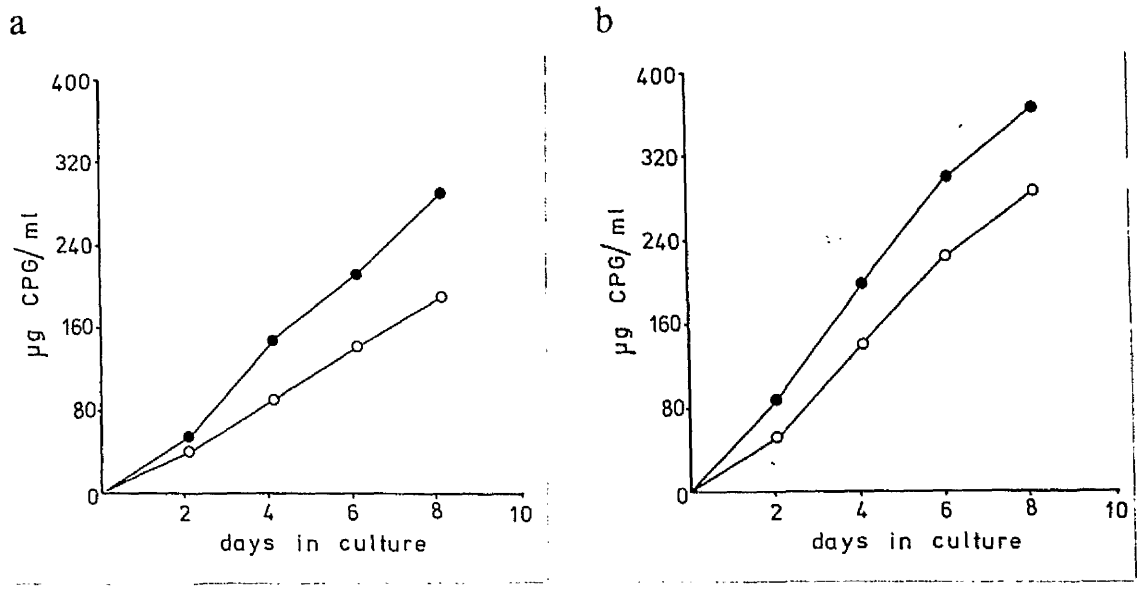
a) Cartilage and synovium cultured in contact for 8 days in the presence of 3 x 10^6 monocyte-depleted blood leucocytes. (+). Tol. blue. x 15 .
b) Paired explant of cartilage with synovium after 8 days in culture with 3 x 10^6 blood mononuclear leucocytes. (+++). Tol. blue. x 15 .

Fig. 32



a) Cartilage and synovium cultured in contact for 8 days in the used medium from 48 hour cultures of 1 x 10^6/ ml blood mononuclear leucocytes. (++++). Tol. blue. x 15 .
b) Paired explant of cartilage with synovium after 8 days in control medium. (+). Tol. blue. x 15 .

Fig.30



a. Cumulative release of proteoglycan after 8 days in culture from paired explants of cartilage and synovium in contact:

●——● 3×10^6 blood mononuclear leucocytes added to the cultures.

○——○ control cultures with no leucocytes.

b Cumulative release of proteoglycan after 8 days in culture from paired explants of cartilage and synovium in contact:

●——● 3×10^6 blood mononuclear leucocytes added to the cultures.

○——○ 3×10^6 monocyte-depleted blood leucocytes added to the cultures.

Used medium from leucocyte cultures added to the organ culture In one experiment blood leucocytes were added either on top of the synovium or directly into the medium. The extent of breakdown was the same in each group suggesting that the effect of the leucocytes was due to an alteration in the culture medium and not to a direct action on the synovial cells.

A series of experiments was made to examine the effect of used medium decanted from 48 hour cultures of blood leucocytes on cartilage and synovium in contact (Table 15, Fig. 32). The used medium from the leucocytes caused an increased breakdown of the cartilage.

(b) Mononuclear Leucocytes from Lymph Glands

The results so far have failed to demonstrate any effect of the peripheral blood lymphocytes. The experiments were repeated, therefore, with leucocytes from a different source (mesenteric lymph gland) to see if this made any difference.

Leucocytes added to the organ culture When the experiments were repeated with lymph gland leucocytes no effect could be seen (Table 14) and the presence of these cells failed to enhance the breakdown of cartilage in contact with synovium.

Used medium from leucocyte cultures added to the organ cultures A series of experiments was made to examine the effect of medium from 48 hour cultures of leucocytes (Table 15). Again the lymph gland leucocytes failed to alter the breakdown of the cartilage by the synovium.

(c) Effect of PHA on the Leucocytes, Cartilage and Synovium

The results reported in the previous Sections demonstrated that blood monocytes affect cartilage and synovium in culture, but failed

Used culture medium	Number of explants	Loss of Proteoglycan (toluidine blue)					
		++ ++	+ ++	++	+	±	0
Control	31			8	18	5	
Blood leucocytes	16	7	6	3			
Lymph gland leucocytes	8			2	6		
Blood leucocytes 2 hr pre-incubation with PHA	40	21	12	5	2		
Lymph gland 2 hr pre-incubation with PHA	28		1	5	19	3	

TABLE 15

EFFECT OF USED MEDIUM FROM 48 HOUR CULTURES OF BLOOD AND LYMPH GLAND LEUCOCYTES ON LOSS OF PROTEOGLYCAN FROM CULTURES OF CARTILAGE IN CONTACT WITH SYNOVIUM FOR 8 DAYS IN CULTURE. ANALYSIS OF HISTOLOGY

to show any destructive role for blood lymphocytes or lymph gland leucocytes. The experiments were repeated after treating the leucocytes with PHA to see whether or not activated lymphocytes had an effect on the breakdown of the matrix.

PHA and lymph gland leucocytes in the organ culture In one group explants of cartilage and synovium were cultured for 48 hours in the presence of lymph gland leucocytes in medium containing PHA; controls were grown in medium with PHA but without leucocytes. After 8 days histological examination showed that cartilage in contact with synovium cultured with leucocytes plus PHA had lost much more metachromatic material and had released more proteoglycan into the medium than the controls cultured with PHA alone (Table 16, Figs. 33,34).

PHA alone in the organ culture PHA itself had an effect on the cartilage and synovial explants and when added to the culture medium it caused more degradation of the matrix than in paired controls (Fig. 35).

Pre-incubation of leucocytes with PHA In order to minimise the effect of PHA on the cartilage and synovium the experiments were repeated with blood and lymph gland leucocytes which had been pre-incubated with PHA for 2 hours. Other work suggested that lymphocytes can be activated by exposure to PHA for this length of time (312).

After their 2 hour incubation with PHA the leucocytes were washed and then either added to organ cultures or cultured separately. When added directly to the cultures neither blood nor lymph gland leucocytes had any effect on the breakdown of the matrix as compared with controls to which untreated leucocytes had been added (Table 14). In other

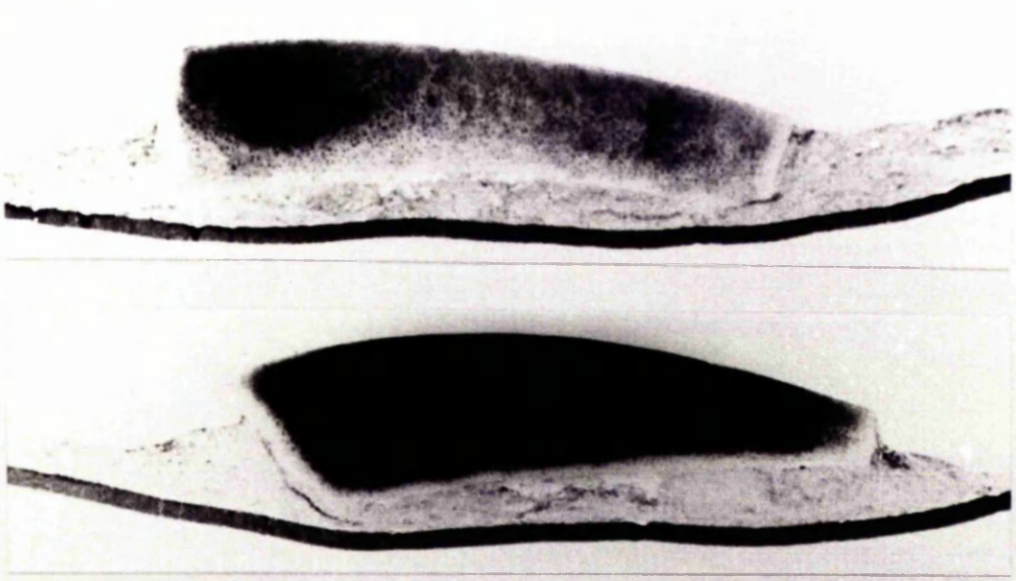
3×10^6 leucocytes added to cultures	Number of explants	Loss of Proteoglycan (toluidine blue) ++ ++	+ ++	++	+	±	0
Lymph gland leucocytes	16	4	4	8			
Control (+ PHA)	16		2	2	11	1	

Lymph gland leucocytes vs Control $p < 0.001$
(student's 't' test)

TABLE 16

EFFECT OF LYMPH GLAND LEUCOCYTES ON THE LOSS OF PROTEOGLYCAN FROM CULTURES OF CARTILAGE IN CONTACT WITH SYNOVIUM MAINTAINED FOR 8 DAYS IN CULTURE WITH PHA FOR THE FIRST 2 DAYS

Fig. 33

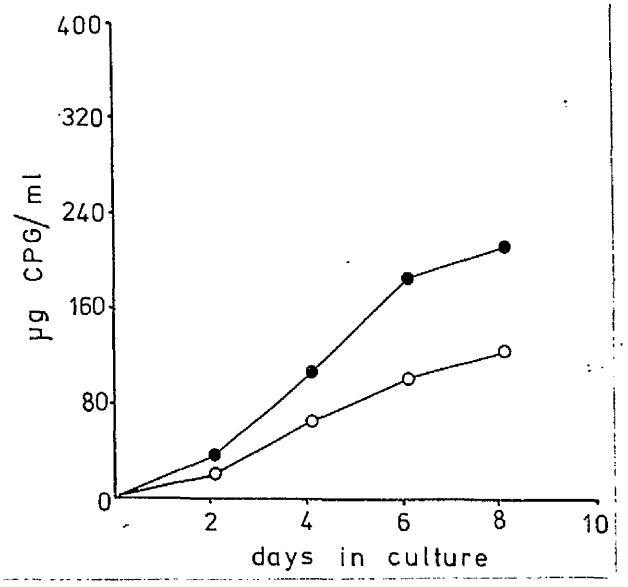


a) Cartilage and synovium cultured in contact for 8 days in the presence of 3 x 10^6 lymph gland leucocytes with phytohaemagglutinin for the first 2 days. (+++). Tol. blue. x 15 .
b) Paired explant of cartilage with synovium after 8 days in culture with phytohaemagglutinin for the first 2 days. (+). Tol. blue. x 15 .

Fig. 35

a) Cartilage and synovium cultured in contact for 8 days with phytohaemagglutinin in the medium for the first 2 days. (++). Tol. blue. x 15 .
b) Paired explant of cartilage with synovium after 8 days in control medium. (+). Tol. blue. x 15 .

Fig.34



Cumulative release of proteoglycan after 8 days in culture from paired explants of cartilage and synovium in contact in the presence of phytohaemagglutinin for the first 2 days :

●——● 3×10^6 lymph gland leucocytes added to the cultures.

○——○ control cultures with no leucocytes.

experiments the two sources of leucocytes were cultured for 48 hours and the cartilage and synovium were maintained in the used medium for 8 days; there was still no enhanced breakdown due to the PHA (Table 15).

III SUMMARY

Leucocytes have the following effects on the breakdown of pig cartilage grown for 8 days in contact with synovium:

1. Blood mononuclear leucocytes enhance the loss of cartilage proteoglycan.
2. Monocyte-depleted blood leucocytes have no effect.
3. The effect of the blood mononuclear leucocytes is due to an alteration in the culture medium.
4. Lymph gland leucocytes have no effect.
5. Lymph gland leucocytes exposed to PHA for 48 hours increase the loss of proteoglycan.
6. Two hour exposure of blood or lymph gland leucocytes to PHA does not enhance the breakdown of matrix.

IV CONCLUSION

Peripheral blood mononuclear leucocytes, PHA stimulated lymph gland leucocytes, and PHA can all enhance the breakdown of cartilage when in contact with synovium. The effect of the peripheral blood's cells is monocyte dependent.

CHAPTER 9

EFFECT OF SYNOVIAL TISSUE ON THE UPTAKE OF ^{35}S INTO CARTILAGE

One of the earlier experiments (Fig. 3) suggested that the synovium not only caused the breakdown of the existing matrix but also inhibited the synthesis of new proteoglycan. It was found that isolated living cartilage, while retaining its metachromasia, released much more proteoglycan into the medium than isolated dead cartilage (Fig. 3a); this implied that new proteoglycan was being synthesised by the living chondrocytes. When such explants were grown in contact with synovial tissue, both the living and dead fragments lost their metachromasia, and both shed virtually the same amount of proteoglycan into the medium (Fig. 3c); this suggested that the synovium was inhibiting the living chondrocytes from forming new proteoglycan.

I OBJECT OF EXPERIMENTS

Experiments were made to investigate the rate of synthesis of proteoglycan by studying the uptake of radio-active sulphate ($^{35}SO_4$) by articular cartilage after cultivation with and without synovium.

II RESULTS

(a) Uptake of ^{35}S Into Isolated Cartilage

Comparison of paired explants Since the effects of different experimental conditions were always compared on paired explants, it was important to test the reliability of this procedure.

The uptake of $^{35}SO_4$ by paired explants, each pair taken from the same strip of cartilage, was measured under four different labelling conditions (Table 17). There was no significant difference between the means of the pairs in any of the groups. On the other hand in each group there was wide variation in both the uptake of $^{35}SO_4$ (four-fold) and the wet weights (range of 3–19 mg) of explants from different trotters; there was no correlation between uptake and wet weight.

In paired explants cultured for 8 days and exposed either to 1.0 µCi $^{35}SO_4$/ml for the last 48 hours of the culture period (4 pairs) or to 10 µCi $^{35}SO_4$/ml for the final 2 hours (8 pairs), the intensity and distribution of the autoradiograph were similar in both members of a pair. The autoradiograph was weakest over the articular zone 1, increased over zone 2 and suddenly became very intense over the border between zones 2 and 3 (Fig. 37a). It declined over the deeper, hypertrophic cells of zone 3. The uptake was mainly over the cells but especially in the explants exposed for 48 hours, there was also a diffuse deposit of silver over the matrix. There was some variation in the amounts of zones 2 and 3 included in the two explants of a pair, and since the incorporation of ^{35}S differs at different levels in the cartilage, this probably accounted for some scatter in the biochemical assay.

Explants cultured (a) in reverse orientation and (b) immersed in medium

(a) To find whether the amount and pattern of uptake of $^{35}SO_4$ was affected by the orientation of the cartilage on the grid, paired explants were prepared in which one member of each pair was placed with the articular surface upwards in the usual way and the other with this surface downwards; they were incubated for 24 hours in

Labelling Conditions				Test Explant		Control Explant			
Length of culture (days)	$^{35}SO_4$ pulse (hrs)	Do $^{35}SO_4$ (μCi/ml)	Number of pairs	Culture	Mean uptake (cpm)	Culture	Mean uptake (cpm)	%* Difference test/control	p+
1	24	1	20	Cartilage on grid	4224	Cartilage on grid	4284	- 1%	ns
2	48	1	10	"	5859	"	5427	+ 8%	ns
8	48	1	12	"	4989	"	5328	- 6%	ns
8	2	10	18	"	1998	"	2046	- 2%	ns
1	24	1	8	Cartilage articular surface down	4614	Cartilage on grid	4710	- 2%	ns
1	24	1	8	Cartilage immersed	4164	"	4404	- 6%	ns
2	48	1	12	Dead cartilage	0	"	5673	-	

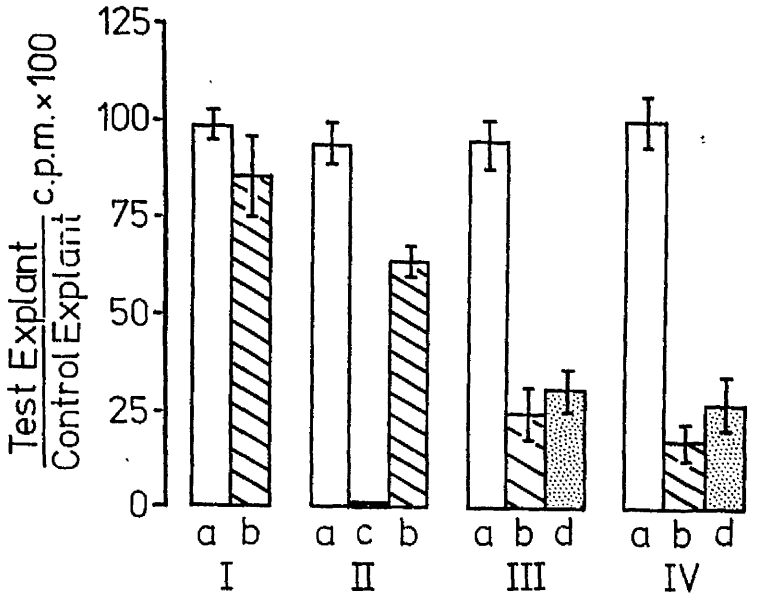
* $\frac{\text{Mean difference between pairs (test - control)}}{\text{Mean of control explants}} \times 100$

+ Paired student's 't' test ns = not significant

TABLE 17

UPTAKE OF ^{35}S INTO PROTEOGLYCAN: COMPARISON OF PAIRED CARTILAGE EXPLANTS UNDER IDENTICAL AND DIFFERENT EXPERIMENTAL CONDITIONS. THE CARTILAGE WAS EXPLANTED ARTICULAR SURFACE UPWARDS UNLESS OTHERWISE STATED

Fig. 36



Histogram showing inhibition by synovial tissue of uptake of ^{35}S into cartilage proteoglycan (CPG) under four different labelling conditions; bars represent standard error of the mean.

I culture period: 24 hrs with 1 µCi $^{35}SO_4$/ml for 24 hrs

II culture period: 48 hrs with 1 µCi $^{35}SO_4$/ml for 48 hrs

III culture period: 8 days with 1 µCi $^{35}SO_4$/ml for last 48 hrs

IV culture period: 8 days with 10 µCi $^{35}SO_4$/ml for last 2 hrs

There were four different culture conditions:-

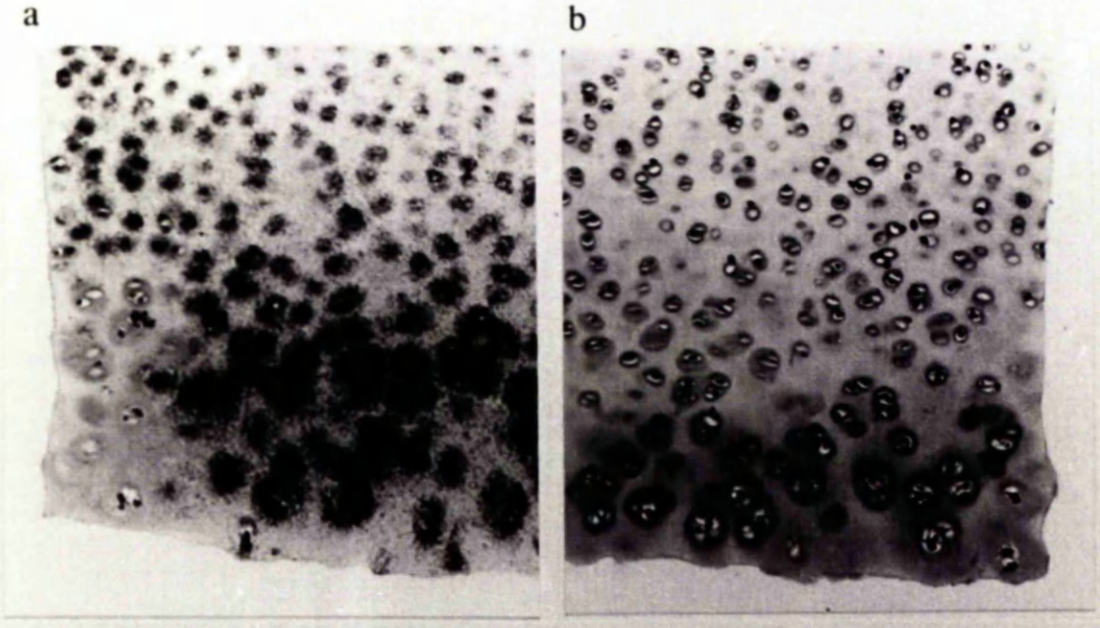
a. test explant: isolated cartilage.
   control explant: paired isolated cartilage.

b. test explant: cartilage in contact with synovial tissue.
   control explant: paired isolated cartilage.

c. test explant: dead cartilage.
   control explant: paired living isolated cartilage.

d. test explant: cartilage separated from synovial tissue.
   control explant: paired isolated cartilage.

Fig.37

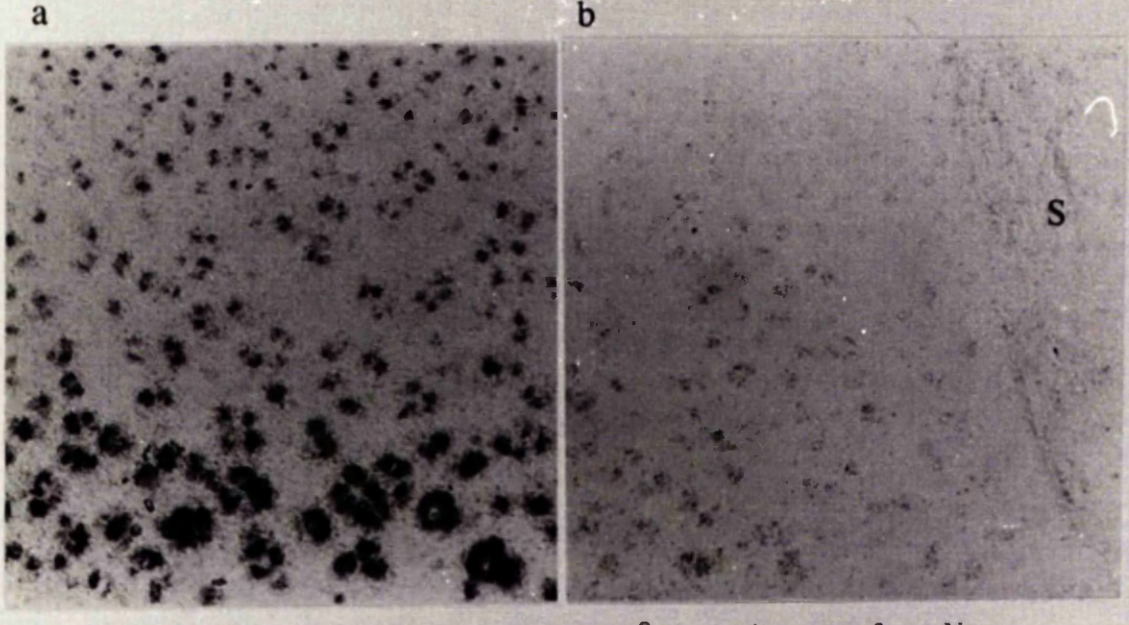


Paired cartilage explants incubated for 2 days in medium containing 1 µCi $^{35}SO_4$ per ml.

a) Living explant; intense uptake except in a dead corner. x 100.

b) Dead explant; no uptake. Carazzi's haematoxytin. x 100.

Fig.38



Paired cartilage explants incubated for 8 days in normal medium plus 2 hrs with 10 µCi $^{35}SO_4$ per ml.

a) Isolated cartilage. x 100.

b) Cartilage in contact with synovial tissue (S). x 100

medium containing 1.0 µCi $^{35}SO_4$/ml. The orientation had no obvious effect on either the amount (Table 17) or pattern of uptake.

(b) Explants placed on the surface of the grid are covered by only a thin layer of fluid. To find whether total immersion of the cartilage would increase or alter the pattern of its uptake, one of each pair of explants was placed articular surface upwards on the top of the grid and the other, with the same orientation, was completely submerged in the medium which contained 1 µCi $^{35}SO_4$. After 24 hour's incubation, there was no significant difference in either the uptake (Table 17) or pattern of uptake between the two sets of explants.

From these results it was concluded that the amount and pattern of uptake was determined by the intrinsic activity of the chondrocytes in the different zones of the cartilage.

Comparison of living and dead cartilage Paired living and dead explants were incubated for 48 hours in medium containing 1.0 µCi $^{35}SO_4$/ml. No uptake could be demonstrated in the dead explants by either biochemical assay (Table 17, Fig. 36b) or autoradiography (Fig. 37).

(b) The Effect of Synovial Tissue on the Uptake of $^{35}SO_4$

Paired explants with and without synovial tissue were cultivated for different periods and exposed to either 1.0 µCi $^{35}SO_4$ for 24 or 48 hours, or to 10 µCi $^{35}SO_4$/ml for 2 hours.

Paired cartilage explants, one of each pair being grown in contact with synovium and the other in isolation, were cultured in medium containing 1 µCi $^{35}SO_4$/ml; biochemical assay (Table 18, Fig. 36) showed that the synovial tissue strongly inhibited the

Labelling Conditions				Test Explant		Control Explant			
Length of culture (days)	$^{35}SO_4$ pulse (hrs)	Do $^{35}SO_4$ (μCi/ml)	Number of pairs	Culture	Mean uptake (cpm)	Culture	Mean uptake (cpm)	%* Diff. Test/ control	p+
1	24	1	8	Synovium in contact	1728	Isolated cartilage	2097	- 16%	<0.1
2	48	1	8	"	2109	"	3165	- 33%	<0.001
8	48	1	8	"	861	"	3504	- 75%	<0.0005
8	2	10	8	"	522	"	2931	- 82%	<0.0025
8	48	1	8	Synovium separated	1956	"	6207	- 69%	<0.01
8	2	10	6	"	360	"	1221	- 71%	<0.0005

* $\frac{\text{Mean difference between pairs (test - control)}}{\text{Mean of control explants}} \times 100$

\+ Paired student's 't' test

TABLE 18

EFFECT OF SYNOVIAL TISSUE ON UPTAKE OF ^{35}S INTO CARTILAGE PROTEOGLYCAN. COMPARISON OF PAIRED CARTILAGE EXPLANTS. ALL EXPLANTS ON CULTURE GRID WITH ARTICULAR SURFACE UPWARDS

incorporation of $^{35}SO_4$ by the cartilage. After only 24 hours the mean uptake by the cartilage in contact with synovium was reduced to 85% of that of the isolated control (Fig. 36I), after 48 hours to 75% (Fig. 36II), and by 8 days to only 25% (Fig. 36III). Since it was possible that the chondrocytes were incorporating $^{35}SO_4$ at the normal rate and that the synovial tissue was causing its release from the cartilage during the long labelling period, the experiment was repeated with exposure to 10 µCi $^{35}SO_4$ for only 2 hours to reduce any error due to loss of proteoglycan during the labelling period. Exactly the same inhibitory effect was obtained (Table 18, Fig. 36IV).

Another series of explants from the same experiments was fixed for autoradiography. The preparations showed that at 24 hours (8 pairs; 1.0 µCi $^{35}SO_4$/ml for 24 hours) the chondrocytes near the junction with the synovium had taken up less ^{35}S than comparable cells in their isolated controls; in sections stained with toluidine blue, a narrow region of non-metachromatic matrix had appeared at the same site, indicating that depletion of the existing matrix had begun. After 48 hours, (8 pairs; 1.0 µCi $^{35}SO_4$/ml for 48 hours) both decline in uptake and depletion of proteoglycan had spread over a wider area. After 8 days (8 pairs; 1.0 µCi $^{35}SO_4$/ml for 48 hours; 8 pairs 10 µCi $^{35}SO_4$/ml for 2 hours) the metachromasia was further reduced especially in the proximal part of the explants (Fig. 38); in the distal portion the inner cells sometimes continued to give a fairly intense uptake though less than in the controls, but elsewhere the incorporation of $^{35}SO_4$ was very weak.

Even when the cartilage was explanted on the same grid but at a distance from the synovial tissue, biochemical assay demonstrated

a great reduction in uptake after 8 days (Table 18; Fig. 36) as compared with isolated paired controls. This difference was confirmed by autoradiography (Fig. 39) (2 pairs; 10 µCi $^{35}SO_4$/ml for 2 hrs; 4 pairs; 1.0 µCi $^{35}SO_4$/ml for 2 days).

In many explants grown for 8 days either in contact with or at a distance from the synovial tissue, a region immediately above the cut surface became depleted of both proteoglycan and collagen (Fig. 39f). As soon as breakdown of the collagen appeared, the chondrocytes began to multiply to form clusters. Some of these groups redifferentiated, secreted new metachromatic matrix and gave a strong autoradiograph with $^{35}SO_4$ (Fig. 40). Other adjacent groups displayed much less uptake and as destruction of the collagen progressed they assumed a fibroblastic form. Despite the presence of synovium, emigrated chondrocytes often proliferated and formed new proteoglycan below the cut surface (Fig. 39c,f).

(c) The Recovery of Cartilage Explants After Removal of the Synovial Tissue

Sulphate-uptake Experiments were made to find whether articular cartilage cultivated in the same dish as but not in contact with synovial tissue would recover its capacity to incorporate $^{35}SO_4$ if it were transferred to medium without synovium. Sets of paired explants were cultivated according to the scheme shown in Table 19. It will be seen that in all 4 groups the uptake of $^{35}SO_4$ was considerably greater in the cartilage that had been cultured for 8 days in the presence of synovial tissue and then maintained for 4 days without synovium, than in the various controls (Table 19). This was even true of the experiments of group 3 where the paired controls had been grown in isolation for 12 days.

An equal number of pairs for the 4 groups were fixed for autoradiography (Fig. 41). In groups 1, 2 and 4 there was complete correspondence between the autoradiographs and the biochemical

Group	Number of pairs	Test Explant: Culture condition	Test Explant: Mean uptake (cpm)	Control Explant: Culture condition	Control Explant: Mean uptake (cpm)	%* Diff. test/control	p+
1	8	Synovium (8 days) → No synovium (4 days)	867	Synovium (8 days)	339	+ 155%	<0.01
2	8	" → "	1029	Synovium (12 days)	249	+ 313%	<0.005
3	8	" → "	1227	Isolated cartilage (12 days)	786	+ 56%	<0.005
4	4	Synovium (8 days) → No synovium (8 days)	864	Synovium (8 days)	237	+ 264%	<0.01

* $\frac{\text{Mean difference between pairs (test - control)}}{\text{Mean of control explants}} \times 100$

\+ Paired student's 't' test

TABLE 19

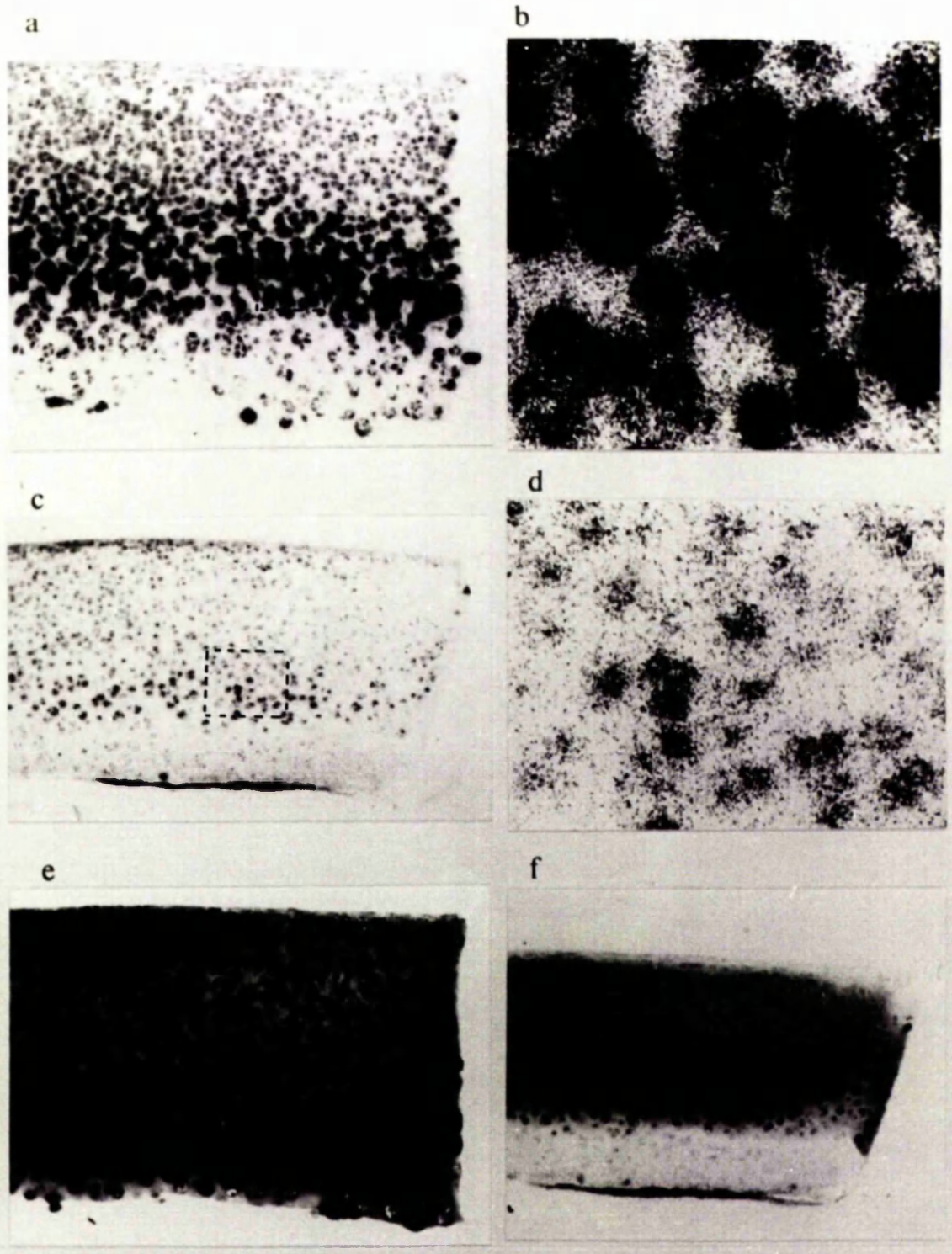
RECOVERY OF CARTILAGE FROM THE INHIBITORY EFFECT OF SYNOVIUM ON THE UPTAKE OF ^{35}S INTO PROTEOGLYCAN

SYNOVIUM IN THE SAME DISH BUT SEPARATED FROM THE CARTILAGE . COMPARISON OF PAIRED CARTILAGE

EXPLANTS. ALL EXPLANTS ON CULTURE GRID WITH ARTICULAR SURFACE UPWARDS. PULSE OF

10 µCi/ml $^{35}SO_4$ FOR 2 HRS GIVEN AT END OF CULTURE PERIOD

Fig. 39



Paired cartilage explants grown for 8 days in normal medium plus 2 hrs with 10 µCi $^{35}SO_4$ per ml.

a) Isolated cartilage; note 3 zones of uptake. x 40.

b) High power of marked area in (a). x 250.

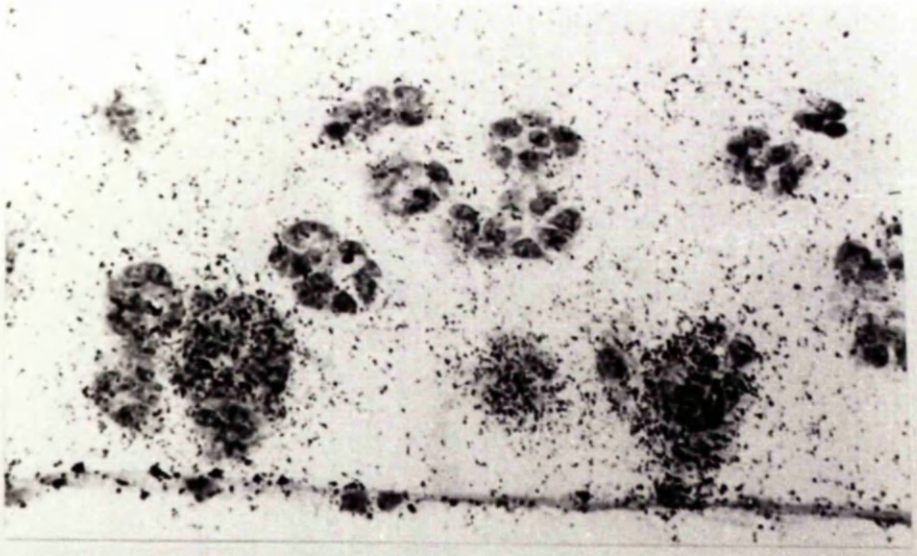
c) Cartilage separated from synovium; the cut surface is partly covered by emigrated cells which give an intense uptake. x 40.

d) High power of marked area in (c). x 250.

e) Isolated cartilage as in (a). Tol. blue. x 40.

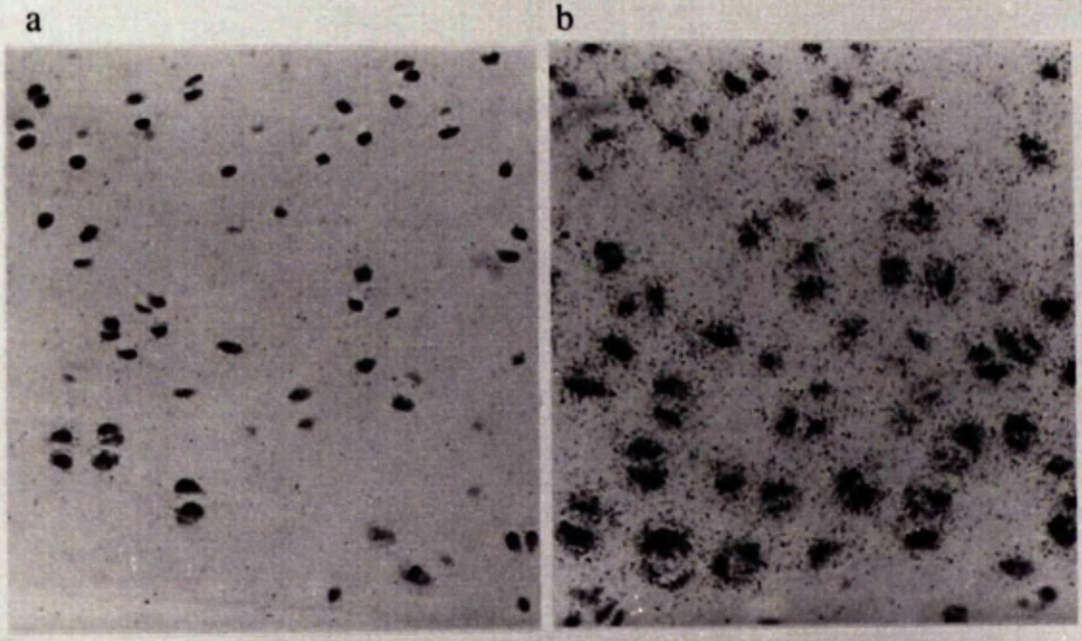
f) Cartilage separated from synovium as in (c). Tol. blue. x 40.

Fig. 40



Islets of cells in cartilage grown in contact with synovium for 8 days and exposed to 10 µCi $^{35}SO_4$ per ml for 2 hrs. Some islets display an intense uptake while others very little. Carazzi's haematoxylin. x 400.

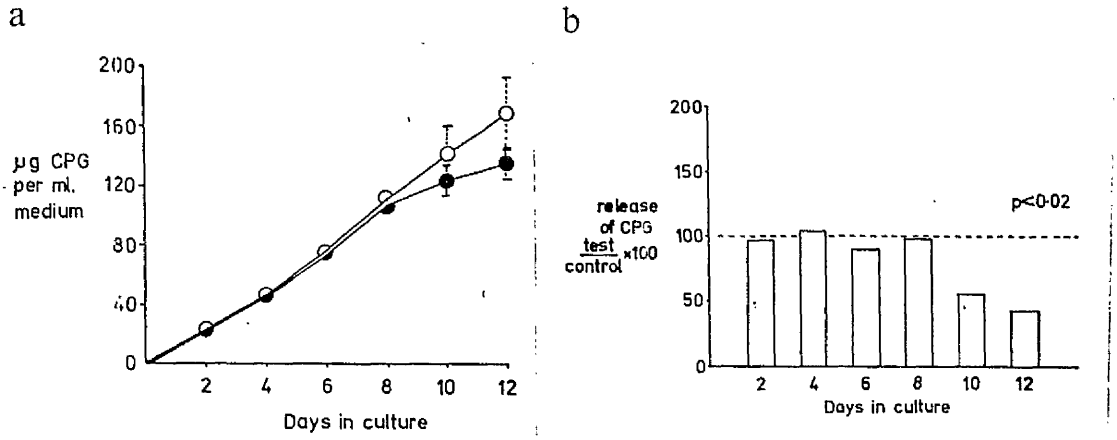
Fig. 41



a) Cartilage explant grown for 8 days in the presence of but not in contact with synovial tissue plus 2 hrs with 10 µCi $^{35}SO_4$ per ml. Carazzi's haematoxylin. x 250.

b) Paired explant cultivated for 8 days with synovium followed by 4 days in isolation plus 2 hrs with 10 µCi $^{35}SO_4$ per ml. Carazzi's haematoxylin. x 250.

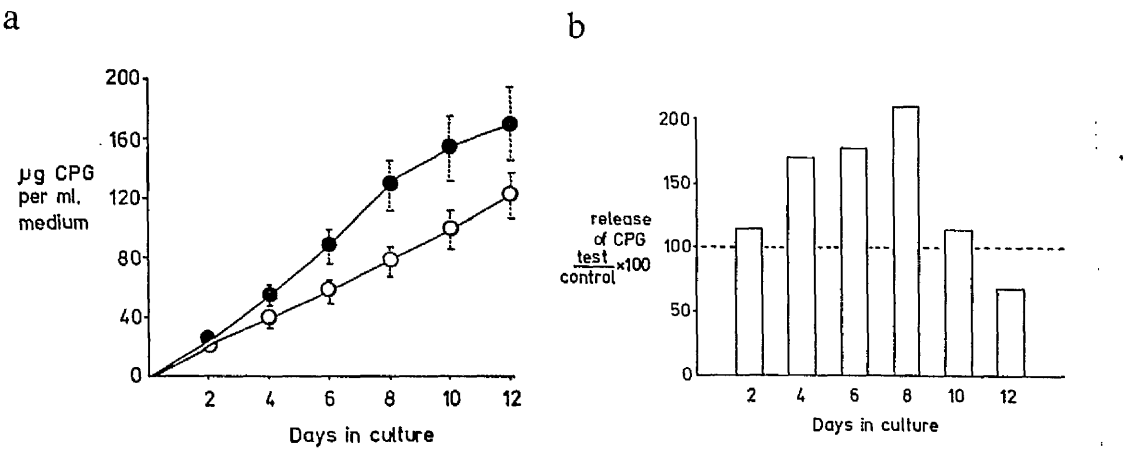
Fig. 42



(a) Cumulative release of CPG into the medium. Each curve is the mean of 4 experiments. The bars represent the S.E.M. ●——● cartilage grown in the presence of synovium for 8 days and in isolation for a further 4 days (Test group); O——O paired explants cultured in the presence of synovium for 12 days (Control group).

(b) Histogram of release of CPG into the medium at 2 day intervals. The release from the test group expressed as a percentage of the release from the control group. Note significant reduction in the loss of CPG when the synovial tissue is removed.

Fig. 43



(a) Cumulative release of CPG into the medium. Each curve is the mean of 4 experiments. ●——● cartilage grown in the presence of synovium for 8 days and in isolation for a further 4 days (Test group); O——O paired explants grown in isolation for 12 days (Control group).

(b) Histogram of release of CPG into the medium at 2 day intervals. The release from the test group expressed as a percentage of the release from the control group. Note reduction in the release of CPG from the test cultures below that from isolated cartilage.

findings with a more intense uptake in the explants grown in isolation for 4-8 days following 8 days' inhibition by synovium, than in their paired controls; the only exception was a pair in group 2 in which no difference could be detected.

In one experiment of group 3, there was no significant difference in uptake between the two sets of explants (4 pairs), but in the second experiment (4 pairs) there was much more uptake in the explants grown for 8 days with and 4 days without synovium than in the controls.

Release of proteoglycan In 14 recovery experiments removal of the synovium not only restored the incorporation of $^{35}SO_4$, but immediately diminished the release of proteoglycan from the cartilage. Four days after removal of the synovium there was significantly less proteoglycan being released than if the synovium was present for the 12 days (Fig. 42). The release of proteoglycan even fell below that of isolated cartilage at 12 days (Fig. 43).

III SUMMARY

1. Pig articular cartilage incorporates $^{35}SO_4$ into proteoglycan in organ culture.
2. There is a zonal variation in the incorporation of the ^{35}S.
3. Synovial tissue, either in contact with or separated from the cartilage, inhibits the incorporation of ^{35}S into the matrix.
4. Some chondrocytes synthesise proteoglycan despite the presence of synovial tissue.
5. The inhibitory effect of synovial tissue is reversible.

IV CONCLUSION

Synovial tissue can cause a reversible inhibition of proteoglycan synthesis by articular chondrocytes.

CHAPTER 10

EFFECT OF SYNOVIUM ON HUMAN ARTICULAR CARTILAGE

The experiments so far reported have been on cartilage from immature pigs. It was important to extend the study and to relate the observations not only to adult cartilage but also to human tissues as there might be important age or species differences. As demonstrated in earlier Chapters and in other work (313) pig synovium produces humoral factors in culture which affect both pig and bovine chondrocytes. The humoral effects of pig synovium on cartilage are, therefore, not specific to one species and it was decided to study the response of human chondrocytes to pig synovium. There was still a risk of species specificity so that pig factors would not affect human chondrocytes, but equally it was not known whether human synovium could be induced to produce similar factors in culture.

I OBJECT OF EXPERIMENTS

Experiments were made to study the response of chondrocytes in normal adult cartilage obtained from the articular head of human femurs to pig synovium.

II RESULTS

(a) Isolated Human Cartilage

Cartilage explants (wet weight 10-20 mg) from the head of the femur were maintained for 16 days in organ culture. Care was taken to cut as far as, but not into, the calcified cartilage. 1 μCi $^{35}SO_4$/ml was added to the cultures for the last 48 hours. During the period of culture there was a linear release of proteoglycan

into the medium (Fig. 44a). Biochemical analysis showed that the explants incorporated ^{35}S into the proteoglycan (Table 20); this was confirmed by autoradiographic examination of sections (Fig. 45b). The degree of metachromatic staining of the matrix was the same as in explants fixed without being cultured.

Dead cartilage maintained for 16 days under similar conditions of culture showed no evidence of incorporation of ^{35}S into the explant either in autoradiographs (8 explants) or analysis of extracted proteoglycan (8 explants). The cartilage, however, released proteoglycan (Fig. 44b).

(b) Cartilage in Contact with Synovium

Living cartilage The effect of synovium on cartilage when the tissues were in contact was studied in material from 4 femoral heads. After 16 days' culture with ^{35}S for the last 48 hours the uptake was inhibited by 68% as compared with paired explants grown in isolation (Table 20). In all samples examined histologically (19 explants) there was less uptake in the autoradiograph of the cartilage in contact with synovium (Fig. 45).

Evidence for loss of proteoglycan was less clear. Fifteen of 27 pairs showed less staining for proteoglycan in the presence of synovium (Table 21; Fig. 45c,d) and biochemical analysis suggested an average loss of about 10% (Table 22). These results are not statistically significant.

Analysis of the used medium was unhelpful since little proteoglycan could be detected in the medium by the alcian blue assay (Fig. 44a). It was not clear whether this meant that there was no release of proteoglycan or that the synovial medium was interacting with the proteoglycan such that it did not react with the

Explant	Number of pairs	Mean uptake ^{35}S/explant (cpm)	Test explant / Paired control x 100		
			per explant	per mg wet wt	per mg protein
Cartilage + synovium overlapped	12	162	32%	30%	31%
Paired isolated cartilage		570			
Cartilage + synovium separated	12	213	34%	34%	33%
Paired isolated cartilage		657			

TABLE 20

EFFECT OF LIVING PIG SYNOVIUM ON THE UPTAKE OF ^{35}S INTO PROTEOGLYCAN IN LIVING EXPLANTS OF HUMAN ARTICULAR CARTILAGE. EXPLANTS MAINTAINED IN ORGAN CULTURE FOR 16 DAYS WITH 1 µCi/ML SO_4 FOR THE FINAL 48 HOURS

Explant	Number of explants	Loss of Proteoglycan (toluidine blue)					
		++ ++	+ ++	++	+	±	0
Living Cartilage:							
Isolated	35					12	23
+ synovium overlap	27			1	9	11	5
+ synovium separated	8						8
Dead Cartilage:							
Isolated	24				2	2	20
+ synovium overlap	16				9	7	
+ synovium separated	8					2	6

Degree of depletion as in Table 1

TABLE 21

EFFECT OF PIG SYNOVIUM ON PROTEOGLYCAN IN HUMAN ARTICULAR CARTILAGE - ANALYSIS OF HISTOLOGY

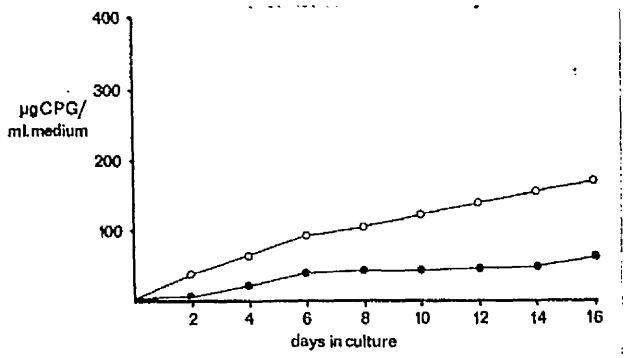
Explant	Number of pairs	Mean µg CPG/ explant	Test explant / Paired control x 100		
			per explant	per mg wet wt	per mg protein
Living cartilage					
+ synovium overlapped	12	811	96%	89%	91%
isolated		881			
Dead cartilage					
+ synovium overlapped	8	934	79%	87%	-
isolated		1164			
Living cartilage					
+ synovium separated	12	1156	104%	100%	97%
isolated		1137			

TABLE 22

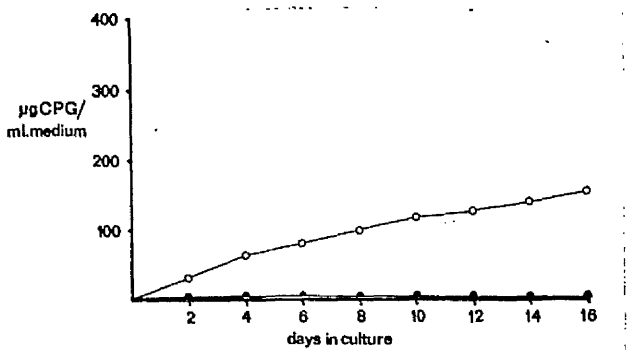
EFFECT OF LIVING SYNOVIUM ON TOTAL PROTEOGLYCAN IN EXPLANTS OF HUMAN ARTICULAR CARTILAGE MAINTAINED IN CULTURE FOR 16 DAYS

Fig. 44

a



b



Cumulative release of proteoglycan after 16 days in culture,

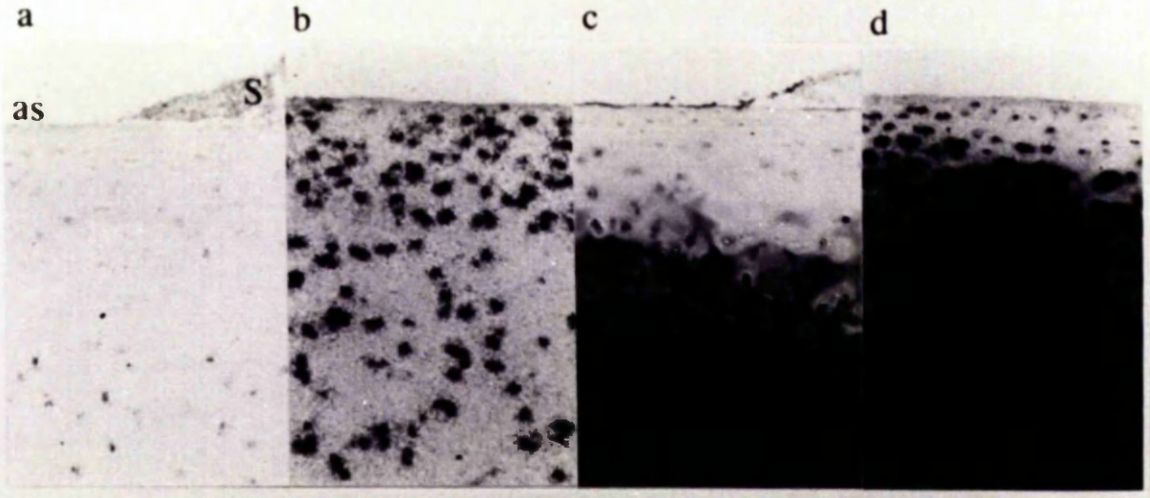
a) ●——● Living human cartilage in contact with pig synovium.

○——○ paired living human cartilage.

b) ●——● Dead human cartilage in contact with pig synovium.

○——○ paired dead human cartilage.

Fig.45



Effect of pig synovium on human articular cartilage after 16 days in culture with 1 μCi $^{35}SO_4$ /ml for the last 2 days.

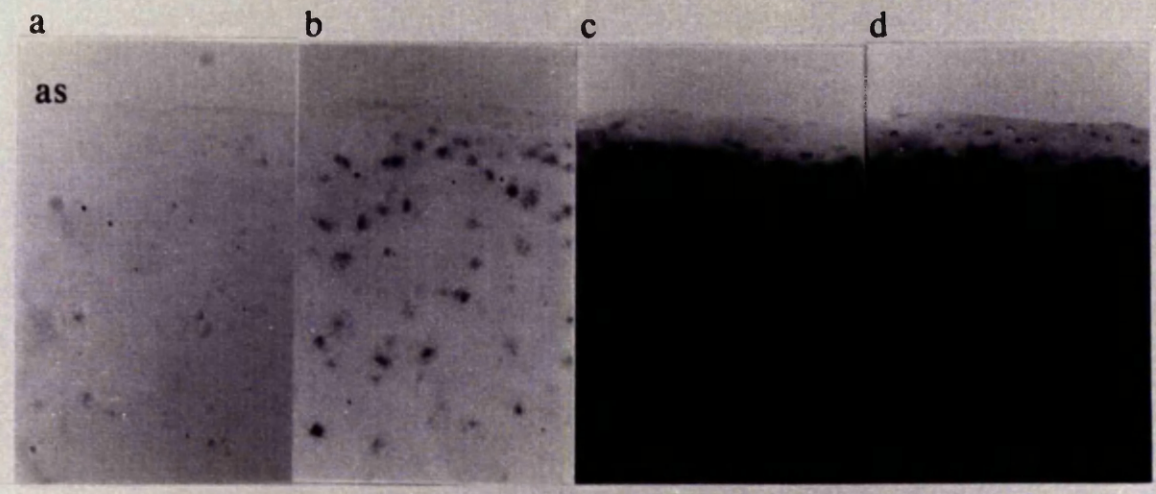
a) Autoradiograph of cartilage explant maintained in contact with pig synovium (S). Note very poor uptake of ^{35}S. as = articular surface. x 50.

b) Autoradiograph of paired isolated cartilage. x 50.

c) Same explant as (a). Note some loss of metachromasia from around the chondrocytes. Tol. blue. x 50.

d) Same explant as (b). Tol. blue. x 50.

Fig.46



Effect of pig synovium on human articular cartilage after 16 days in culture with 1 μCi $^{35}SO_4$ /ml for the last 2 days.

a) Autoradiograph of cartilage explant maintained at a distance from pig synovial mince. Note very little uptake of ^{35}S.
as = articular surface. x 50.

b) Autoradiograph of paired isolated cartilage. x 50.

c) Same explant as (a). Note no evidence of loss of metachromasia. Tol.blue. x 50.

d) Same explant as (b). Tol. blue. x 50.

alcian blue (for further discussion of this, see Appendix). There was no detectable loss of collagen.

Dead cartilage Dead cartilage with synovium did not incorporate any ^{35}S. Histological analysis after 16 days suggested that the synovium was causing some loss of proteoglycan (Table 21). Fourteen of 16 paired explants showed less metachromasia than the isolated controls and biochemical analysis of the tissue indicated 20% less proteoglycan in the cartilage associated with the synovium (Table 22). Analysis of the medium, however, failed to confirm this observation (Fig. 44b) (see Appendix).

(c) Cartilage Separated from Synovium

Living cartilage from 3 femoral heads was maintained for 16 days in the same culture dish as synovium but not in contact. The incorporation of ^{35}S into proteoglycan was inhibited by 66% by the synovial tissue, which was the same degree of inhibition as when the tissues were in contact (Table 20). All samples analysed by autoradiography (8 pairs) showed less uptake into the cartilage with the synovium (Fig. 46).

Biochemical and histological analysis failed to provide any evidence for loss of proteoglycan after 16 days in culture (Table 22; Fig. 46).

(d) Cartilage with Retinol

A pilot experiment on human cartilage grown for 16 days in culture medium containing retinol (10 iu/ml) indicated that the uptake of ^{35}S during the last 2 days of culture was inhibited by the vitamin (Table 23b; Fig. 47a,b).

There was some histological evidence for depletion of proteoglycan (Fig. 47c,d) but biochemical analysis of the cartilage explant gave equivocal results (Table 23a).

Explant	Number of pairs	Mean µg CPG/ explant	Test explant / Paired control x 100		
			per explant	per mg wet wt	per mg protein
Retinol	8	652	83%	95%	100%
Control		770			

(a) Loss of Proteoglycan

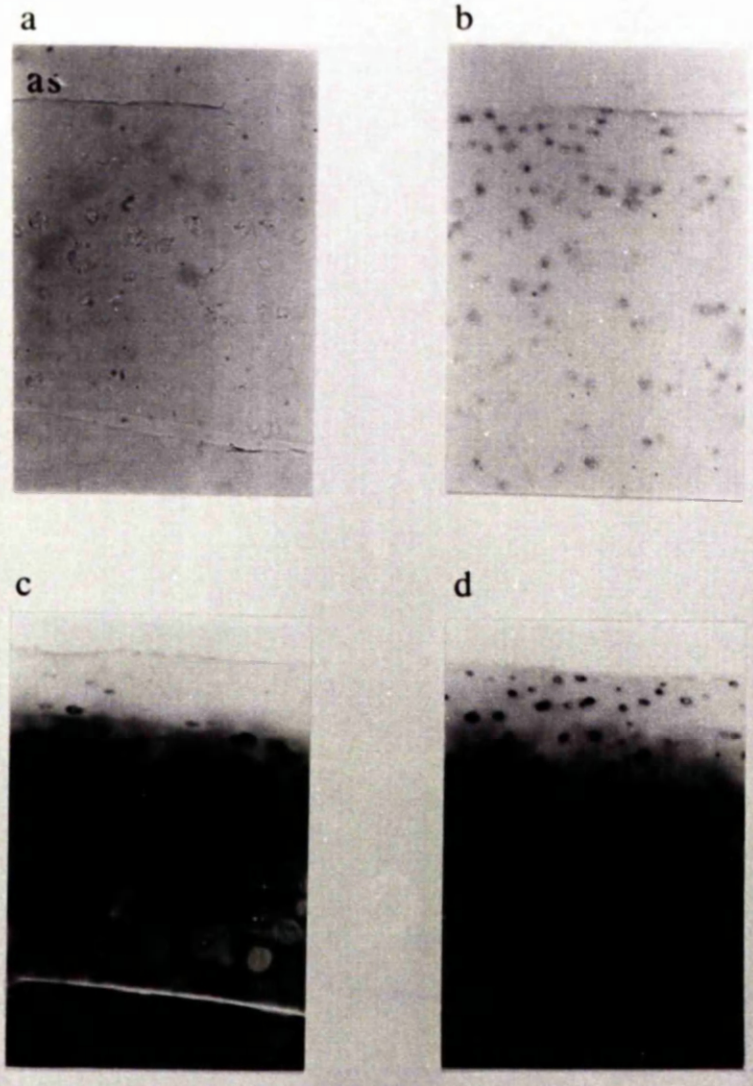
Explant	Number of pairs	Mean uptake ^{35}S/explant (cpm)	Test explant / Paired control x 100		
			per explant	per mg wet wt	per mg protein
Retinol	8	318	48%	54%	55%
Control		678			

(b) Uptake of ^{35}S into Proteoglycan

TABLE 23

EFFECT OF RETINOL (10 IU/ML) ON HUMAN ARTICULAR CARTILAGE AFTER 16 DAYS IN CULTURE. ^{35}S IN CULTURE FOR LAST 48 HOURS.

Fig. 47



Effect of retinol on human articular cartilage during 16 days in culture with 1 μCi $^{35}SO_4$ /ml for the last 2 days.

a) Autoradiograph of cartilage + 10 iu retinol /ml. as = articular surface. x 50.

b) Autoradiograph of paired control cartilage. x 50.

c) Same explant as (a). Note some loss of metachromasia from around the chondrocytes situated near the cut in the cartilage. Tol. blue. x 50.

d) Same explant as (b). Tol. blue. x 50.

III SUMMARY

1. Aged human cartilage synthesises proteoglycan in organ culture.
2. Pig synovium in contact with human cartilage inhibits the synthesis of proteoglycan by the cartilage.
3. When in contact with dead human cartilage, pig synovium causes loss of proteoglycan from the cartilage matrix.
4. There was no evidence of loss of proteoglycan from human cartilage not in contact with synovium.
5. Retinol inhibits the synthesis of proteoglycan in human cartilage (preliminary observation).

IV CONCLUSION

Pig synovium can cause the inhibition of proteoglycan synthesis by adult chondrocytes in human cartilage. Under the conditions of the experiments the synovium did not have a destructive effect that was mediated via the chondrocytes.

CHAPTER 11

DISCUSSION

Although the results have been described in seven separate Chapters, the experiments have all been performed with the same object, namely to try to elucidate the mechanisms concerned in the breakdown of cartilage. The Chapters, therefore, are closely interrelated and the Discussion will be subdivided not according to Chapter, but according to topics to which the results described in the different Chapters are relevant.

I THE ROLE OF SYNOVIAL TISSUE IN THE BREAKDOWN OF CARTILAGE

(a) Minced Synovial Tissue

The minced synovial tissue used in the experiments reported in this thesis was obtained from normal pigs and was therefore virtually devoid of inflammatory cells. The minced tissue caused the total destruction of the matrix of articular cartilage but without killing the chondrocytes. A crucial question is why the synovium has such a destructive action in the cultures. In earlier experiments (206, 210) explants of intact synovium, associated with some subsynovial capsular tissue, had much less effect on adjacent cartilage explants than the mince used in the present work. The minced tissue underwent changes during culture such as reduction in size of the explant, proliferation of cells and release of collagen and collagenolytic enzymes. Recent ultrastructural studies (Glauert, Fell, personal communication) have shown that after culture many cells in the minced synovium, though otherwise normal, contain large amounts of intracellular collagen.

The increased destructive effect may have been partly due to the gross morphological disturbance of the minced synovium, but partly also to the fact that the mince, unlike the intact explants, contained many synovial villi which *in vivo* appear to play an important role in the breakdown of cartilage in various arthritides. When minced fibrous tissue was cultured in association with cartilage, however, the matrix was degraded to exactly the same extent as in paired explants associated with synovium. This fibrous tissue, however, contained very few synoviocytes. The abnormal environment of the culture system may also contribute to the destructive action of the synovium.

(b) Breakdown of Cartilage Matrix by Minced Synovial Tissue

The observations on living and dead cartilage indicate that in this experimental system two apparently distinct processes are concerned in the destructive action of the synovial tissue on cartilage matrix: (a) a direct effect on the matrix of both living and dead cartilage placed in contact with the synovial mince, and (b) an indirect action mediated through the chondrocytes, which affects living cartilage only.

Neither the direct nor the indirect effect of the synovium on cartilage explants is properly understood. As regards the direct action, it is probable that the synovial cells release enzymes that degrade both the proteoglycan and the collagen of the matrix, since both components were depleted in dead as well as in living cartilage. Synovium can release enzymes capable of causing the loss of proteoglycan (68,69) and collagen (70). In the experiments on the effect of hyperoxia on cartilage and synovium, an increased release of collagenolytic activity by the synovium was associated with an increased loss of collagen from the cartilage explants in contact.

This was not due to stimulation of the indirect effect which was inhibited by the raised oxygen concentration.

Recently the indirect action of the synoviocytes on living cartilage has been extensively studied. An active fraction has been partially purified from used synovial culture medium (313). This fraction contains proteins with a molecular weight of around 20,000 daltons. The fraction has no detectable enzyme activity which makes it unlikely that inactive synovial enzymes in the culture medium are activated in the immediate neighbourhood of the chondrocytes. It is not clear whether the factor, which has been tentatively called Catabolin (313) induces the chondrocytes to synthesise hydrolytic enzymes or to activate enzymes already present in the cartilage.

(c) Influence of Minced Synovial Tissue on Proteoglycan Synthesis

Dingle has emphasized the importance of "the concept of a dynamic balance in skeletal tissues between synthesis and degradation. Disturbance of either aspect of this balance can lead to pathological changes instead of normal turnover", (314).

The results presented in this thesis demonstrate that synovial tissue in culture has a disastrous effect on this balance. Not only does it enhance the breakdown of the existing matrix by at least two mechanisms but, as assessed by the incorporation of $^{35}SO_4$, it also inhibits the synthesis of new material. The latter effect appears very early before there is any histological evidence of depletion of the matrix. The difference in uptake between paired cartilage explants cultured with or without synovium was the same whether the labelling period was 48 hours or 2 hours. This suggests that there is inhibition of synthesis of sulphated proteoglycan and not just inhibition of incorporation of the proteoglycan into the matrix.

The experiments do not establish at which stage the synthesis is inhibited but only that lesssulphated proteoglycan appears in the matrix. Further experiments are needed to clarify this important point.

It remains to be determined whether or not the synovial factor released into the medium which induces the breakdown of matrix via the chondrocytes is the same as the factor that inhibits the synthesis of proteoglycan. The inhibition of uptake was seen throughout the whole cartilage explant whereas the degradation of the matrix by the chondrocytes started at the periphery and gradually progressed toward the interior. This suggests that the actions are mediated by different agents.

Proteoglycan synthesis in rabbit articular cartilage (224) and bovine nasal cartilage (225) is inhibited by rheumatoid synovial tissue in culture but not by synovial tissue from normal humans (224). Lippiello and co-workers (224) have presented evidence that the active agent from rheumatoid synovium that inhibits proteoglycan synthesis in rabbit cartilage is a prostaglandin.

It is interesting that in the experiments reported here the inhibition of proteoglycan synthesis was reversible, and removal of the synovium allowed the synthesis to be resumed and even increased to levels above that of isolated cartilage. This is consistent with other evidence (209) that in pig cartilage the depletion of proteoglycan by exogenous enzymes stimulates the reserve capacity of the chondrocytes to restore the proteoglycan in the matrix. At present nothing is known about the molecular structure of the regenerated proteoglycan, whether it is the same as that of the original proteoglycan or of a different chemical form.

Although living synovial tissue is destructive to cartilage matrix, when the cartilage was cultured on the top of dead synovium, the matrix was not depleted and histological preparations showed that in some of the explants there was an increased formation of chondroid tissue beneath the cut surface. The explanation for this is unknown, but the autolytic products of dead synovium may contain factors that promote chondrogenesis.

(d) Tissue and Species Specificity of Breakdown of Cartilage

The experiments with minced blood vessel and living and dead cartilage demonstrate that the intimal synovial cells are not essential for either the direct or the indirect destructive effect on the cartilage matrix. The minced fibrous tissue that was used was from tissue associated with the joint capsule and hence it is closely related to the synovial tissue. Nevertheless, all three sources of tissue had produced the same amount of breakdown in the cartilage at the end of the culture period. It is not clear whether this is due to release of similar amounts of catabolic factors or to a maximum response by the cartilage. All the tissues used in the study contained a variety of cell types and it is not known which cells are important. Monocytes from pig peripheral blood have recently been shown to have a catabolic effect mediated via the chondrocytes in pig articular cartilage (Fell, Schofield, personal communication).

Minced pig synovial tissue had little destructive effect on human articular cartilage. There was some loss of proteoglycan when the tissues were in contact but no evidence could be found for an indirect effect mediated via the chondrocytes. This might be due to species specificity, age related differences, or unsuitable culture conditions. There was, however, inhibition of proteoglycan synthesis

which provides further evidence that destruction of existing matrix by the chondrocytes and inhibition of proteoglycan synthesis are not mediated by the same factor.

The catabolic factor that affects chondrocytes shows some species specificity. Pig synovial factor affects chondrocytes in pig articular cartilage and bovine nasal cartilage (Tyler, Saklatvala, personal communication) but not in human articular cartilage, whereas human synovial factor causes loss of proteoglycan from human articular cartilage, pig articular cartilage and bovine nasal cartilage (Tyler, Saklatvala, personal communication). The data from experiments with bovine nasal cartilage, however, have to be interpreted with caution as there are channels of soft connective tissue penetrating the cartilage which might contribute to any catabolic effect.

II FACTORS THAT INFLUENCE THE BREAKDOWN OF CARTILAGE IN CULTURE

(a) Retinol

For many years, chondrocytes have been known to contain enzymes that are capable of degrading cartilage proteoglycan (26,27,28,29) but when the work for this thesis was started there was little evidence that these cells could degrade collagen. Recently collagenolytic activity to type I collagen has been demonstrated in cultures of human osteoarthritic cartilage by Ehrlich et al. (123), but, in their study, the possibility of any breakdown of cartilage collagen was not investigated.

The present experiments on the effect of retinol on isolated cartilage clearly show that, in culture, the chondrocytes can cause the loss of both proteoglycan and collagen from the matrix. Earlier

work suggested that retinol did not greatly affect isolated pig articular cartilage in culture (206), but a later study demonstrated complete loss of proteoglycan and a slight loss of collagen in response to retinol (207). The experiments reported in this thesis have shown a marked collagenolytic effect with transformation of the chondrocytes. The reason for these differences is not clear but there are minor changes in the culture conditions in the three series. Barratt used pig serum while in the other two series rabbit serum was used. Also in the work by Dingle and co-workers, it is possible that the deeper zone of the cartilage was missed. This region is the most susceptible to the collagenolytic effect of the retinol.

In many of the experiments the breakdown of matrix was confined to the hypertrophic zone during the 16 days in culture; this zone is not present in mature articular cartilage. In vivo the hypertrophic zone is the region of cartilage that will be invaded by marrow sinuses and replaced by bone, which might account for its susceptibility. This zone also synthesises less proteoglycan than the more superficial regions. Although the cells of the hypertrophic zone were very sensitive to the action of retinol the chondrocytes in all regions were able to respond to this agent.

The exact mechanism of the retinol's action is unknown. It can labilise lysosomal membranes causing the release of degradative enzymes (194) but it is not known whether this is relevant to the loss of the matrix collagen. Collagenolytic activity is increased in bone cultures by the addition of retinol (315). As yet the factor responsible for the collagenolytic activity of the chondrocytes has not been characterised.

(b) Hyperoxia

In the organ cultures of isolated articular cartilage from the pig, hyperoxia (55% and 95% oxygen) neither caused loss of metachromasia nor increased the amount of proteoglycan shed into the medium; indeed slightly less was released than by the controls in 20% oxygen. There was no evidence of loss of collagen. Jacoby and Jayson (235) noted some loss of proteoglycan from adult human articular cartilage after 8 days' cultivation in an atmosphere of 95% oxygen, but more than three-quarters of the nuclei were pyknotic, which raises the possibility that the degradation of proteoglycan might have been due to the liberation of autolytic enzymes from the necrotic cells.

In the present experiments isolated synovial tissue released a collagenolytic agent into the medium. Although some was found in the medium at normal (20%) oxygen tension, the amount was increased by hyperoxia. Williams (316) found that the optimum concentrations of oxygen were between 20% and 50% for human synovial tissue. Probably the tissue, which has an aerobic metabolism (46), is physiologically more active at the higher level.

Hyperoxia (55% oxygen) greatly enhanced the breakdown of cartilage in contact with synovium, apparently by stimulating the direct enzymatic action of the synovium on the matrix. When the direct effect was eliminated by cultivating the cartilage at a distance from the synovial explant, the partial breakdown of the matrix seen at 20% oxygen was reduced by exposure to 55% oxygen. Thus the indirect action of the synovial cells on the matrix, in contrast to their direct effect, was inhibited by the raised oxygen. It is possible that either the hyperoxia prevented the synovial tissue from producing the changes in the culture medium that stimulated

the chondrocytes to destroy their matrix, or that it inhibited the chondrocytes from reacting to the stimulus. The latter seems the more likely explanation. Unlike the synovium, cartilage has a largely anaerobic metabolism (46).

That hyperoxia has an inhibitory effect on at least one synthetic activity of the chondrocytes has been shown by Lemperg et al. (50), who found that exposure to 50% oxygen reduced the uptake of $^{35}SO_4$ into calf articular cartilage in organ culture. It may well be that the synthesis of the enzymes responsible for the degradation of the matrix is similarly inhibited in living cartilage.

Sledge and Dingle (196) cultivated the cartilaginous limb-bone rudiments from chicks in an atmosphere of 85% oxygen and found that the matrix was severely depleted of proteoglycan. In the present experiments, however, hyperoxia failed to cause the breakdown of isolated articular cartilage. This apparent discrepancy between the two sets of results may be attributed to the fact that the limb-bone rudiments were enclosed in a sheath of connective tissue. It may well be that Sledge and Dingle's experimental system corresponded to the affronted explants of synovium and articular cartilage in which hyperoxia caused severe breakdown, rather than to the explants of isolated articular cartilage devoid of soft connective tissue. Alternatively the chondrocytes of embryonic limb-bones may respond differently to hyperoxia in organ culture.

(c) Mononuclear Leucocytes

Both peripheral blood monocytes and lectin-stimulated lymphocytes enhanced the breakdown of cartilage in cultures of affronted explants of cartilage and synovium. Soluble mediators were involved but the target cell or tissue is not known. From the data presented it is

not clear whether the monocytes and lymphocytes act by the same mechanism. The leucocyte factors might act on the synovial tissue, the chondrocytes, or directly on the cartilage matrix. Further experiments are being undertaken to answer these important questions.

Phytohaemagglutinin (PHA) seems to affect the breakdown of cartilage in several ways. When added to affronted cultures of cartilage and synovium for 48 hours it enhanced the loss of proteoglycan from the cartilage; the PHA might act either on the chondrocytes or on the synovial cells. The addition of lymphocytes further enhanced the breakdown. When the lymphocytes were pre-incubated with PHA for 2 hours, however, the culture medium from 48 hour cultures of lymphocytes failed to enhance the breakdown of the matrix. This might be because the lymphocytes had not been activated but other work has shown that a short incubation with PHA is sufficient for activation (312,318,319). Possibly the relevant action of the PHA in the present system is on the cartilage or synovium and not on the lymphocytes.

The lack of response by the blood lymphocytes to PHA might be due to the monocytes obscuring any effect. The difference in effect between mononuclear leucocytes derived from peripheral blood and those derived from lymph gland might merely reflect the different proportions of cells. Thus there were very few monocyte-like cells in the samples from the lymph glands and few macrophage-like cells were present at the end of the culture period.

III TRANSFORMATION OF THE CHONDROCYTES

A striking feature of the breakdown of the cartilage in these experiments was the morphological change that many of the chondro-

cytes undergo. At a certain stage in the degradation of the cartilage the cells took on the appearance of fibroblasts. As the proteoglycan disappeared the chondrocytes became irregular in outline, very basophilic and often underwent mitosis. With the loss of collagen they became fusiform, oval or triangular in shape with smooth, slightly concave borders. The taxonomy of fibroblasts is a complex problem (for discussion, see Abercrombie, 320) and these transformed chondrocytes can only be described as fibroblast-like cells. They seem to arise whenever there is loss of the collagenous component of the matrix as indicated by van Gieson staining. As the process advances new fibres, which stain with van Gieson, appear round the fibroblast-like cells. This gives the affected area the appearance of fibrous tissue and it would be interesting to know what type of collagen is being synthesised; the presence of type I collagen would provide evidence that these cells, as well as resembling fibroblasts, had acquired a fibroblastic phenotype. Retinol, synovial tissue and blood vessel all produce this cellular transformation within the cartilage explant. In experiments designed to study the ability of the cartilage to recover (211) it appears that as long as the collagen network is intact the cells behave as chondrocytes, and replace the lost proteoglycan. If, however, the collagen network is largely destroyed and the cells have assumed a fibroblastic appearance then the cartilage fails to redifferentiate and the cells do not revert from the fibroblastic to the chondrocytic form.

Cells often wander out from isolated articular cartilage in culture, and under the cut surface of many explants there are areas of fibroblast-like cells and also chondrifying tissue.

On the floor of the culture dish colonies of typical fibroblast-like cells and of rounded or polygonal cells with intercellular metachromatic material were often seen as well as colonies of intermediate cell types. These cells were all derived from emigrated chondrocytes. It is not known what factors control these modulations of the chondrocytes; retinol in culture prevents the formation of chondrifying colonies, whereas chondrogenesis is enhanced beneath living cartilage explanted on dead synovium. While the catabolic factors from living synovium may induce fibroblastic transformation of many of the chondrocytes, they do not prevent the formation of islets of new chondroid tissue within depleted areas of cartilage. Other experimental work has shown that embryo extract (271) or mechanical factors during culture (273) can alter the phenotypic expression of the chondrocyte. It is not clear how relevant these observations are to the present culture conditions.

Thus, in explants of cartilage associated with synovium the original chondrocytes may have at least four different fates; (1) the original cells may remain as chondrocytes within the matrix; (2) transform into fibroblast-like cells still within the depleted matrix; (3) emigrate from the cartilage and either assume a fibroblastic-like form or (4) emigrate but remain as chondrocytes and synthesise pericellular proteoglycan and collagen.

IV THE VALUE OF ORGAN CULTURE IN THE STUDY OF ARTHRITIS

The question arises of the relevance of the observations reported in this thesis on articular cartilage in organ culture to the destruction of cartilage in arthritis. Although data obtained from organ

culture experiments cannot be directly related to changes found in arthritis, they indicate possible mechanisms in the pathogenesis of these diseases which might then be amenable to experimental study.

Degradation of both the proteoglycan and the collagen of the cartilage matrix has been demonstrated at and near the interface between the pannus and the cartilage in rheumatoid arthritis (106, 87,321). The rheumatoid joint is so heavily infiltrated with inflammatory cells (polymorphs, lymphocytes and plasma cells), however, that it is impossible to determine to what extent the synoviocytes themselves are concerned in the breakdown of the cartilage matrix.

In this connection the work of Salter, McNeill and Carbin (187) is very interesting. In clinical and experimental studies they found that in long standing joint deformity, the articular cartilage was grossly abnormal and sometimes absent over that part of the joint surface which, because of the deformity, was no longer in contact with the opposing joint surface but was in continuous contact with synovial membrane. The authors found no evidence of "an aggressive action" of the synovial tissue and their figures indicate no leucocytic infiltration or synovial hypertrophy. They attribute the destruction of the cartilage to "local obliteration of the synovial space with resultant loss of nutrition to the articular cartilage". In the light of the results reported here, however, it seems possible that when the synoviocytes are brought into contact with the cartilage by the deformity, they exert an enzymatic action on the matrix and that this is at least a contributory factor in the breakdown of the cartilage.

The role of the chondrocytes in the destruction of cartilage in inflammatory arthritis is difficult to ascertain. Whereas the

fibroblastic transformation of these cells can be readily interpreted in culture where explants of pure cartilage are grown at a distance from synovial tissue, in a pathological joint it would be impossible to know whether a fibroblast-like cell in disintegrating matrix was an invading fibroblast or a transformed chondrocyte. Mills (104) has reported invasion of fibroblasts into cartilage in the absence of pannus and it is possible that the cells are transformed chondrocytes. Other workers have suggested this possibility (74,105,111) but until now no suitable mechanism has been demonstrated.

It is vital to ascertain whether this mechanism of chondrocyte activation has any clinical significance. To this end the catabolic factor must be isolated before its role in arthritis can be clarified. The first step in this process has already been taken (313), by the isolation and partial purification of an active fraction from the used medium of pig synovium which contains proteins with a molecular weight of around 20,000 daltons.

It has been suggested that factors, such as immune complexes, which may reside in pathological cartilage induce the invasion of the pannus (98). The results reported here demonstrate that in culture the synovium can alter normal cartilage by inducing the fibroblastic transformation of the chondrocytes to produce a histological picture, that resembles the invading edge of pannus. The presence of immune complexes, therefore, is not a prerequisite for an invasive process.

Several studies have demonstrated inhibition of proteoglycan synthesis by chondrocytes in experimental inflammatory arthritis (226,227). The evidence that the synthesis is altered in rheumatoid arthritis is unsatisfactory. It has been shown that the rate of

synthesis of proteoglycan is greater in a severely eroded area than in an apparently unaffected area in the same joint (223). It is difficult to relate these data to the rate of synthesis in a comparable normal joint. Lippiello et al. (224) have reported that synovial tissue from rheumatoid but not normal joints inhibits the synthesis of proteoglycan in vitro.

Further studies in culture on the mechanisms of chondrocyte transformation, on the factors controlling the release of the catabolic factors and on proteoglycan synthesis should help in the understanding of experimental and pathological inflammatory arthritis.

In osteoarthritis it is not clear what factors initiate the breakdown of the matrix. The disease can be induced by mechanical factors but this does not seem to account for the majority of cases (115). A hypothetical mechanism can be devised in which the catabolic factor from the synovium has a central role in the breakdown of cartilage in osteoarthritis and further information about this possibility may be of considerable importance.

A feature of osteoarthritis is the concurrent existence of areas of breakdown and regeneration. This is particularly prominent in osteoarthritis associated with acromegaly. In this disease the balance is initially in favour of regeneration with thickened hypertrophic cartilage, but later the degradative features may supervene with the appearance of gross osteoarthritic changes. As seen from the results reported here a similar co-existence of destruction and regeneration appeared in the affronted explants of cartilage and synovium in culture; groups of chondrocytes in an otherwise depleted area of matrix showed increased uptake of $^{35}SO_4$ and intercellular metachromatic material. Further studies might answer the question of why the isolated groups of chondrocytes are not affected

by the catabolic factor from the synovium. A better understanding of this phenomenon might enable the chondrogenic cells to be stimulated to repair and replace damaged cartilage. At the moment, however, nothing is known about the quality of the matrix produced by these cells.

Experimental osteoarthritis has been induced by retinol (175). The intra-articular injection of the vitamin into the knee joint of rabbits produced chronic lesions in the joint which closely resembled those seen in osteoarthritis. It is impossible to tell from the data which tissues in the joint are affected by the retinol. The breakdown of the collagen might be due to enzymatic degradation by the synovium or the cartilage or to mechanical factors. In human articular cartilage, Ehrlich et al. (123) noted that osteoarthritic cartilage produced more collagenolytic activity than normal cartilage; this was shown by culturing the cartilage and assaying the used medium. The results of in vivo experiments with retinol are compatible with the effects of retinol on cartilage in culture and suggest that further study of the mechanism of breakdown in culture might be useful for the understanding of cartilage breakdown in osteoarthritis.

The results from the organ culture experiments reported in this thesis have shown that synoviocytes, chondrocytes, monocytes, lymphocytes and cells from blood vessels and fibrous tissue are all capable of contributing to the breakdown of cartilage matrix. All these cell types are present in the joint during an episode of inflammatory arthritis. An increase in the availability of oxygen also increases the breakdown of the matrix, which might be significant during episodes of hyperaemia in the arthritic joint. The experimental data has also shown some parallels with the pathological changes in osteoarthritis.

This work provides the basis for further investigation into the pathogenesis of both inflammatory and degenerative arthritis. In particular, the demonstration of soluble factors produced by the synovium that can induce the fibroblastic transformation of chondrocytes is the foundation for the hypothesis that such a process is a feature of inflammatory arthritis. This hypothesis is amenable to experimental verification and if shown to be correct it would have important therapeutic consequences. The study, therefore, of articular tissues in organ culture helps towards a better understanding of the pathogenesis of arthritic diseases.

APPENDIX

THE EFFECT OF USED SYNOVIAL MEDIUM ON THE ALCIAN BLUE ASSAY

When the results of the organ culture experiments were analysed, there was a disparity between the amount of proteoglycan released from cartilage into the medium and that remaining in the tissue as indicated by sections stained with toluidine blue, when synovium was present in the cultures. Thus after 14 days in culture, histological preparations showed a complete loss of metachromasia in cartilage that had been in contact with synovium, but analysis of the medium by the alcian blue assay (see Methods), revealed only a small amount, much less than from paired explants of isolated cartilage, which still remained deeply metachromatic (Fig. 48). It seemed that the alcian blue assay failed to measure the released proteoglycan when synovium was present in the cultures. If purified chondroitin sulphate was added to used medium from cultures of isolated synovium it also was undetectable by the alcian blue assay (Table 24).

The alcian blue assay is based on the ability of the cationic alcian blue molecule to bind to polyanionic molecules and form neutral, insoluble complexes (298). The assay might fail to detect chondroitin sulphate for several reasons. Firstly, the chondroitin sulphate might be degraded to sub-units that would not precipitate with the dye. Secondly, the synovium might release some substance that blocked the interaction between the chondroitin sulphate and the alcian blue by binding to the chondroitin sulphate. Thirdly, the synovial medium might interact directly with the alcian blue

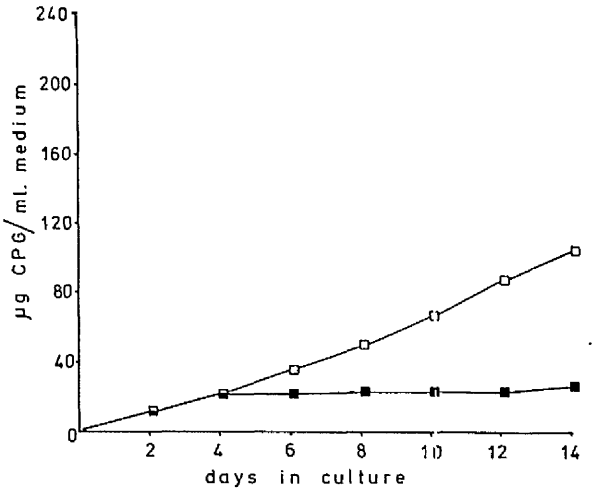
	Chondroitin sulphate µg/ml			
	0	12.5	25	50
Phosphate buffered saline (PBS)	0	12.6 (0.5)*	25.7 (1.5)	50.3 (1.5)
Synovial culture medium (SCM)	0	0	5.5 (2.8)	34.8 (2.9)
PBS + 10 mg/ml trypsin	0	13.4 (0.7)	26.7 (0.5)	50.7 (1.5)
SCM + 10 mg/ml trypsin	4.2 (0.6)	18.2 (1.0)	32.5 (0.8)	56.6 (1.6)

* Standard deviation

TABLE 24

EFFECT OF USED SYNOVIAL CULTURE MEDIUM AND TRYPSIN ON THE ALCIAN BLUE ASSAY

Fig.48



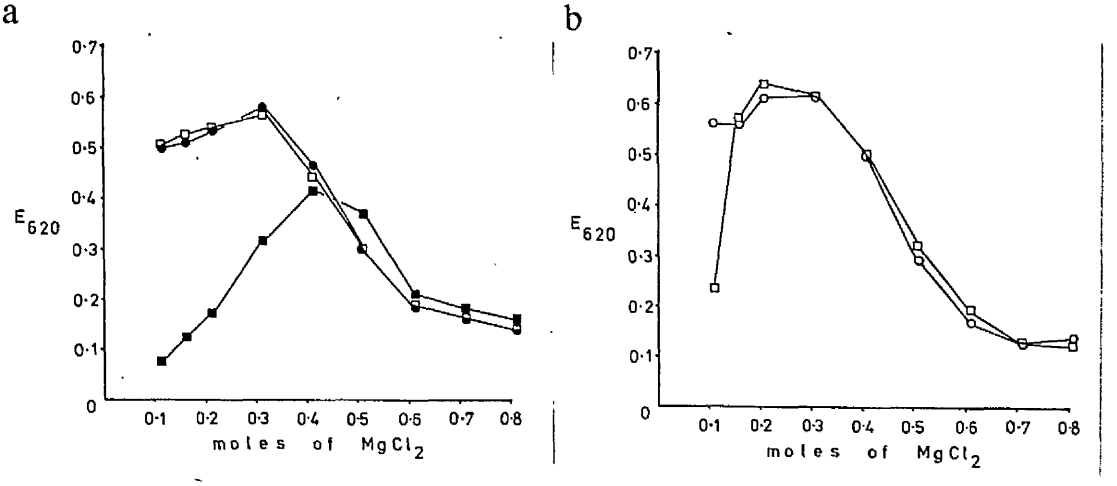
Cumulative release of proteoglycan during 14 days in culture.

Alcian blue assay at salt concentration of 0.15 M $MgCl_2$.

□——□ Isolated cartilage.

■——■ Cartilage in contact with synovium.

Fig. 49



a. Graph of the amount of complexed alcian blue with increasing concentrations of $MgCl_2$.

●——● Saline with 50μg/ml chondroitin sulphate (CS).

■——■ 14 day synovial medium with 50μg/ml CS.

□——□ 14 day synovial medium with 50μg/ml CS and 1mg/ml Trypsin.

b. Graph of amount of complexed alcian blue with increasing concentrations of $MgCl_2$.

□——□ Uncultured medium with 50μg/ml CS.

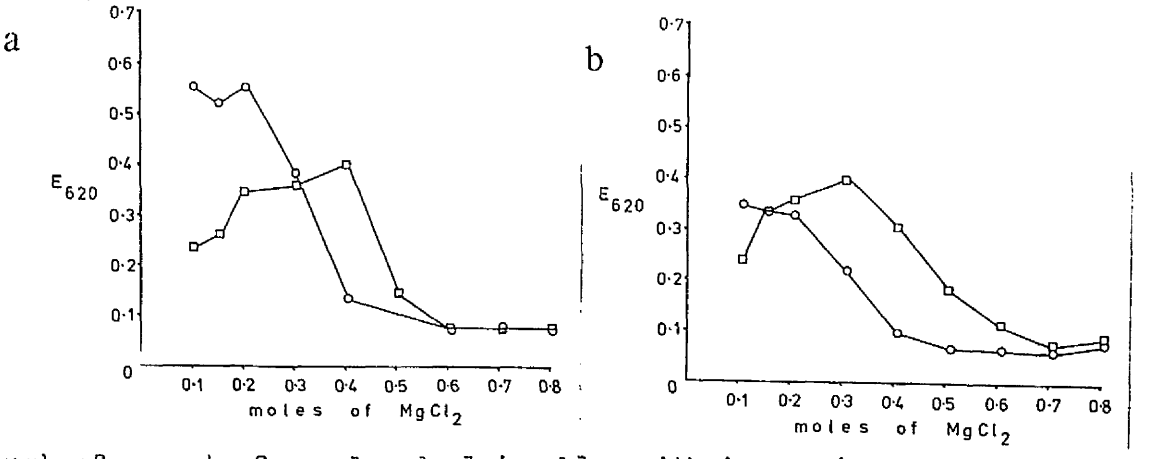
○——○ As above with 1mg/ml trypsin.

and hence block the formation of complexes of alcian blue and chondroitin sulphate.

The extent to which alcian blue binds to chondroitin sulphate depends on the concentration of competing cations. The standard assay solution contains 0.15 M $MgCl_2$. As the concentration of magnesium chloride in the assay solution is increased there is progressive dissociation of the complex of alcian blue and chondroitin sulphate (298). This means that a graph can be derived from the amount of alcian blue complexed to chondroitin sulphate in different salt concentrations (Fig. 49a). When a similar curve is drawn for the amount of alcian blue complexed to chondroitin sulphate in synovial medium, there is an unmasking of the chondroitin sulphate as the salt concentration increases enabling it to bind to the alcian blue (Fig. 49a). At about 0.4 M $MgCl_2$ the curve joins the curve of chondroitin sulphate in phosphate buffered saline and thereafter follows the same pattern. This suggests that the medium contains some compound that can bind reversibly to the chondroitin sulphate. Uncultured medium gives the same pattern of complex-formation of chondroitin sulphate with alcian blue as saline, except in a very low salt concentration (Fig. 49b). Similar salt concentration curves can be produced for the proteoglycan released into the culture medium of affronted explants of cartilage and synovium (Fig. 50a) and also from isolated cartilage (Fig. 50b).

Since chondroitin sulphate and cartilage proteoglycan are polyanionic it is probable that the substance in the synovial medium is cationic, possibly a protein. This was also suggested by the effect of treating synovial medium after culture with trypsin (Table 24). Large quantities of this enzyme had to be used owing to inactivation of the enzyme by the culture medium. Only when there was tryptic

Fig. 50



a. Graph of amount of complexed alcian blue with increasing concentrations of $MgCl_2$.

□——□ Used medium from 8-12 day cultures of cartilage and synovium in contact.

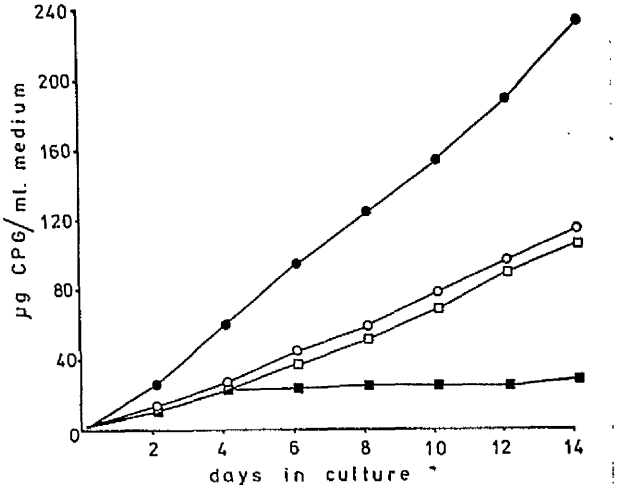
○——○ As above with 1mg/ml trypsin.

b. Graph of amount of complexed alcian blue with increasing concentrations of $MgCl_2$.

□——□ Used medium from 8-12 day cultures of isolated cartilage.

○——○ As above but after addition of 1mg/ml trypsin.

Fig. 51



Graph of cumulative release of proteoglycan during 14 days in culture, before and after treatment of the used medium with 1mg/ml trypsin. Alcian blue assay at salt concentration of 0.15 M $MgCl_2$.

□——□ Medium from cultures of isolated cartilage.

○——○ As above with 1mg/ml trypsin.

▣——▣ Medium from cultures of cartilage in contact with synovium.

◎——◎ As above with 1mg/ml trypsin.

activity, as measured by the BZAN assay (322) was the chondroitin sulphate unmasked (Table 25). The addition of trypsin immediately unmasked the chondroitin sulphate so that the whole of the added amount could be identified by the standard alcian blue assay (Fig. 49a). As expected trypsin treatment of the uncultured medium had no effect on the assay (Fig. 49b).

Trypsin treatment of medium from cultures of isolated cartilage had no effect on the assay at a salt concentration of 0.15 M $MgCl_2$ (Fig. 50b) although at a higher concentration there was dissociation of the curves. This dissociation was probably due to an alteration in the protein component of the proteoglycan. The medium from combined explants of cartilage and synovium contained large amounts of measurable proteoglycan after treatment of the used medium with trypsin (Fig. 50a, Fig. 51).

Chymotrypsin had a similar unmasking effect on the chondroitin sulphate added to used synovial medium (Table 25). The production of this compound is not confined to minced synovial tissue. Both minced blood vessel and fibrous tissue caused a similar masking of proteoglycan in the medium which was corrected by trypsinisation.

The nature of this substance in the synovial medium is not known. The alcian blue assay is not affected by the addition of albumin, type I collagen, rabbit serum or the basic amino acids lysine and arginine. The effect of the synovial medium is not lost by dialysing the medium against saline.

Discussion

The results of the preceding experiments suggest that the synovial mince in organ culture is either releasing a cationic protein into the medium or altering a protein already present in

		Tryptic activity BZAN assay)	CS (alcian blue) µg/ml
Synovial medium with 25 ug/ml CS		0	3
+ trypsin	10 mg/ml	+	29
+ "	1 mg/ml	+	28
+ "	0.1 mg/ml	0	1
+ "	0.01 mg/ml	0	1
+ chymotrypsin	10 mg/ml	0	28
+ "	1 mg/ml	0	28
+ "	0.1 mg/ml	0	20
+ "	0.01 mg/ml	0	1
Uncultured medium with 25 µg/ml CS		0	24
+ trypsin	10 mg/ml	+	26
+ "	1 mg/ml	+	25
+ "	0.1 mg/ml	0	25
+ "	0.01 mg/ml	0	24

TABLE 25

EFFECT OF PROTEOLYTIC ENZYMES ON THE MEASUREMENT OF CHONDROITIN SULPHATE (CS) IN USED SYNOVIAL MEDIUM BY THE ALCIAN BLUE ASSAY. SYNOVIAL MEDIUM FROM CULTURES OF ISOLATED SYNOVIUM COLLECTED AT DAYS 4 AND 8

the culture medium. This protein is able to form reversible complexes with either purified chondroitin sulphate or cartilage proteoglycan. Synovium is known to produce glycoproteins (16) but the biological significance of the substance found in the culture medium is unknown. The production of a similar effect by minced blood vessels suggests that it is not a unique factor produced by the synovium for joint lubrication. The blocking by uncultured medium at very low salt concentrations might suggest that the blocking factor is already present in the medium and that the synovium alters it in some way.

It is puzzling that little proteoglycan could be detected in cultures of pig tissue with human cartilage. Isolated human cartilage released reasonable quantities but when pig synovium was present very little could be detected despite trypsinisation of the medium. The explanation for this is not clear and time did not permit a more detailed study.

For most of the organ culture experiments trypsinisation of the used medium was routinely incorporated into the assay.

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