MECHANICAL AND OTHER FACTORS AFFECTING MATRIX SYNTHESIS
BY CARTILAGE AND BONE CELLS

By
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SUMMARY

Discs of articular cartilage from the humeral heads of adult goats have been compressed in vitro by a method which allows diffusion of liquid and solutes into the matrix. The ability of the chondrocytes to incorporate $^3$S sulphate into glycosaminoglycans during mechanical loading has been examined both by autoradiography and after the chemical separation of the labelled matrix components.

Static loads of greater than 15 kg./cm.$^2$ were sufficient to inhibit synthesis almost completely. Fluctuating loads of 50 kg./cm.$^2$ only partially inhibited sulphate incorporation. In both the loaded and unloaded specimens, most of the radioactivity was associated with the chondroitin sulphate. The cartilage was able to recover most of its ability to manufacture chondroitin sulphate after 2 h. continuous compression by loads of 50 kg./cm.$^2$.

Chondrocytes were isolated from their matrix by enzymic digestion and cultivated for periods of up to 21 days in a chemically defined medium. The cell population remained constant or increased slightly during this period. By the twelfth day, the cells, which had originally been randomly dispersed, had become aggregated into clumps. The appearances of the cell cultures at various stages were examined both by light and by scanning electron microscopy. The cells incorporated sulphate and proline. Small quantities of hydroxyproline were produced. The synthesis of
sulphated glycosaminoglycans was not inhibited by the addition of chondroitin sulphate to the medium.

Cells were isolated from the calcified matrix of cancellous bone of adult goats and osteoarthritic humans by partial decalcification in ethylenediamine tetraacetic acid (E.D.T.A.) followed by enzymic digestion. The isolated cells fluoresced brilliantly when stained with Euchrysine JR and viewed by transmitted fluorescent illumination. The cells also excluded eosin.

The isolated bone cells were cultivated for periods of up to four weeks. The cells clumped during cultivation, and the population remained constant. Osteoclasts could be identified after four weeks cultivation. The isolated cells incorporated $^{14}$C labelled proline and hydroxylated small quantities of it.
INTRODUCTION

Hyaline articular cartilage covers the main load-bearing surfaces of synovial joints. Although apparently subjected to large mechanical forces, normal articular cartilage is capable of maintaining its appearance and function for many years. In some instances, however, degenerative changes take place in it leading eventually to its total loss. These changes may occur in the absence of pathogenic microorganisms or any apparent systemic disorder. This condition has been classified as osteo-arthritis, (referred to by some authors as osteo-arthrosis) or simply degenerative joint disease.

The frequent occurrence of osteo-arthritis in some of the load-bearing joints of the lower limbs, and the association of certain forms of the disease with the occupation of the patient has suggested to many workers that osteo-arthritis may be caused, in part, by abnormal mechanical stresses. At the same time, it has become apparent that symptomatic osteo-arthritis is preceded by certain biochemical changes in the matrix.

Several excellent studies have examined the effects of mechanical loading on the joint in vivo (Salter & Field, 1960, Evans et al, 1960, Trice, 1961, Crolin & Southwick, 1964, Thompson & Bassett, 1970, Radin, 1972). These experiments were, however, of long duration, ranging from three days to many months. Since they involved intact
joints they were not able to distinguish easily between changes initiated in the cartilage or those which had their origins in other tissues of the joint.

Recently, a method has been devised, of applying mechanical loads reproducibly to discs of cartilage which have been separated from the underlying bone (Edwards, 1967, 1970). A modification of this apparatus has been used in this project to examine the effects of loading on the ability of chondrocytes to manufacture protein-polysaccharide.

Osteo-arthritis, as its name implies also affects the cancellous bone underlying the articular cartilage. The examination of the cells in fully calcified bone has been hampered by the technical difficulties of examining bone cells and of cultivating them. A method has been devised for isolating cells from mature bone (Bard et al, 1972). The cells isolated both from arthritic human and normal goat bone have been cultivated in a chemically defined medium. The ability of the cells to convert proline to hydroxyproline during cultivation has been examined.
CHAPTER 1
HISTOLOGY, HISTOCHEMISTRY, CYTOLOGY & BIOCHEMICAL COMPOSITION
OF ARTICULAR CARTILAGE.

Normal articular cartilage is a smooth, translucent tissue of mesenchymal origin which covers the articulating surfaces of bones and acts mechanically as the chief bearing surface in the joint. It is an avascular tissue consisting of chondrocytes embedded in an organic matrix.

Articular cartilage was divided by early workers into three, ill-defined layers, the superficial, intermediate, and deep layers. In the superficial layer, the cells are flattened and arranged parallel to the articular surface, whereas in the intermediate and deep zones the cells tend to be rounded and arranged in columns perpendicular to the surface. In the deep layer the cells are rather larger than in the other two layers. The lower part of this deepest layer is calcified. The calcified areas of the matrix can be seen in some preparations to be separated from the rest of the matrix by a wavy basophilic line. (Ham, 1965, Davies, 1969). (Plate I-1)

The matrix appears to be without any definite structure in a conventionally prepared section which has been fixed in formalin and stained with haematoxylin and eosin. In freeze dried, paraformaldehyde vapour fixed
Section through articular cartilage and some of the underlying cancellous bone. Haematoxylin and Eosin, X 170.
paraffin sections however, bundles of fibres are apparent, running parallel to the surface in the superficial zone and thicker bundles arranged perpendicular to the surface in the deeper layers. In the intermediate zone no clear pattern is apparent. By electron microscopy, the fibres comprising these bundles may be identified as collagen by their characteristic 640 Å banding. The fibres near the surface are approximately 340 Å in diameter. They become thicker with depth and fibres as thick as 1,400 Å can be identified in the deepest layers. (Muir et al, 1970).

All normally possess the characteristic 640 Å banding of collagen although there are reports of the abnormal 'Fibre Long Spacing' (F.L.S.) form of collagen in the vicinity of disintegrating cells in the superficial zone. (Silberberg et al, 1963, Silberberg & Hasler, 1970).

The fibrous architecture of articular cartilage has also been studied by scanning electron microscopy. (McCall, 1969, Clarke, 1971 a). These studies confirm both the change in direction and coarsening of the fibres with depth.

The surface of the articular cartilage has been examined with the aid of scanning electron microscopy (Clarke, 1971 b). In the preparations studied, it appeared to be covered with small depressions, which, from their size and shape, were identified by the author as the outlines of the cell lacunae immediately below the surface. The surface
of normal cartilage did not appear to be fibrous.

The matrix of the cartilage contains mucopolysaccharide (glycosaminoglycan) in addition to collagen. Mucopolysaccharide can be demonstrated histochemically by the periodic acid - Schiff (P.A.S.) test for carbohydrates or the more specific metachromatic stains such as toluidine blue, the phthalocyanin dyes such as alcian blue and green, and the ability to bind colloidal iron. The chemistry of these, and other staining reactions has been the subject of several excellent recent reviews. (Pearse, 1968, Quintarelli, 1968, Stone, 1970).

The P.A.S. reaction is based on the periodate oxidation of carbohydrates. The carbohydrate must have at least two adjacent unsubstituted hydroxyl groups for the reaction to occur. These hydroxyl groups are oxidised to aldehyde groups with a splitting of the C-C bond. The aldehyde groups are identified by combination with Schiff's reagent (fuchsin sulphurous acid) to give a red dye. Most of the glycosaminoglycans would be expected to react on structural grounds. It has been reported however, that hexosamine remains intact and glucuronic acid residues do not react to give aldehydes (Hoogwinkel & Smits, 1957). This excludes most of the glycosaminoglycans of articular cartilage. Articular cartilage does, however, stain with P.A.S., the staining being most intense in the deeper layers of the cartilage.
The chemistry of the metachromatic dyes is a subject of continuing controversy. The reaction appears to involve a polymerisation of dye molecules in association with negatively charged radicals (Pearse, 1968). These metachromatic dyes will react with all the sulphated glycosaminoglycans found in articular cartilage. Azure A, methylene blue, cresyl blue, crystal violet, basic fuchsin and thionine fall within this category in addition to toluidine blue.

The copper phthalocyanin dyes are thought to form ligands with negatively charged groups. The reaction can be made specific for sulphate or uronic acid groups by varying the ionic strength of the medium (Scott & Borling, 1965, 1966). They can be made to stain most of the glycosaminoglycans of cartilage by choosing the appropriate reaction conditions.

Specific proteins and protein-polysaccharides have been demonstrated by the use of fluorescent antibody techniques with cartilage (Barland, 1966). The method involves the production of antibodies for specific proteins and protein-polysaccharides. The antibodies are then covalently linked to a suitable fluorescent dye. This method unlike the others can therefore be used to detect individual, or very closely related compounds.
The Chondrocyte

The chondrocytes are arranged in columns of two to five in the deeper layers of normal, mature articular cartilage. Nearer the surface, they tend to be separate and to be smaller and more elongated. In conventionally prepared sections the cells are seen to lie in lacunae which are rather larger than the cell. This may be a shrinkage artefact and it is much less apparent in sections of freeze dried cartilage and in cartilage which has been fixed in glutaraldehyde and osmium tetroxide for transmission electron microscopy. Studies employing the scanning electron microscope have shown that the chondrocytes appear to be surrounded by a sheath of coarse fibres (McCall, 1969, Dickens personal communication).

The average diameter of intermediate zone chondrocytes show little variation between species, ranging from 10 μ in the mouse to 14 μ in man. Cell density appears to be related to cartilage thickness. The number of cells below a given area of the articular surface appears to have a constant value of 30,000 ± 8,500 cells/mm². Differences in thickness are mainly accounted for by varying quantities of matrix (Stoekwell, 1971).

The cytology of the chondrocyte is conveniently studied in preparations of cells isolated from their matrix by enzymic digestion. Such preparations may
be viewed by phase contrast or fluorescence microscopy (Smith, A.U., 1965). By phase contrast microscopy they can be seen to retain their rounded shape and to possess a round nucleus surrounded by a granular cytoplasm. When the cells are stained with the acridine orange dye, Buchrysine 3R, and viewed by transmitted ultraviolet illumination using a dark ground condenser, the nuclei are seen to fluoresce a vivid green. The cytoplasm contains red fluorescing granules of various sizes which fade to yellow on exposure to the ultraviolet light. Most of these red granules are removed by treatment of the cells with ribonuclease (Young & Smith, 1964). The remainder can be removed by hyaluronidase.

Mature chondrocytes of the middle zone can be seen, by transmission electron microscopy, to contain copious rough endoplasmic reticulum, microtubules, large mitochondria, and a well developed Golgi area (Palfrey & Davis, 1966). Many microprocesses are revealed. They are approximately 1 μ long and extend from the cells into the matrix. Occasionally desmosomes are seen between adjacent cells. Most of the cells are, however, separated from one another by the matrix (Palfrey & Davis, 1966). Granules of glycogen, and lipid filled vesicles are often encountered within the cytoplasm of chondrocytes especially in the deeper zones. (Collins et al., 1965, Silberberg et al., 1964). Some chondrocytes contain fibrous areas within the cytoplasm. These fibres may come to occupy most of the cytoplasm.
Their presence may be a sign of senescence or degeneration (Barnett et al., 1963, Meachim & Roy, 1967).

Nearer the surface, the cells are more flattened and contain less rough endoplasmic reticulum. In the deepest layers the cells often contain much glycogen and prominent lipid filled vesicles (Collins et al., 1965). Some may appear degenerate.

The development of an undifferentiated mesenchymal cell into a chondrocyte is documented (Fell, 1925, Silberberg et al., 1961). It consists of a progressive enlargement of the cytoplasm and an increase in the number and size of the mitochondria. At the same time the cell becomes rounder and develops its microprocesses. The rough endoplasmic reticulum becomes more extensive. During the whole of this process, the cell is synthesising matrix and becomes separated from its neighbours by these metabolic products. As the cells age and eventually atrophy, the amount of rough endoplasmic reticulum and size of the Golgi area decrease. The granules of glycogen and the lipid filled vesicles become more apparent (Silberberg, 1961, 1970 b).

In calcifying epiphyseal plate cartilage, small, osmophilic, P.A.S. reactive, membrane bound microbodies have been identified which are separated from the cellular cytoplasm of the chondrocytes (Bonucci, 1967). These microbodies give a positive ultrahistochemical reaction
for alkaline phosphatase and contain minute crystals. They are thought to act as centres of calcification (Anderson, 1968). They can be separated from the cartilage by homogenisation followed by gradient centrifugation and form a fraction physically and enzymatically distinct from the microsomal fraction (Ali et al., 1970, 1971). Attempts to identify these particles in articular cartilage during pathological calcification are at present being undertaken (Ali, personal communication).

Biochemical Composition of the Matrix

a) Protein-Polysaccharides

The principal organic constituents of the matrix of articular cartilage are collagen and a series of protein-polysaccharide complexes. Although it was realised as early as 1889 (Hörner, 1889) that the polysaccharide components of cartilage were closely associated with protein, most of the early workers concentrated on the carbohydrate portions, and it is only in relatively recent years that attempts have been made to characterise intact protein-polysaccharide complexes.

The polysaccharide components are chondroitin 4 and 6 sulphates, keratan sulphate (keratosulphate) and small quantities of hyaluronic acid. Traces of sialic acid, probably associated with the keratan sulphate, have also been identified. The chondroitin sulphates were the first
FIG. 1.1: THE GLYCOSAMINOGLYCANS OF CARTILAGE:

Formulae of Repeating Disaccharides

Chondroitin Sulphate A (chondroitin-4-sulphate):

3-O-(β-D-glucopyranosyluronic acid)-
2-acetamido-2-deoxy-D-galactopyranosyl-
4-sulphate.

Chondroitin Sulphate C (chondroitin-6-sulphate)

3-O-(β-D-glucopyranosyluronic acid)-
2-acetamido-2-deoxy-D-galactopyranosyl-
6-sulphate.

Keratan Sulphate

4-O-β-D-galactopyranosyl-3-O-(2-acetamido-
2-deoxy-β-D-glucopyranosyl-6-O-sulphate).

Hyaluronic Acid

3-O-β-D-glucopyranosyluronic acid-
4-O-(2-acetamido-2-deoxy-β-D-
glucopyranose.
to be characterised. They were shown to consist predominantly of repeating disaccharide units involving glucuronic acid and galactosamine. The structure of this repeating disaccharide was shown to be: 3-0-(β-D-glucopyranosyluronic acid)-2-deoxy-D-galactopyranosyl-4 (or 6)-sulphate (Fig. 1-1). The two chondroitin sulphates found in articular cartilage differ in the position of the sulphate group. The disaccharide units are linked together by β-1-4-glycosidic linkages (Wolfson et al., 1952, Davidson & Meyer, 1954). The regular repeating structure is interrupted at the potentially reducing end by a trisaccharide. This trisaccharide acts as a link between the rest of the chondroitin molecule and the protein, to which it is covalently linked. The structure of this part of the molecule is: 3-0-β-D-galactosyl-4-0-β-D-galactosyl-D-xylose (Fig. 1-2). The galactose is linked to the first galactosamine residue of the repeating disaccharide chain and the xylose is covalently bound to the protein, by a β-0 glycosidic linkage. Chondroitin sulphate appears to be linked almost exclusively to the hydroxy amino acid, serine, as only this amino acid is modified during the treatment of chondroitin sulphate proteins with alkali (Rodén & Armand, 1966, Lindahl & Rodén, 1966, Rodén & Smith, 1966, Helting & Rodén, 1968).

Little is known about the non-reducing end of the molecule. The preliminary results of Rodén (1970)
FIG. 1-2
THE CARBOHYDRATE - PROTEIN LINKAGE REGION OF CHONDROITIN SULPHATE.*

* After Rodén (1970)
suggest that the terminal position is occupied mainly by hexosamine. About 10% of the non-reducing end positions are occupied by glucuronic acid. These studies were hampered by the difficulty of obtaining reliably undegraded extracts (Rodén, 1970).


Keratan sulphate, like chondroitin sulphate, consists mainly of a repeating disaccharide subunit. Unlike the chondroitin sulphates, however, it consists of galactose
and glucosamine. There is no uronic acid. The structure of the disaccharide is 4-0-β-D-galactopyranosyl-3-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl-6-0-sulphate) (Fig. 1-1) (Hirano et al., 1960). The disaccharide subunits are linked by β-1-3 linkages. Preparations of skeletal keratan sulphate have also been found to contain small quantities of sialic acid (Meyer et al., 1964), xylose, mannose and galactose (Castellani et al., 1962).

Skeletal keratan sulphate resembles chondroitin sulphate in being linked to protein by the reducing end of the carbohydrate chain. The mode of linkage is, however, less well understood than in the case of chondroitin sulphate. Threonine, as well as serine, is destroyed by the treatment of protein-keratan sulphates with alkali (Seno et al., 1965, Bray et al., 1967, Seno & Toda, 1970). Indeed, Seno & Toda (1970) showed that 75% of the threonine in a preparation of whale skeletal keratan sulphate was converted to α-aminobutyric acid whereas only 54% of the serine was converted to dehydroalanine by alkaline hydrolysis. 70% of the galactosamine was destroyed at the same time. These data suggest that galactosamine is bound predominantly to serine or threonine by glycosidic linkages. (Greiling et al., 1970) have suggested that mannose might be involved in the linkage to serine. They noted that the ratio of serine to N-acetylgalactosamine to mannose was
approximately 1:1.06:0.86 and concluded that mannose occurred between N-acetylgalactosamine and the main repeating disaccharide part of skeletal keratan sulphate. Little is known about the linkage to threonine. It is possible that there may be several types of linkage region amongst the skeletal keratan sulphates which involve threonine. The position and mode of linkage of the sialic acid is also obscure. It has been suggested that it may be situated at the non-reducing ends of short side chains (Roden, 1970).

The molecular weight of protein free preparations of skeletal keratan sulphate appear to fall within the same range as do the molecular weights of similar preparations of chondroitin sulphate.

Keratan sulphate preparations from different sources may show an average number of sulphate groups to each disaccharide unit which is greater or less than one. Sulphate may be attached to the six position of the galactose as well as to the six position of N-acetylgalcosamine. (Bhavananden & Meyer, 1966, 1967, 1968). Preparations containing an average of up to 1.6 sulphate groups to each disaccharide unit have been described (Mathews & Ciffonelli, 1965). (Seno et al., 1965).

No chondroitin sulphate has yet been reported, however, which contains more than one sulphate group to each disaccharide subunit. Some preparations contain less. (Meyer et al., 1964). It is possible that although chondroitin
4 and 6 sulphates can be separated completely (Antonopoulos & Gardell, 1963). 'Hybrid' chondroitin sulphates occur in which some N-acetylgalactosamine residues are sulphated in the 4 and some in the 6 position. (Hjertquist, 1964, Antonopoulos et al., 1965).

Hyaluronic acid has also been detected in small quantities in extracts of articular cartilage (Lowther, cited by, Silpanata et al., 1967). Hyaluronic acid also consists predominantly of a repeating disaccharide. This disaccharide contains glucuronic acid and glucosamine and has the structure: \(3\)-\(\beta\)-\(D\)-Glucopyranosyluronic acid-\(4\)-\(\beta\)-(2-acetamido-2-deoxy-\(\beta\)-\(D\)-glucopyranose) (Fig. 1-1) (Jeanloz, 1952). The disaccharide units are linked by \(\beta\)-1-3 linkages. Hyaluronic acid is not sulphated. It also occurs in hyaline cartilage from other sources (Hardingham & Muir, 1973). In these preparations it does not appear to be covalently bound to protein (Hardingham & Muir, 1973). In pig laryngeal cartilage it accounts for approximately 1% of the total, extractable uronic acid.

The structure of the protein portions of the protein-polysaccharides remains rather obscure. Several amino acid analyses have been performed on protein-polysaccharides extracted from various sources (Serafini-Fracassini et al., 1967, Serafini-Fracassini, 1968, Sajdera & Hascall, 1969, Hascall & Sajdera, 1969, Tsiganos & Muir, 1969).
Most preparations are free of hydroxyproline and are rich in glutamic acid, glycine, proline and serine. Cystine has been detected in small quantities (6-14 residues/1,000) by some workers (Pal et al., 1966, Hascall & Sajdera, 1969, Sajdera & Hascall, 1969, Brandt & Muir, 1971). In other preparations, however, it appears to be absent (Serafini-Fracassini et al., 1967, Tsiganos & Muir, 1969).

Several attempts have been made to identify the amino acids nearest to the linkage with the polysaccharide (Muir, 1958, Gregory et al., 1964, Anderson et al., 1965, Johnson & Baker, 1973). In addition to serine, glycine, glutamic acid, proline, alanine, valine, leucine, aspartic acid, isoleucine and threonine remain associated with chondroitin sulphate. After digestion of this partially degraded preparation with a series of different proteolytic enzymes and removal of the carbohydrate, a series of short peptides can be isolated. Sequence determinations on some of these peptides show that ser-gly, ala-ser-gly, glu-ala-ser-gly, ala-ser and leu-ser are present (Johnson & Baker, 1973). Whether there is any difference between the amino acids flanking the serine residues which are bound to polysaccharide and those which are not, is at present unknown.

The isolation and characterisation of intact protein-polysaccharides is bedevilled by their susceptibility to degradation by most conventional extraction techniques, and their tendency to form aggregates in aqueous solution.
Two main types of extraction procedure have been employed. Earlier workers used methods involving high speed homogenisation (Gerber et al., 1960, Schubert, 1966, Pal et al., 1966). This type of extract, on centrifugation at 10,000g in aqueous suspension yielded a precipitate, called PP-H and a soluble fraction, called PP-L. PP-H had a carbohydrate:protein ratio of 50:50 whereas this ratio for PP-L was 65:15. The PP-L had a sedimentation constant of 10.5 S. PP-H, by its high hydroxyproline content, was shown to contain much collagen. Both fractions contained chondroitin sulphate (Gerber et al., 1960).

Most of the protein-polysaccharide in hyaline cartilage may alternatively be extracted by aqueous solutions of varying ionic strengths. Sajdera & Hascall, 1969) have compared protein-polysaccharides from bovine nasal cartilage prepared by high speed homogenisation, and by extraction in guanidinium chloride solution. They concluded that homogenisation yielded an appreciably degraded product. Rosenberg et al., 1970a, however, claim that these effects are minimal. Sajdera & Hascall, 1969, found that reduction of the disulphide bonds was much easier in preparations which had been subjected to homogenisation. This observation might suggest that the tertiary and quaternary structures of the protein-polysaccharide may be altered by homogenisation.
There is considerable evidence for supposing that protein-polysaccharide is heterogeneous. Pal et al (1966), Hascall & Sajdera (1969), Tsiganos & Muir, (1969, 1970) and Brandt & Muir, (1971 a, b) have obtained, by a variety of techniques, fractions which differ appreciably in amino acid composition, protein:carbohydrate ratio and N-terminal amino acid analysis. Immunological studies also suggest that there are several types of protein-polysaccharide molecules (Tsiganos & Muir, 1969).

Molecular weight determinations of protein-polysaccharides show considerable variation. Studies on PP-L preparations from fresh cartilage give molecular weights of $1 - 7 \times 10^6$ (Partridge et al, 1961, Fitton-Jackson, 1965, Partridge, 1968, Öbrink & Wasteson, 1971). It is possible, however, that these figures represent the molecular weights of large aggregates of protein polysaccharide. Partridge and his co-workers (1961) were able to show a reduction in the apparent molecular weight of protein-polysaccharide after hyaluronidase digestion much greater than could be accounted for by the loss of carbohydrate from the protein chain. Other workers have shown similar changes on treatment with solutions of differing pH or ionic strength (Hascall & Sajdera, 1969, Brandt & Muir, 1971 b). There is also electron microscopic evidence for the formation of aggregates by protein-polysaccharides (Fitton-Jackson, 1965, Rosenberg et al, 1970 b). Partridge (1968) quotes a
molecular weight of 240,000 (number average) for a purified preparation of a chondroitin sulphate-protein complex from bovine nasal cartilage. It is probable, however, that there are considerable variations in molecular weight between different species, different age groups, different anatomical positions and different fractions from the same sample.

Early light scattering experiments suggested that the protein-polysaccharide consisted of rod-like molecules in aqueous solution (Mathews & Lozaityte, 1958). A structure consisting of a central protein core with polysaccharide side chains has been proposed, both on the basis of these studies and the appearances of smear preparations of protein-polysaccharide stained and viewed by electron microscopy (Mathews & Lozaityte, 1958, Partridge et al, 1961, Fitton-Jackson, 1965, Serafini-Fracassini & Smith, 1966, Serafini-Fracassini et al, 1970, Rosenberg, 1970b). Eyring & Yang (1968) were however, unable to find any evidence, by light scattering, of any ordered molecular structure in a solution of protein-polysaccharide which had been extracted by high speed homogenisation. This finding may suggest that the protein core is flexible, allowing a number of possible configurations. It may, however, be another instance of the effects of homogenisation.
The number of carbohydrate chains attached to a single protein molecule appear to vary considerably between different preparations. Reports vary from 8 - 9 (Partridge, 1968) to as many as 60 (Mathews & Lozaityte, 1958).

Both chondroitin and keratan sulphates may be attached to the same protein core. Seno et al. (1965) have isolated fragments containing both chondroitin and keratan sulphates after the digestion of a protein polysaccharide with papain. The two different carbohydrates could be separated if this preparation were subsequently treated with alkali. No protein polysaccharide has yet been described which contains keratan sulphate alone. Protein-chondroitin sulphates have however been isolated (Roden, 1970).

The protein-polysaccharides extracted from most of the sources so far studied, including pig articular cartilage, appear to contain a low molecular weight fraction. The proportion of protein-polysaccharide accounted for by this fraction appears to increase with age, in pig articular cartilage (Brandt & Muir, 1969). A component of this fraction appears to cause the aggregation of the higher molecular weight protein-polysaccharides (Hascall & Sajdera, 1969, Sajdera et al., 1970). Recent data suggest
that free hyaluronic acid may also be involved in the interaction (Hardingham & Muir, 1975). The formation of this complex was affected by the pH and the ionic strength of the solution. These data suggest that the interaction was predominantly ionic. Complex formation was however inhibited by the reduction or alkylation of the sulphydryl groups of the disaggregated subunits, implying that disulphide bonds are also necessary. These may either be involved directly, or in maintaining the subunits in a conformation favouring the ionic interactions (Hascall & Sajdera, 1969).

b) Collagen.

Collagen accounts for between 40 and 65% of the dry weight of articular cartilage (Mathews, 1953). The proportion tends to increase with age. In articular cartilage, the collagen is associated very closely with the protein-polysaccharide and it is difficult to extract collagen from cartilage in a sufficiently pure state to make its chemical characterisation reliable. Most of the data on collagen is therefore derived from studies on other mammalian connective tissues. The chemistry of collagen has been reviewed by Ramachandran, 1967, Bailey, 1968, and Traub & Piez, 1971.

Amino acid analyses of collagens from a wide variety of mammalian sources show close similarities (Eastoe,
The most notable features of these analyses are the presence of hydroxyproline and hydroxylysine and the high proportion of glycine. Glycine alone accounts for approximately one third of all amino acid residues. Proline is also present in unusually large quantities and constitutes one tenth of the amino acids.

A complete amino acid sequence determination on collagen has been prevented by the large size of the molecule and the apparent regularity of its structure. Sequence studies have, however, been performed on some of the products produced by degradation of collagen by enzymes or by various chemical reagents. The most useful of these chemical reagents appears to be cyanogen bromide, which cleaves peptide chains specifically at methionyl residues (Gross & Witkop, 1961). Since collagen only contains 5 - 9 methionine residues in each peptide chain, a small number of different peptides is formed. These peptides can then be isolated and characterised. The results of these studies confirm the close similarity between collagens from different vertebrate sources. Most of the vertebrate collagens so far studied, with the exception of cod skin collagen, yield similar products on treatment with cyanogen bromide (Traub & Piez, 1971).

Amino acid sequence determinations on the cyanogen bromide products confirms the earlier suppositions
(Grassman et al, 1960) that polar and non-polar amino acids tend to be grouped together in different parts of the chain. Glycine occurs in every third position for quite long lengths of the peptide sequence. This regular structure appears to be interrupted near each end of the original molecule. These sections differ in tertiary structure from the rest of the molecule as they are, for stereochemical reasons, unable to form a helix. It is probable that linkages between different peptides, and the antigenic properties of collagen are located in these areas (Schmitt et al, 1964, Rubin et al, 1965).

Most preparations of collagen contain two different peptides, the $\alpha_1$ and $\alpha_2$ peptides. These are of approximately equal molecular weight but have different amino acid sequences. Some collagens have been isolated from human epiphyseal plate and chicken costal cartilage which contain only one type of peptide. The peptides of the collagen isolated from these cartilages differ in amino acid sequence from collagen peptides isolated from other anatomical sites in the same species (Miller & Matukas, 1969, Miller et al, 1971 a, Miller, 1971).

Collagen fibres can be broken down by treatment with 0.14 - 1.0 M sodium chloride or by treatment with various acidic buffers (Bailey, 1968). The purified acid extract of calf-skin collagen appears to consist of rod shaped molecules approximately 3,000 Å in diameter (Boedtker & Doty, 1956). The results of molecular weight determinations of the calf-skin preparations lie between
310,000 and 345,000 (Boedtker & Doty, 1956, Rice et al, 1964). The molecular weight variation within a single preparation appears to be quite small. This product has been termed 'tropocollagen'. It is assumed to be the fundamental collagen molecule.

When solutions of tropocollagen are heated the tropocollagen denatures within a narrow temperature range. The denaturation is marked by a large drop in the viscosity of the solution and in its optical rotation (Harrington & von Hippel, 1967, Gallop et al, 1972). If the denatured preparation is subjected to density gradient centrifugation three separate bands can be identified. The undenatured tropocollagen shows only one band. The weight average molecular weight of the tropocollagen is reduced during heat denaturation by a factor of approximately 2.5. These data can be interpreted by assuming that the native tropocollagen molecule consists of three peptide chains of similar molecular weight and that some of these polypeptide subunits are linked by covalent bonds. During heat denaturation, it is assumed that the quaternary structure of the collagen is destroyed, releasing single polypeptides, the α peptides, dimers, the β peptides and trimers, the γ peptides. The β and γ peptides consist of α peptides joined by covalent crosslinks. Normally two α₁ peptides are combined with one α₂ peptide in a single tropocollagen molecule. Tropocollagens have however been
isolated from human epiphyseal plate and chick costal cartilages which appear to consist of three $\alpha_1$ peptides (Miller, 1971, Miller et al, 1971).

If the solution of denatured collagen is cooled below the transition temperature, the original properties of the tropocollagen are partially regained. The extent to which the reaction is reversible and the rate at which it occurs, depends on the conditions.

More information about the molecular conformation of collagen can be derived from its high-angle X-ray diffraction pattern. The pattern has been described by many authors, Ramachandran (1967) and Traub & Piez (1971) being among the more recent. The pattern is sharpened considerably by stretching the fibre by approximately 10\% (Cowan et al, 1955). It can be interpreted by assuming that the tropocollagen has a mainly helical structure, and from measurements of the pattern, the dimensions of the helices can be calculated.

It is assumed on stereochemical grounds, that the helical portions of the peptides correspond to those parts which have the regular repeating Gly-X-Y amino acid sequence. The most recent determinations of the dimensions of the unstretched helix indicate that each tripeptide unit accounts for a rotation of $108^\circ \pm 2^\circ$ around the central axis of the helix. The longitudinal displacement, parallel to the axis, accounted for by each tripeptide is $2.91 \pm 0.01$ A.
X-ray diffraction yields little information about the dispositions of individual amino acids and this information can only be derived from stereochemical speculation based on what is known of the amino acid sequence.

Opinion, at present, is divided between two models. Both are based on three separate helical peptides with their three axes running parallel. The structure is held together by inter- and intra-chain hydrogen bonds. The models differ in the number of inter-chain hydrogen bonds which each tripeptide can enter into with its neighbours. In the collagen II structure of Rich & Crick (1961) each tripeptide can participate in only one hydrogen bond, whereas according to the model of Ramachandran (1967) two hydrogen bonds can be formed. The Rich & Crick structure is in closer accord with the bond angles and dimensions proposed by Pauling & Corey, 1951. It is also supported by studies on synthetic polypeptides resembling collagen (Traub & Pies, 1971). The model of Ramachandran is, however in better agreement with measurements of infra-red dichroism and tritium and deuterium exchange rates of collagen (Harrington et al, 1966, Ramachandran, 1967). Thermodynamic measurements based on the shrinkage and denaturation temperatures of collagen also support a structure involving two inter-chain hydrogen bonds to each tripeptide (Harrington, 1964). The controversy continues.

The peptides of tropocollagen may also be
joined by covalent linkages. The chemical nature of these
crosslinks has been a source of much conjecture. Much of
the evidence suggests that lysine and hydroxyllysine are
involved in the linkage. The involvement of aldehyde groups
has also been suspected for some time since lathyritic
collagen which is deficient in crosslinks also contains
The presence of allysine, in which the ε-amino group of
lysine has been replaced with an aldehyde group, has been
detected in several collagens (Kong et al, 1969, Stark et
al, 1971). Peptides which contain dehydrohydroxylsinylnorleucine have been isolated from borohydride reduced
insoluble (but not soluble) calf-skin collagen (Bailey
& Peach, 1971). These data suggest that crosslinkages
could be formed by aldol condensation of two separate
allysine or hydroxyallysine residues on adjacent chains.
(Fig. 1-3).

Evidence also suggests that the ε-amino
group of lysino may react with aldehyde groups by a Schiff
addition. The reduced product of this reaction
(dehydrohydroxylsinylnorleucine) has been isolated
from bovine achilles tendon collagen which had been treated
with borohydride. (Mechanic & Tanzer, 1970). (Fig. 1-4).
All the possible condensation products between lysino,
hydroxylsine and their aldehydes have been identified in
collagen, apart from the aldol condensation product of two
residues of hydroxyallylsine. (Traub & Pies, 1971).

Evidence has recently been produced to suggest that the aldol linkage may undergo a further addition reaction with a histidine residue (Fairweather et al, 1972). Such a reaction could result in a tri-functional crosslink. The possibility of other basic amino acids undergoing addition reactions to aldol linkages has not been excluded.

The evidence for other types of crosslink is less well established. Aldol linkages involving derivatives of glutamine and aspartate as well as lysine have been proposed (Gallop et al, 1972). Cystine appears to be absent in vertebrate collagens so far studied and disulphide linkages have only been detected in some invertebrate collagens. (Pies, 1967). The chemistry of collagen crosslinking has been reviewed recently (Gallop et al, 1972).

Although collagen possesses carbohydrate bound to some of the hydroxyl groups of hydroxylysine residues, it is unlikely that these are involved in crosslinking (Bailey, 1968, Pies, 1963). In most collagens the carbohydrate appears to consist of the disaccharide, glucosylgalactose (Butler & Cunningham, 1966). In collagen isolated from chick cartilage, however, longer polysaccharide chains of sixteen to eighteen monosaccharide units have been identified. This carbohydrate appears to consist of
FIGURE 1-3
The formation of an aldol crosslink

FIGURE 1-4
Dehydrohydroxylsinohydroxynorleucine

P1-CH2-CH2-CH-CH = N-CH2-CH-CH2-CH2-P2
OH OH
glucose and galactose. In chick cartilage collagen, approximately 40% of hydroxylsine residues are involved in glycosidic linkages (Miller, 1971).

The presence of intermolecular linkages in collagen has been deduced from the isolation of \( \gamma \) components containing three \( \alpha_2 \) peptides or \( \beta \) components containing two \( \alpha_2 \) peptides. (Bormstein & Pies, 1964, Vois & Aneey, 1965). The linkages so far studied appear similar in chemistry to the intramolecular linkages. Both types of intra and inter molecular linkage occur either near the C-terminal ends of the peptides or between a quarter and half the distance along the peptide from the N-terminus. (Rojkind et al, 1970). The number of both inter and intra molecular crosslinks tends to increase as the collagen fibres age (Pies et al, 1965).

Native collagen fibres, stained with uranyl acetate, and viewed by transmission electron microscopy, normally show a regular pattern of light and dark bands which is repeated every 640 Å (Hall et al, 1942). Artificial, segment long spacing (SLS) forms of collagen have been prepared by allowing tropocollagen to precipitate in the presence of ATP or chondroitin sulphate. SLS collagen is thought to consist of aggregates in which the tropocollagen molecules lie side by side with their ends in register. Measurements of SLS preparations confirm that the tropocollagen molecule is 20,000 - 30,000 Å long, or
approximately four times the periodicity of the native fibrils (Schmitt, 1956). These observations led Schmitt, 1956 to propose that the 640 Å pattern of the natural collagen was caused by a 'quarter stagger' arrangement of tropocollagen molecules. According to Schmitt each tropocollagen molecule was arranged parallel to the long axis of the fibre and was displaced by a quarter of its length in relation to its nearest neighbour. Further evidence for this arrangement was provided by Hodge & Schmitt (1960) and Kuhn and his co-workers (1960). They were able to reconstruct the 640 Å pattern by adding four identical images of SLS collagen. The pattern could only be simulated if three of the SLS images were displaced by one quarter, one half, and three quarters in relation to the first one. More precise measurement indicated that the length of the tropocollagen molecule was 4.4 times the length of the 640 Å fibril period and the model had to be modified to include gaps between the ends of the tropocollagen molecules (Hodge & Petrushka, 1963). In a three dimensional structure, it would be impossible for each tropocollagen molecule to be displaced by a quarter of its length in relation to all of its neighbours (Smith, J. W., 1965). Several three dimensional structures which retain various features of the 'quarter stagger' arrangement have been proposed (Traub & Piez, 1971).

An alternative model has been proposed by
Grant et al (1965) and Cox et al (1967) and their co-authors. According to them, the tropocollagen molecule is divided into five regions in which intermolecular attraction is strong and four in which it is weak. Aggregation, they suggest, is largely random. It is restricted only by the necessity of bonding zones forming linkages with other bonding zones in different molecules.

Interactions Between Collagen and Protein-Polysaccharides

Fibril formation by solutions of tropocollagen is considerably accelerated by the presence of protein-polysaccharide and to a lesser extent by high molecular weight chondroitin sulphate and hyaluronic acid (Mathews, 1965, Mathews & Becker, 1968). The rapidity of the reaction and the structure of the product depend both on the pH and ionic strength of the medium. These data suggest that the interactions are ionic (Lowther et al, 1970).

The relationship between protein-polysaccharide and collagen in FSH has been studied by transmission electron microscopy (Serafini-Fracassini & Smith, 1966, Serafini-Fracassini et al, 1970). Their data suggest that protein-polysaccharide is associated with collagen in a highly specific manner. Two protein-polysaccharide molecules appear to be associated with each collagen 640 Å period in their preparations (Serafini-Fracassini & Smith, 1966).

The role of this type of reaction in the
organisation of tissues in vivo has caused considerable speculation. Mathews (1968) has proposed a model in which collagen fibres and the protein cores of protein-polysaccharides run parallel. The pictures published by Serafini-Fracassini and colleagues (1970) suggest however, that the protein cores of the protein-polysaccharides may lie at right angles to the axes of the collagen fibrils. It is possible that several types of association may be involved.
CHAPTER 2
THE METABOLISM OF PROTEIN-POLYSACCHARIDE & COLLAGEN

Synthesis of Protein-Polysaccharides
The carbohydrate precursors:

The immediate precursors of the glycosaminoglycans of articular cartilage are the uridine diphosphate (UDP) derivatives of their monosaccharide components. The pathways by which these UDP sugars appear to be synthesised from glucose is summarised in figure 2-1. Although not all the enzymes involved have been isolated from cartilage, there is little reason to suppose that the routes by which the UDP sugars are manufactured differ vastly between different tissues. The UDP-N-acetylglucosamine epimerase reaction has been demonstrated in cartilage. Evidence for the glutamine-fructose-6-phosphate aminotransferase reaction is implied by the observation that amino sugar synthesis in cartilage is inhibited by glutamine analogues. The evidence for the various stages in these pathways has been reviewed by Davidson (1966).

The N-acetylhexosamine pathway is probably subject to feedback control. Kornfeld et al (1964) demonstrated that rat liver glutamine-fructose-6-phosphate aminotransferase (E.C. 2.6.1.16) is inhibited by UDP-N-acetylglucosamine and to a lesser extent by UDP-glucuronic acid. N-acetylglucosamine had no inhibitory effect. Similar effects have been demonstrated with bovine thyroid gland
The UDP-glucose dehydrogenase reaction, by which UDP-glucuronic acid is produced, is inhibited by UDP-xylose in chick cartilage (Neufeld & Hall, 1965). The addition of UDP-xylose to a cell-free preparation of bovine cornea causes a reduction in chondroitin sulphate synthesis and a slight increase in keratan sulphate synthesis (Castellani et al, 1970).

The synthesis of complete glycosaminoglycans is dependent on protein synthesis and is stopped entirely by inhibitors of protein synthesis such as puromycin (Telser et al, 1965). It is therefore probable that the core protein must be at least partially synthesised before the glycosaminoglycan chains can be initiated.

Nearly all the studies of protein-glycosaminoglycan synthesis have concentrated on protein-chondroitin sulphate. The enzyme which initiates the glycosaminoglycan chain, UDP xylotransferase has been partially purified from the soluble fractions of homogenates of chick cartilage and partially characterised (Robinson et al, 1966) (Roden & Schwartz, 1975). Carbohydrate free protein derived from protein-polysaccharide by the Smith degradation is an excellent substrate for this enzyme (Baker et al, 1971). Some activity is also shown with the tripeptide serylglycylglycine, but not serine (Baker et al, 1972). No activity is shown with the unmodified protein-glycosaminoglycans,
Figure 2-1 The Biosynthesis of the UDP Sugars (after Barrett, 1968)
FIGURE 2-2 THE ENZYMES OF UDP-SUGAR SYNTHESIS

1) Hexokinase EC.2.7.1.1.
2) Glucose phosphate isomerase EC.5.3.1.9.
3) Glutamine-fructose-6-phosphate aminotransferase EC.2.6.1.16
   (Ghosh et al, 1960)
4) Glucosamine phosphate isomerase EC.5.3.1.10
5) Phosphoglucomannose transacetylase EC.2.3.1.4.
   (Davidson et al, 1957, Pattabiraman & Bachawat, 1962)
6) Acetylglucosamine phosphomutase EC.2.7.5.2.
7) UDP-Glucosamine pyrophosphorylase EC.2.7.7.23.
8) UDP-Acetylglucosamine epimerase EC.5.1.3.7.
9) Phosphoglucomutase EC.2.7.5.1.
10) Glucose-1-phosphate uridylyltransferase EC.2.7.7.9.
11) UDP-Glucose dehydrogenase EC.1.1.1.22.
12) UDP-Glucuronate deacetylase EC.4.1.1.35.
13) UDP-Glucose epimerase EC.5.1.3.2.
   (also Hexose-1-phosphate uridylyltransferase EC.2.7.7.12)
14) Galactose-1-phosphate uridylyltransferase EC.2.7.7.10.

+ After Barrot (1968)
although only approximately 50% of the serine residues are substituted (Roden & Schwartz, 1973). This last observation suggests that the identity of the amino acids flanking the appropriate serine residues may be important in determining the activity of the enzyme.

The remainder of the UDP-glycosyl transferases are membrane bound and this complication has hindered their isolation and characterisation. The problem has been partially overcome by the use of Tween 20 (Roden & Schwartz, 1973).

Two UDP-galactosyl transferases have been identified by experiments using different substrates (Roden, 1970). One enzyme, shows activity with 0-β-D-xylosyl-L-serine and less activity with free xylose. This enzyme is presumably responsible for the addition of the first galactose to xylose. It shows no activity with 4-0-β-D-galactosyl-L-xylose and is probably not therefore capable of adding the second galactose of the linkage region.

The second UDP-galactosyl transferase on the other hand shows no activity with free xylose or β-D-xylosyl-L-serine. It will however, add galactose to 4-0-β-D-galactosyl-L-xylose. This is presumably the enzyme which adds the second galactose of the linkage region.

There are also two UDP-glucuronosyl transferases. One shows maximum activity with 3-0-β-galactosyl-D-galactose and slight activity with free galactose. The other can only add glucuronic acid to an oligosaccharide with N-acetyl-
galactosamine at its non-reducing end. The
N-acetylgalactosamine may be sulphated in the sixth position. Free N-acetylgalactosamine is not a substrate for this enzyme.

N-acetylgalactosamine appears to be added to the growing oligosaccharide by only one enzyme. This enzyme requires an oligosaccharide with glucuronic acid at its non-reducing end. (Rodén, 1970; Rodén & Schwartz, 1973).

Glycosaminoglycan synthesis therefore seems to require six different UDP-glycosyl transferases. The hypothetical process is summarized in figure 2-3.

Sulphation

Sulphate must be incorporated into 3'-phosphoadenylyl-5'-phosphosulphate (PAPS), before it can participate in the sulphation of the glycosaminoglycan. The synthesis of PAPS involves two enzymatic steps.

Sulphate at first reacts with ATP to form adenyl-5'-phosphosulphate (APS) with the release of inorganic phosphate. (1). This reaction is catalysed by sulphate adenylyltransferase (EC.2.7.7.4).

$$\text{SO}_4^2- + \text{ATP} \rightarrow \text{APS} + \text{PP}_i \quad \text{(1)}$$

APS is then phosphorylated, with the simultaneous conversion of one molecule of ATP to ADP. This reaction is catalysed by adenylylsulphate kinase (EC.2.7.1.25.) which requires magnesium as a cofactor.

$$\text{Mg}^{2+}$$

$$\text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP} \quad \text{(2)}$$
FIGURE 2-5
THE SYNTHESIS OF PROTEIN-CHONDROITIN SULPHATE
The enzyme which transfers the sulphate to chondroitin sulphate has been identified in the soluble fraction of homogenates of embryonic chick cartilage (Adams, 1959, 1960, Meesan, 1966), rat liver (Greiling & Bauditz, 1959) and hen oviduct (Suzuki & Strominger, 1959, 1960 a, b). The enzymes from all these sources can add sulphate to either chondroitin 4 or 6 sulphates. The acceptor may be free or linked to protein. None of these enzymes appear to sulphate keratan sulphate. The enzyme from hen oviduct adds sulphate more readily to high molecular weight substrates (Suzuki & Strominger, 1960 b). The enzyme from chick cartilage, however, does not appear to show this preference (Meezan, 1966, Meezan & Davidson, 1967 a). The best acceptor of sulphate for the chick cartilage sulphotransferase so far investigated, seems to be a slightly undersulphated chondroitin-4-sulphate. The sulphate group may be added to the fourth or sixth positions of the galactosamine residues in vitro, depending on whether or not the chondroitin sulphate is attached to protein. The factors which determine which position, if any, takes precedence in vivo is uncertain. Meesan & Davidson, 1967 b have suggested that the conformation of the chondroitin sulphate may play an important role. It is not at present known whether any other sulphotransferases are involved.

**Site of Protein Polysaccharide Synthesis**

The protein portion of the protein-polysaccharide
is presumably manufactured, in common with other proteins in
the rough endoplasmic reticulum. The synthesis of the
polysaccharide, and the addition of sulphate, has been shown by
electron microscopic autoradiography to occur in the Golgi area
(Godman & Lane, 1964). Horowitz & Dorfman, (1968) have however
identified the UDP glycosyltransferase enzymes, capable of
synthesising chondroitin sulphate, in the rough, as well as the
smooth endoplasmic reticulum. This latter observation lends
support to the view that glycosylation of the peptide can
commence before its synthesis is complete.

**Hormonal Control of Protein-Polysaccharide Synthesis**

Protein-polysaccharide synthesis is controlled
by hormones as well as the feedback mechanisms already mentioned.
The most specific hormonal factor is the 'sulphation factor'
which appears in the sera of mammals which have been treated
with growth hormone (Salmon & Daughaday, 1958, Daughaday &
Parker, 1965). Growth hormone itself is, however, inactive.
The sulphation factor increases the production of sulphated
protein-polysaccharide and stimulates DNA synthesis in isolated
embryonic chick chondrocytes (Garland et al, 1972). The
effect of the sulphation factor on protein-polysaccharide
synthesis is opposed by 3',5'-cyclic AMP and theophylline, which
inhibits the breakdown of 3',5'-cyclic AMP (Rendall et al, 1972).

Thyroxine and insulin stimulate the production
of protein-polysaccharide. Cortisone and some other steroids
have inhibitory effects (Fall, 1964).
Biosynthesis of Collagen

The peptide chains of collagen are assembled on the ribosomes in a similar manner to other proteins. The immediate products, precollagen, appears to be rather larger than a normal peptide (Bellamy & Bornstein, 1971, Church et al, 1971). It contains a large proportion of peptide unlike that found in normal collagen. The most striking difference seems to be the presence of five to seven cysteine residues. Precollagen can be converted into a peptide more typical of an α chain by pepsin (Bornstein et al, 1972).

It has been suggested that the 'extra' peptide could assist the formation of the triple helical structure by aligning the three peptides in the configuration most favourable to helix formation (Speakman, 1971, Bellamy & Bornstein, 1971). The extra peptide would then be cleaved by an enzyme resembling pepsin after the helix had formed, but before the molecule was secreted (Bornstein et al, 1972). In the collagen disease, dermatosparaxis, the proteolytic enzyme appears to be absent and defective tropocollagen molecules are secreted (Lensers et al, 1971). These abnormal tropocollagens can be converted into a product resembling normal tropocollagen by pepsin.

An alternative theory suggests that all three α chains are synthesised originally as one peptide and the helical regions brought into register by the folding. The non-collagenous sequences would then be removed, and the three
a peptides separated by the proteolytic enzyme. Such a scheme seems less probable, as in most tropocollagens at least two of the α peptides are identical. No ribosomal complex has yet been isolated which is large enough to account for the synthesis of a peptide of this size (Gallop et al., 1972). It seems unlikely that the α peptides are able to assume their helical configuration without the assistance of one of these, or a similar mechanism. In vitro helix formation takes several hours whereas in vivo it is almost instantaneous.

Hydroxylation of Proline and Lysine

Hydroxyproline and hydroxylysine are formed from proline and lysine which have been already incorporated into polypeptides. Free hydroxyproline and hydroxylysine are not used in peptide synthesis. The enzyme responsible for the hydroxylation of proline has been isolated in a fairly pure state from chick embryos (Kivirikko & Prockop, 1967, Kivirikko et al., 1968) and from the skin of newborn rats (Rhoade & Udenfriend, 1968). It is possible that the hydroxylation of both proline and lysine are catalysed by the same enzyme (Kivirikko & Prockop, 1967). Both reactions appear to require the same cofactors. These are ferrous iron, ascorbate and α-ketoglutarate (Kivirikko & Prockop, 1967). The reaction utilises oxygen molecules and is therefore inhibited by anaerobic conditions. The activity of proline hydroxylase in cell cultures is enhanced by cell crowding and high lactate concentrations (Green & Goldberg,
Lactate accumulates more rapidly in crowded cell cultures and it is therefore probable that these two observations are identical. Lactate has no effect on the activity of cell-free preparations of the enzyme (Comstock & Udenfriend, 1970). McGee et al. (1971) have produced evidence to suggest that collagen proline hydroxylase is produced as an inactive precursor. They have shown that this precursor, which is immunologically identical to the active enzyme, acquires hydroxylase activity when intact fibroblasts containing it are exposed to high concentrations of lactate. It seems probable, therefore, that lactate stimulates the activation step.

Whether or not a particular proline residue is hydroxylated depends in part on the amino acids flanking it. Free proline and poly-L-proline are not hydroxylated, whereas the synthetic peptides (Pro·Gly·Pro)$_n$, (Gly·Ala·Pro)$_n$ and (Pro·Pro·Gly)$_n$ are excellent substrates (Kivirikko et al., 1960). Only one of the three proline residues in bradykinin can be hydroxylated by proline hydroxylase (Shoeds & Udenfriend, 1969). Whether the hydroxylation of lysine shows a similar dependence on amino acid sequence is, at present, unknown.

The stage in the process of collagen synthesis at which hydroxylation takes place is a subject of continuing controversy. Gould (1968) and Rosenbloom & Prockop (1969) have produced evidence to suggest that under their experimental conditions, hydroxylation takes place after the release of the
precollagen from the ribosomes. The possibility that a small amount of hydroxylation takes place while the peptide is still attached to the ribosome has not, however, been excluded. Quite large quantities of unhydroxylated collagen peptides may, however, be secreted by cells in which hydroxylation is blocked. The matter has been reviewed in some detail by Prockop (1970) and by Gallop et al (1972).

Lysyl oxidase, the enzyme which converts lysine to allysine and therefore catalyses crosslink formation, has been identified in extracts of embryonic chick bone (Bailey et al, 1970 a, b) and embryonic chick aorta (Martin et al, 1970). The chick aorta preparation is strongly inhibited by β-aminopropionitrile (Martin et al, 1970). β-aminopropionitrile strongly inhibits the formation of collagen crosslinks, *in vivo*. The resulting condition is known as lathyrisim (Levene, 1962).

The carbohydrate portions of collagen are presumably added by UDP-glycosyl transfer reactions. The process is probably analogous to protein-polysaccharide synthesis (Hagiopian et al, 1968).

The intracellular route of collagen prior to its secretion does not appear to involve the Golgi area. Freshly synthesised tropocollagen is probably released from the cisternae of the rough endoplasmic reticulum either directly through the cell membrane or into the ground cytoplasm (Bhatnagar et al, 1967 a, b), Cooper & Prockop, 1968). If hydroxylation is inhibited,
the precollagen seems to accumulate in the cytoplasm. The stored precollagen is released from the cell when the inhibition is removed (Bhatnagar et al, 1967 a, b). Striated collagen fibrils have been identified in the cytoplasm of intact fibroblasts. It is not certain whether these have been synthesised by the cell or whether they are fragments of collagen which have been ingested.

Fibril formation occurs outside the cell and in the developing embryo the gradual increase in the sizes of the fibrils with age is most apparent (Fitton-Jackson, 1968). The number of collagen crosslinks also increases as cartilage matures. There may well be some extracellular lysyl oxidase activity which catalyses their formation.


Protein-Polysaccharide Synthesis

One of the most convenient and specific measures of protein-polysaccharide is the incorporation into it of inorganic sulphate. The sulphate ion may be conveniently labelled with Sulphur $^{35}$S. This isotope emits $\beta$-particles with a maximum energy of 0.167 MeV. It has a half-life of 87.2 days. The incorporated radioactivity may be measured by liquid scintillation counting of the protein-polysaccharide, after it has been separated from all traces of the free $^{35}$S sulphate. It may also be detected by autoradiography. In this technique, histological sections of the labelled tissue are exposed to a suitable photographic emulsion, after the unbound sulphate has
been washed away.

Sulphate is among the last components to be added to the growing protein-polysaccharide and therefore inhibition of any of the earlier stages should result in a reduction in sulphate incorporation. The sulphation reaction could normally continue, however, for as long as suitable substrates for the reaction remained within the cell. There would probably, therefore, be a lag between the inhibition of one of the early stages of protein-polysaccharide synthesis and the decrease in sulphate incorporation. The sulphotransferase enzyme is intracellular and it seems improbable that sulphate can be added to undersulphated protein-polysaccharide once it has been released from the cell. There is no detectable exchange between free sulphate and the sulphate groups bound to protein-polysaccharide (Bentley, 1970). $^{35}$S sulphate is a particularly convenient label for autoradiography. The $^{35}$S incorporated into protein-polysaccharides accounts for nearly all the $^{35}$S incorporated from sulphate into insoluble matrix components.

The labelled sulphate analogue, $^{75}$Se selenate has also been used as a tracer for protein-polysaccharide synthesis. Although the $^{75}$Se is incorporated, it appears to become associated with the protein rather than the polysaccharide component (Campo et al, 1966, 1967). The incorporation of selenium into cystine and methionine and into proteins as a sulphate analogue has been reviewed (Jauregui-Adell, 1966). The metabolism of selenium is, however, rather obscure and the significance of its incorporation difficult to interpret.
Glucose is a much less specific precursor than sulphate. It may be converted into a number of amino acids in addition to the carbohydrate portions of the protein-polysaccharides. Labelled alanine, glutamate, aspartate, proline, hydroxyproline, serine, glycine and arginine have been found in the collagen produced by bone slices which had been incubated in the presence of $^{14}C$ labelled glucose. (Flanagan & Nichols, 1964). Autoradiographs of tissues labelled with $^{14}C$ or $^3H$ glucose are therefore difficult to interpret. The lower energy of the $\beta$ particles emitted by tritium does, however, enable the sites of its disintegrations to be located more precisely. Glucose is useful in many biochemical studies in which the products are isolated and characterised.

The direct precursors of the polysaccharides, the UDP-sugars are hardly more specific as all may be converted to glucose and other monosaccharides. UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine are separated by a single reversible step.

The Measurement of Collagen Synthesis

The choice of a specific precursor for collagen is more difficult. Glycine and proline have been used in autoradiographic studies (Owen, 1963). Both, however, are incorporated into other proteins and both may be metabolised to other amino acids. There is a strong correlation between the turnover rates of glycine and sulphate in cartilage (Mankin & Lippiello, 1969). These data suggest that most glycine in
cartilage is converted into protein-polysaccharide. Proline and glycine may also be incorporated into precollagen like peptides in the absence of hydroxylation.

The production of hydroxyproline and hydroxylysine have been used as criteria of collagen synthesis in biochemical studies. 4-Hydroxyproline production is most commonly used as a measure of collagen synthesis since it is produced in much larger quantities than 2-Hydroxyproline and hydroxylysine and not subsequently modified. Proline hydroxylation is, however, only a measure of the synthesis of precollagen-like peptides. It does not necessarily imply the formation of native type banded fibrils or even the normal tropocollagen molecule. Slightly under half the proline is converted to hydroxyproline in collagen. Hydroxyproline also occurs in elastin, but in very much smaller quantities (Bastoe, 1967).

The Control of Matrix Synthesis by Extracellular Factors

Articular cartilage in vivo is isolated from a blood supply and it has been suggested that the metabolism of the chondrocytes is partially regulated by the availability of their nutrients (Linn & Sokoloff, 1965). The principal route by which articular chondrocytes obtain their nutrients and dispose of their waste products has been a source of controversy. Their anatomical position suggests two possible routes. Nutrients might diffuse into the cartilage from the synovial fluid in the joint cavity or from the marrow cavities in the underlying cancellous bone. The diffusion of nutrients from the synovial
fluid into articular cartilage has never been seriously questioned. The existence of passages which would allow diffusion between the marrow cavity and articular cartilage however, has been disputed (Collins, 1949; Brower et al, 1962; Maroudas et al, 1968). Numerous experiments have shown that various substances can pass from the marrow cavities of long bones into the cartilage under certain conditions (Ekholm, 1951; Eroedin, 1955; Hodge & McKibben, 1969; Greenwald & Haynes, 1969). Both the species, and the age of the animal appear to be important in determining the relative importance of the two routes. Hodge & McKibben (1969) were able to show that $^{35}$S labelled sulphate which had been injected directly into the subchondral bone, of immature rabbit tibias, appeared in the cartilage within 90 minutes. The articular cartilage of mature animals which had been treated in a similar manner remained, however, unlabelled. Greenwald and Hodge (1969) were able to demonstrate the diffusion of the fluorescent dye sodium 3-oxyprene-5,8,10-trisulphonate from the cancellous bone of healthy adult human femoral heads into the articular cartilage over a sixteen hour period. The anatomy of the subchondral bone in humans however differs markedly from the anatomy of the rabbit bone and it is difficult to draw general conclusions (Greenwald & Hodge, 1969).

The nutrients must first pass through the matrix before reaching the chondrocyte by either of these two routes. The constituents of the matrix are packed too closely to allow intact proteins to pass (Laurent, 1968). The fluid in the matrix probably resembles therefore, an ultrafiltrate of
plasma. Of the low molecular weight constituents of plasma which are possible precursors of collagen and protein-polysaccharide, glucose and glucosamine are relatively abundant. The concentration of both is approximately 800 mg./l. The concentrations of the amino acids are relatively low, about 45 mg./l. The concentration of inorganic sulphate is around 7.8 mg./l. (Hoffman, 1964). These figures probably represent the upper limits of concentrations in the interstitial spaces of the cartilage matrix. Under most physiological conditions, the concentrations may be much lower, especially in the vicinity of cells.

Adamson et al (1966 a, b) have suggested that protein-polysaccharide in embryonic chick cartilage may be regulated by the ability of the cells to take up amino acids by active transport across the cell membrane. They showed that substances which stimulated amino acid uptake such as serum sulphation factor and dibutryl AMP also stimulated sulphate incorporation (Adamson, 1970). Substances such as ouabain, which inhibit amino acid uptake, had the reverse effect. The amino acid concentrations in their incubation medium was several times the physiological concentration which might be expected in adult plasma or synovial fluid. Under their conditions sulphate did not appear to be actively transported into the cell.

Another factor which may control the synthesis of the matrix is the concentration of the matrix outside the cell. It has been recognised for some time that depletion of the
cartilage matrix during osteo-arthritis is accompanied by an increase in sulphate incorporation (Collins & McElligot, 1960). In osteo-arthritis, the increase in sulphate incorporation is however accompanied by a proliferation of chondrocytes (Meachim & Collins, 1962).

More recently, Bosmann (1968) and Fitton-Jackson (1968, 1970) have examined the effects of removing specific matrix components of foetal chick limb rudiments by enzymatic digestion. The limb rudiments were cultivated in the presence of papain (Bosmann, 1968) or hyaluronidase (Fitton-Jackson, 1970). Papain is a relatively unspecific proteolytic enzyme which can cleave a wide variety of peptide bonds. It is not active, however, against the helical parts of collagen. Hyaluronidase cleaves the β(1→4)-N-acetyl-glucosaminide links of chondroitin 4 and 6 sulphates. The principal effects of both these enzymes were therefore to degrade much of the protein-polysaccharide of the matrix.

The limb buds responded to each treatment by greatly enhanced rates of synthesis both of the amino sugars and of protein-polysaccharide. Some of the protein-polysaccharide synthesised by the treated explants was abnormal and much unpolymerised hexosamine was released after each treatment. Hyaluronidase treatment also resulted in a slight increase in hydroxyproline synthesis. Digestion with collagenase, which removed most of the collagen, incidentally destroying much of the matrix, did not increase the rate of collagen synthesis,
although there was an increase in protein-polysaccharide synthesis (O’Dell unpublished, cited by Fitton-Jackson, 1970). These experiments provide strong evidence for the ability of the matrix to influence the process of protein-polysaccharide synthesis.

Enzymic digestion probably renders the matrix considerably more permeable and alters its structure. It is difficult therefore to distinguish between the effects caused by the depletion of the matrix and nutritional effects caused by more rapid diffusion. The matrix may possibly, under some circumstances act as a source of nutrients. The chondrocyte appears to be equipped with the enzymes necessary to utilise it as such.

The rates at which collagen and the protein-polysaccharides are manufactured appear to be controlled independently. Pattnagar and Prockop (1966) have shown that in ten day old chick tibia, α,α’-dipyridyl, which chelates ferrous iron and therefore inhibits proline hydroxylase and thus collagen synthesis, has no effect on protein-polysaccharide production. Similarly, 6-diazo-5-oxonorleucine which inhibits glutamine fructose-6-phosphate aminotransferase and therefore protein-polysaccharide synthesis has no effect on the rate at which collagen is formed. There appears therefore, to be no mechanism under their conditions, by which the rate of production of either collagen or protein-polysaccharide can control the rate of the other.
The various protein-polysaccharides of calf-rib cartilage appear to be synthesised at different rates (Kleine et al., 1971; Kleine, 1975). The factors which control the relative rates of production of the different fractions are however unknown. These findings do, however, suggest control mechanisms more specific than the rates at which amino acids enter the cell.

The Breakdown of the Matrix

Protein-Polysaccharides

Protein-polysaccharides may be broken down by enzymes which attack either the protein core or the carbohydrate side chains. Proteolytic enzymes appear to be the most significant in degrading cartilage under pathological conditions. Ali (1964) has identified a proteolytic enzyme in rabbit ear and both ox and human articular cartilage. The enzyme was capable of degrading the matrix. The proteolytic enzyme, cathepsins B (Ali et al., 1967), B1, D (Woessner, 1967), A and C (Ali & Evans, 1969) have been identified in rabbit ear cartilage. Cathepsin D, however, appears to be the predominant protease of articular cartilage (Ali, 1970; Ali & Evans, 1975).

The cathepsins are a group of proteolytic enzymes which seem to be associated mainly with the lysosomes in normal circumstances. They are distinguished most easily by their specificities for different artificial substrates. They are all able to attack peptide linkages in the centres as well as at the ends of peptide chains. They all exhibit maximum
activity at around pH 5.0. They also attack ester linkages as well as peptide bonds. Unlike other esterases they are not inhibited by E-600 (Galway & Cruess (1972), Pearse, 1972). These last two properties have been used to assist their histochemical identification. The biochemical and histochemical properties of the cathepsins have recently been competently reviewed by Barrett (1969) and Pearse (1972) respectively.

The cathepsins have been shown to be capable of degrading protein-polysaccharide (PP-I) (Ali, 1964, Ali et al, 1967, Woessner, 1967). The loss of protein-polysaccharide (as judged by a decrease in staining with metachromatic dyes) in rabbit knee joints after synovectomy is accompanied by a release of cathepsins and acid phosphatase from the chondrocytes into the matrix (Galway & Cruess, 1972). It seems probable therefore that the cathepsins play a significant rôle in degrading cartilage in vivo. Matrix breakdown may thus be caused by the release of lysosomal enzymes into the matrix.

It is possible that, under pathological conditions, cartilage may be broken down by proteolytic enzymes which arise outside the cartilage. Plasmin is one of the most probable of these enzymes. It is present in blood plasma as the inactive precursor plasminogen and it is converted to the active form by the action of a lysosomal enzyme which is present in the tissues (Ali & Lack, 1965). Injected plasmin and plasminogen have been shown to cause an extensive loss of protein-polysaccharide from rabbits as judged by the decrease in
the stiffness of their ears and the large amounts of glucuronic acid in their urine (Lack et al., 1961). Other proteolytic enzymes such as papain have similar effects when they are injected intravenously. Papain causes the ears of rabbits to become completely limp if it is injected in sufficient quantities (Thomas, 1956). Papain can also cause a loss in the metachromatic staining of articular cartilage and other symptoms resembling those of osteo-arthritis in rabbits (Bentley, 1971, 1972). Proteolytic enzymes in the blood may be able to enter the cartilage either via the synovial fluid or from the marrow cavities.

Enzymes which degrade carbohydrates seem to be much less important in causing the breakdown of articular cartilage. Small quantities of hyaluronidase have been identified in the synovial fluid, but not in articular cartilage (Bollet et al., 1963). β-N-acetylglucosaminidase, β-glucuronidase and β-galactosidase have also been detected in the synovial fluid (Caygill & Pitkeathly, 1966, Caygill & Jevons, 1966). β-glucuronidase has also been detected histochemically in articular cartilage (Pugh & Walker, 1961). Small quantities of a β-xylosidase have also been identified in embryonic chick cartilage. The enzyme appeared to be associated with the lysosomal fraction (Fisher et al., 1966).
The Breakdown of Collagen

Cathepsin B1 has been recently shown to cleave peptide bonds in the helical regions of collagen in addition to its more general proteolytic action. Cathepsin D appears to be inactive against helical collagen (Burleigh, 1973). The specificity of Cathepsin B1, and therefore the products of degradation appear to differ from other mammalian collagenases. Maximum activity was shown between pH 5.6 and 7.0. Although Cathepsin B1 has not been identified in articular cartilage (Ali & Evans, 1973), it might be released from the synovium.

It is possible that other collagenases may be produced by both the synovial membrane and bone in vivo. Synovial cells produce a specific collagenase during cultivation even after subculturing for many generations (Werb et al., 1975). A latent collagenase is also secreted by bone explants during tissue culture. This collagenase precursor can be converted into an active collagenase by trypsin or cathepsin A (Vees, 1971).

Turnover of Cartilage Matrix

The rates of appearance and disappearance of labelled precursors in the matrix of various types of cartilage have been studied by several workers (Gross et al., 1960, Bowness, 1961, Davidson & Small, 1965, Bankin & Lippiello, 1969). After a single intravenous injection of $^{14}C$ glucose, activity appears much more rapidly in galactosamine than glucosamine, (Davidson & Small, 1963). These data suggest that protein-polysaccharides containing only chondroitin sulphate are synthesised more
rapidly than those containing keratan sulphate. \(^{35}S\) labelled sulphate and \(^{14}C\) labelled leucine show similar rates of incorporation and subsequent disappearance from the cartilage. It would appear therefore, that protein-polysaccharide accounts for nearly all the protein produced by the chondrocyte. Approximately half the incorporated activity had disappeared from rat costal cartilage after eight days (Gross et al, 1960, Mankin & Lippiello, 1969).

The different protein-polysaccharides appear to be metabolised at different rates. A protein-polysaccharide fraction isolated by extraction in water had a much higher specific activity than a less readily soluble fraction which could only be extracted by a sodium hydroxide solution (Gross et al, 1960, Bowness, 1961). The different rates of tracer incorporation into different fractions of protein-polysaccharide has been studied in greater detail by Kleine and his collaborators (Kleine et al, 1971, Kleine, 1973). They produced evidence to suggest the presence in calf rib cartilage, of at least four metabolic pools of protein-chondroitin sulphate with different turnover rates.
CHAPTER 3
OSTEO-ARTHRITIS AND MECHANICAL FACTORS

The Pathology of Osteo-arthritis

The first morphological changes in osteo-arthritis appear to be in the articular cartilage. The surface of the cartilage loses its smooth glistening appearance and comes to resemble felt, in the areas affected. On histological examination, the surface of the cartilage, in these regions, can be seen to contain fissures and portions of the surface appear to be flaking away. As the condition becomes more severe, the area affected spreads and the fissures become deeper. Eventually, the fissures may extend throughout the depth of the cartilage. At this stage, chondrocytes in the deeper layers appear to proliferate and form small clumps in the cartilage. The chondrocytes in the superficial layer do not seem to divide and many appear degenerate (Meachim & Collins, 1962, Gardner, 1965, Sharp, 1969, Sokoloff, 1969).

These changes are accompanied by a reduction in the ability of the matrix to stain with alcian blue and toluidene blue. The staining reaction is decreased first in the superficial layer, and later throughout the whole depth of the cartilage. Eventually all the uncalcified cartilage is lost, leaving a denuded surface of burnished bone.

While fissures appear in the uncalcified cartilage, the calcified zone begins to thicken and to become invaded by capillaries. These capillaries may arise from the
marrow cavities of the cancellous bone, or the cartilage may become invaded by capillaries from the synovial membrane. These changes result in a thickening of the subchondral bone. This thickening has become quite pronounced by the time that most of the cartilage has disappeared. The process continues after the cartilage has gone, and at the same time bony outgrowths, the osteophytes, appear at the edges of the articular surface. The osteophytes may contain trabeculae and resemble normal cancellous bone. They are often covered by a layer of fibrocartilage. Hyaline cartilage, however, has limited powers of repair.

The thickened layer of burnished bone resembles compact bone. It often, however, contains dead areas, and areas containing fibrocartilage-like cells. It may also contain cysts. The cysts may contain loose cells of various types embedded in a protein-polysaccharide or fibrous matrix. Some cysts may be connected by channels to the surface of the bone and the joint cavity.

The cancellous bone beneath the compact surface layer may contain abnormally large quantities of fat in its marrow cavities. Often the bone in this region is softer than normal bone. Further away from the surface, however, the bone resembles the cancellous bone of normal joints much more closely. The shape of the joint varies considerably during these changes and there is undoubtedly extensive remodelling within the bone (Gardner, 1965, Sharp, 1969, Sokoloff, 1969).
Ultrastructural Changes in Osteoarthritis

Various ultrastructural changes during the osteo-arthritis degeneration of articular cartilage (Silberberg & Silberberg, 1964) have been studied by several authors (Little et al, 1958, Meachim et al, 1965, Rütter & Spycher, 1968, Meachim & Roy, 1969, Weiss & Hirow, 1972). The subject has been reviewed by Ghadially and Roy (1969) and by Weiss (1973).

The first ultrastructural changes in osteo-arthritis cartilage are probably to be found in the superficial layer. The normally smooth surface of the articular cartilage may, in the joints of adult humans, show signs of flaking or splitting. In areas which show the first signs of osteo-arthritis, there may be clefts in the surface of the cartilage. Blister-like cavities are sometimes seen in the superficial layer. Prominent lipid globules may also be encountered immediately below the articular surface. The chondrocytes in the superficial layer sometimes appear to be degenerate in specimens from mildly arthritic areas (Meachim & Roy, 1969).

Specimens from more severely arthritic cartilage show deeper clefts in the matrix. In the superficial zone, collagen fibres lose their normal orientation parallel to the articular surface and become more randomly arranged. There is less 'interfibrillar ground substance' probably protein-polysaccharide and the collagen fibres become more widely spaced. The collagen fibres in these specimens appear to be rather finer than those from the same zone in normal cartilage. The lipid
globules are more numerous (Weiss & Mirow, 1972).

The chondrocytes of the superficial zone appear, at this stage to be more rounded and to have a more developed Golgi area, more rough endoplasmic reticulum and more mitochondria than is common in cells from the superficial layer of normal cartilage. Cells are often found in clusters near the clefts in the matrix. In more advanced lesions, most of the superficial cells appear degenerate with scant endoplasmic reticulum, prominent lysosomes and clumped nuclear chromatin (Weiss, 1973).

The matrix of the mid-zone is less electron-dense in early osteo-arthritis than in normal cartilage. In more severely affected cartilage, the collagen fibres come to lie perpendicular to the surface. The clefts in the cartilage deepen.

The cells of the superficial zone in moderate osteo-arthritis are often found in groups (Weiss & Mirow, 1972). The cells are rather larger than the chondrocytes of the superficial zone in normal cartilage. They also have a well developed rough endoplasmic reticulum, a prominent Golgi area and many mitochondria. Centrioles are also more common than in the cells of normal cartilage.

In addition to these cells, which show apparent signs of enhanced activity, there are many degenerate cells in the intermediate zone even in moderately advanced osteo-arthritis. The degenerate cells come to account for an increasingly large proportion of the cell population as the
osteo-arthritis becomes more severe.

In the deep zone, the cells appear degenerate even in early osteo-arthritis. These cells may contain intra-cytoplasmic filaments and have only a sparse endoplasmic reticulum. These changes become more apparent as the disease advances.

Biochemical Changes in Osteo-arthritis

The decreased staining reaction of arthritic articular cartilage with alcian blue and toluidene blue suggests that there may be a loss of glycosaminoglycan from the matrix. Most biochemical studies of osteo-arthritis cartilage support this view (Matthews, 1953, Bollet et al, 1963, Anderson et al, 1964, Benhaman et al, 1969, Mankin & Lippiello, 1970, 1971, Mankin et al, 1971, Bjelle et al, 1972, Hjertquist & Lemperg, 1972, Mankin, 1973). These authors have all noted a decrease in the hexosamine content of the cartilage, which is approximately proportional to the histological severity. Opinion is divided, however, on whether chondroitin 4 or 6 sulphate or keratan sulphate or all three are lost from the cartilage. Bollet and his co-workers (1963) reported a progressive decrease in the uronic acid concentration of the cartilage, roughly proportional to the severity of the lesion. The decrease in the uronic acid was very much greater than the decrease in hexosamine content. The authors therefore concluded that the predominant glycosaminoglycans lost were the chondroitin sulphates. Bjelle and his colleagues (1972) and Hjertquist and Lemperg (1972) have both fractionated the glycosaminoglycans of normal and arthritic cartilage on columns of
cellulose impregnated with cetylpyridinium chloride. They found that all their fractions appeared to be reduced by similar proportions in the osteo-arthritic specimens. Both mildly and more severely arthritic specimens of cartilage were depleted of glycosaminoglycans to approximately the same extent. Mankin and Lippiello (1971) also studied osteo-arthritic cartilage by a similar fractionation technique. They reported however, a decrease only in keratan sulphate. Chondroitins 4 and 6 sulphates, in their experiments, actually appeared to have higher concentrations in the arthritic than in the normal specimens.

These discrepancies may possibly be explained by different sampling techniques and ascribed to different criteria for assessing the severity of osteo-arthritis. The results may also be influenced by whether the cartilage of the control groups is taken from unaffected joints or from the apparently unaffected areas of arthritic joints.

The glycosaminoglycans of osteo-arthritic cartilage differ from those of normal cartilage in other respects. The chain length (Bollet & Nance, 1966) and the number average molecular weight (Hjertquist & Wasteson, 1972) of chondroitin sulphate appear to be reduced in osteo-arthritic cartilage. Chain length is closely related to the number average molecular weight in this instance and hence these results confirm one another.

Mankin and Lippiello (1971) report the identification of a highly sulphated chondroitin-4-sulphate in osteo-arthritic cartilage, not present in normal cartilage from
patients of the same age group. Hjertquist and Lemperg, (1972) were not able, however, to confirm this finding.

Collagen concentration appears to be much less affected by osteo-arthritis. Several authors have noticed slight decreases in the hydroxyproline concentration of arthritic cartilage (Matthews, 1953, Bollet et al, 1963). These are normally confined, however, to the most severe lesions (Bollet et al, 1963). Other authors have been unable to detect any significant changes (Mankin & Lippiello, 1970).

Collagen fibres in early osteo-arthritis retain their characteristic appearances as revealed by transmission electron microscopy. In more severely diseased specimens however, the fibres in the remnants of the superficial layer tend to be finer than is characteristic for specimens of normal cartilage from patients of the same age group (Weiss, 1973).

DNA, but not RNA, concentration appears to be elevated in articular cartilage affected with osteo-arthritis, and the increase seems to be related to the amount of histological alteration (Mankin & Lippiello, 1971). The increase in DNA content is most probably a reflection of the cellular proliferation observed in histological studies (Meachin & Collins, 1962).
Metabolic Changes

Protein-Polysaccharide Synthesis

The decrease in glycosaminoglycan concentration in the matrix of osteo-arthritis cartilage is accompanied by an increase in the incorporation of $^{35}$S labelled sulphate (Collins & McElligot, 1960, Collins & Meachim, 1961, Meachim & Collins, 1962, Mankin & Lippiello, 1970, 1971). The rate of sulphate incorporation is related to the severity of the histological changes. It appears to bear an inverse exponential relationship to hexosamine concentration (Mankin & Lippiello, 1970, 1971). Glycine incorporation is also increased in osteo-arthritis cartilage as compared with the normal figure. (Mankin & Lippiello, 1970). The close relationship between glycine and sulphate incorporation suggests that most of the glycine is converted into protein-polysaccharide rather than collagen.

Collagen Synthesis

Comparatively few studies have been made of the synthesis of collagen by articular cartilage and the ways in which it might be altered by osteo-arthritis. This is due in part, to the extremely slow rate at which collagen is synthesised in adult cartilage. A slight increase in the production of hydroxyproline has however been noted in rabbit joints in which the cartilage had been experimentally damaged (Repo & Mitchell, 1971). Even in the damaged cartilage, however, detectable quantities of $^3$H hydroxyproline were not produced until four to six days after the injection of $^3$H proline. It seems therefore,
that articular cartilage may be capable of responding to injury by synthesising increased quantities of collagen. Whether it does so during osteo-arthritis is still a matter of conjecture.

**DNA Synthesis**

The increased DNA concentration of osteo-arthritis cartilage is accompanied initially by an increased rate of DNA synthesis as measured by $^3$H thymidine incorporation (Mankin & Lippiello, 1971). This increased rate of synthesis seems to be limited to the less severe lesions. In the advanced stages of osteo-arthritis as estimated by a complex system based on various morphological and histochemical criteria, there appeared to be a fall off in the DNA synthesis. These biochemical data confirm earlier morphological observation that chondrocytes may proliferate during osteo-arthritis. The fall off in DNA synthesis is possibly a reflection of the death and disintegration of the cells of severely arthritic cartilage as seen by transmission electron microscopy (Weiss & Mirow, 1972).

**The Differences Between Osteo-arthritis and Aging.**

The biochemical changes and metabolic changes in osteo-arthritic cartilage are sufficiently different from those caused by aging alone to suggest definite pathological changes. The glycosaminoglycan content of normal articular cartilage does not appear to decrease in concentration appreciably with age in unaffected joints (Mathews, 1965). There is, similarly, no tendency for its synthesis to be increased (Collins
& McElligot, 1960). Mitoses of chondrocytes do not normally occur with increased frequency in aged cartilage. Byers and his collaborators (1970) after an extensive study involving the post-mortem examination of over 500 hip joints concluded, that the cartilage of the femoral head commonly underwent some degeneration with increasing age. These changes appeared, however, to differ from and to be independent of arthritic changes.

**Biochemical Factors which might Initiate Osteo-arthritis**

The primary biochemical change in articular cartilage in the early stages of osteo-arthritis appears to be the loss of protein-polysaccharide from the matrix. Cathepsin D seems to play an important role in osteo-arthritis. Both Ali & Evans (1973) and Saposchik and co-workers (1973) have shown that the activity of a cathepsin D like enzyme is higher in osteo-arthritic cartilage than in normal articular cartilage. The release of the enzyme from the cells into the matrix may, however be as important as its total concentration in the tissue.

Galway and Cruess (1972) have demonstrated the simultaneous appearance of cathepsin-like esterase activity and acid phosphatase in the articular cartilage of rabbit knees which had been subjected to synovectomy. The extracellular appearance of these lysosomal enzymes was accompanied by a loss of toluidene blue metachromatic staining from the matrix.

The decrease in the molecular weight (chain length) of chondroitin sulphate during osteo-arthritis has led some investigators to suggest that a hyaluronidase-like enzyme
might be responsible for the breakdown of the matrix (Bollet et al, 1966, Bollet, 1969, Bollet & Mitchell, 1969). Attempts to identify this enzyme in cartilage have, however, been largely unfruitful (Sokoloff, 1969). There is some evidence for hyaluronidase activity in synovial fluid, and some other carbohydrate degrading enzymes have been identified in both synovial fluid and cartilage (p.63). The shorter chain length may alternatively be the result of abnormal synthesis. Protein-polysaccharides with shorter carbohydrate side chains than normal are synthesised by embryonic limb buds which have had some of the protein-polysaccharide removed by enzymic digestion (Bosman, 1968, Fitton-Jackson, 1970) (p.59).

The protein-polysaccharides of cartilage have measurable turnover rates and therefore they must be degraded even in normal cartilage. The decrease in protein-polysaccharide content in early osteo-arthritis might therefore be influenced by changes in the rate of their synthesis as well as in the rate of their breakdown.

Mechanical Factors in Osteo-arthritis

It has been thought for many years, on the basis of clinical and morphological data, that mechanical forces may play some part in causing osteo-arthritis (Sokoloff, 1969). Osteo-arthritis has been said, for example, to be more severe in the hips and knees of obese patients (Lawrence et al, 1966, Sharp, 1969) although this view has been questioned (Nöhring, 1966, Danielsson, 1964) and dietary factors cannot be eliminated. Slight
deformities of the hip incurred during adolescence may lead to osteo-arthritis in later life (Murray & Duncan, 1971). The first arthritic changes in the human knee occur in those areas of the cartilage which appear to be subject to the greatest mechanical stresses (Collins, 1949). In the hip, however, the reverse seems to be generally true (Harrison et al, 1965).

The circumstantial evidence for mechanical causes of osteo-arthritis has received some support from studies of animal joints which had been compressed or immobilised for long periods (Salter & Field, 1960, Evans et al, 1960, Trias, 1961, Crelin & Southwick, 1964, Thompson and Bassett, 1970, Radin, 1972). The continuous compression of rabbit knees by a clamp, for periods of more than three days produces degenerative changes in the matrix. These are softening of the cartilage matrix, leading to its eventual loss, a decrease in the nuclear staining by the chondrocytes and hypertrophy of the underlying bone (Salter & Field, 1960). The metachromasia of the matrix is lost in the course of the degeneration of the cartilage (Thompson and Bassett, 1970). The immobilisation of rat knees in partial flexion for periods of up to ninety days apparently causes similar degenerative changes in the articular cartilage and signs of bone remodelling. The joint cavity also became partially filled with fatty connective tissue arising from the synovium (Evans et al, 1960). Only after immobilisation for eight weeks does there appear to be vascular invasion of the rat cartilage (Evans et al, 1960). In rabbits, no evidence of
vascular invasion could be seen in the articular cartilage of knees which had been compressed for up to six weeks (Salter & Field, 1960, Thompson & Bassett, 1970).

Intermittent impact loading applied to the hind legs of guinea pigs for a short period each day appears to produce changes resembling osteo-arthritis. Loss of protein-polysaccharide was evident after six days and within twenty-one days the cartilage had undergone extensive degeneration. These changes were preceded by an increase in the stiffness of the underlying bone which was most apparent between the fifth and seventh days of the treatment. The stiffness of the bone returned to near normal values if the experiment was continued (Radin, 1972). The stiffness of the subchondral bone is also higher than normal in human post-mortem specimens which show signs of mild osteo-arthritis. Specimens showing more severe degeneration have stiffness values not significantly different from normal specimens (Radin, 1972).

The effects of the lack of compression have been studied in rabbits by removing one of the two femoral condyles. The tibial cartilage opposite the absent condyle showed signs of vascular invasion within fourteen days of the operation. The cartilage also lost most of its metachromasia during this period (Thompson & Bassett, 1970, Hall, 1964). Destruction of synovial tissue, in experiments in which the joint is opened, may influence the response of the cartilage. Galway & Cruess (1972) have shown an almost total loss in the metachromasia of the articular cartilage of rabbit knees which had had most of their
synovial tissue removed. The loss of metachromasia after synovectomy extended to most of the articular cartilage in both weight and non-weight bearing areas. The change was reversible and the cartilage had regained a normal histological appearance by the time that the synovium had regenerated. There were no signs of cellular changes or vascular invasion. The ability of chondrocytes to incorporate $^{35}S_4$ after synovectomy is largely unimpaired (Mitchell & Cruess, 1967).

It would appear from these data that both excessive pressure, and the lack of it may lead to symptoms resembling some of the changes in osteo-arthritis. It has been suggested that the flow of liquid and dissolved nutrients through the matrix may be assisted by compression and decompression of the cartilage (Salter & Field, 1960, Sokoloff, 1969, Thompson & Bassett, 1970). This mechanism may partially explain these conflicting sets of data. The composition of the fluid may, however, be as important as its rate of movement.

The precise effects of pressure in human joints, have been far from clear. In some joints, the relationship between loading and osteo-arthritis may appear fairly obvious, in many others it does not (Sokoloff, 1969). Osteo-arthritis of the finger joints, for example, is relatively common, whereas osteo-arthritis of the ankle, which apparently carries much greater loads, is not.

Part of the problem lies in the difficulty of assessing the precise contact areas of each joint and the
forces acting on them. In addition to the weight of the body, the forces exerted by the muscles may well make a significant contribution. Rydell (1966), by the use of femoral head prostheses fitted with strain gauges, has measured forces of between 1.8 and 3.3 times body weight during walking. Muscular activity may also influence the direction in which the force is applied.

The mechanical behaviour of the cartilage itself is also important in an understanding of any changes in its metabolism in response to mechanical stress. Of particular interest is the extent to which cartilage may be deformed by mechanical loading, and the extent to which this deformation affects its permeability to solutions of nutrients. Changes in these mechanical properties during aging and disease might shed some light on possible mechanisms by which mechanical pressure might cause or aggravate osteo-arthritis. Sokoloff (1963) (1966) and Kenpseon et al (1968, 1970, 1971) have measured the deformation of articular cartilage still attached to bone, caused by the application of metal probes of various shapes. Articular cartilage deforms rapidly when a metal probe is first forced into it. This initial rapid deformation is followed by a much slower, non-linear, decrease in thickness if the pressure is maintained. A constant thickness is eventually reached. The cartilage normally swells to almost its original thickness, if the pressure is removed and the cartilage is kept immersed in an isotonic medium (Sokoloff, 1963, 1966). Both the initial deformation and the subsequent rate of consolidation are much larger in osteo-arthritic
than in normal cartilage (Sokoloff, 1966, Kempson et al, 1971). There also appear to be similar differences between cartilage from apparently normal areas of osteo-arthritisic femoral heads and cartilage from the same anatomical positions in unaffected joints (Kempson et al, 1971). The areas of cartilage which deform more rapidly in this test also coincide with areas of decreased hexosamine and uronic acid content (Kempson et al, 1970).

The distance to which a probe may be forced into articular cartilage is dependent not only on the force applied to it and the properties of the cartilage but also on the shape of the probe and the thickness of the tissue. The relationship between the thickness of the cartilage and the amount by which it deforms is mathematically complex. Above a certain value, the thickness has no effect on the extent of the deformation. The size of this value is, however, dependent on the shape of the probe and the stiffness of the cartilage (Sokoloff, 1966). 'Stiffness' values obtained from indentation studies are probably only comparable if they are obtained by workers with apparatus of exactly the same design. They may also be influenced by the mechanical properties of the underlying bone.

In an attempt to eliminate these variables Edwards (1967, 1970) designed a 'standard test' for cartilage. This test used discs of articular cartilage separated from their bony backing. A disc of cartilage was placed in a closely fitting metal dish, which effectively prevented the lateral creep of the cartilage during compression. Pressure was applied by
a plunger acting vertically on a porous metal disc which covered the entire surface of the cartilage. The cartilage was immersed in a physiological medium. The permeability coefficient of the metal disc was about $10^7$ greater than the permeability coefficient of the cartilage. The movement of fluid in and out of the cartilage was probably not, therefore, seriously impeded by the metal disc. The plunger was powered by a pneumatic piston and its movements measured by a linear differential variable transformer (L.D.V.T.) connected to a chart recorder.

Cartilage undergoes a similar type of deformation during both the standard test of Edwards and in the indentation tests. An initial, almost instantaneous decrease in thickness when the load is first applied is followed by further deformation at a much slower rate. A constant thickness is eventually reached. The amount of compression, however, (defined as Initial Equilibrium Thickness) unlike the indentation tests, is directly related to the pressure applied to the cartilage (Edwards, 1967, 1970). The decrease in the thickness of the cartilage is approximately proportional to the amount of liquid squeezed out of it. The behaviour of the cartilage in the standard test is more amenable to mathematical analysis than its behaviour in indentation tests. Its deformation can be shown to bear an approximately exponential relationship to time (Edwards, 1970). Most of the chondrocytes can survive compression in the standard test apparatus with loads of up to 70 kg./cm.$^2$ for periods of at least an hour. Approximately 95% of the chondrocytes
isolated by enzymic digestion from cartilage which had been 
compressed under these conditions appeared to be alive as judged 

The permeability of cartilage to the flow 
of liquid under pressure has been measured by Maroudas et al 
(1968) and Edwards (1970). The values obtained by these workers 
for the permeability coefficient of uncompressed articular 
cartilage vary between $4.3 \times 10^{-13}$ and $6.2 \times 10^{-13}$ cm.$^4$ dyne$^{-1}$sec$^{-1}$. 
There appears to be a decrease in permeability in the deeper 
zones of the articular cartilage (McCutchon, 1962). Values 
obtained using the articular cartilage of dogs and cows appear 
remarkably similar (Edwards, 1970). There does not seem to be 
any significant relationship between permeability and the 
hydrostatic pressure forcing liquid through the cartilage 
(Edwards, 1970). Hydrostatic pressure, of course, differs from 
the mechanical pressure of the standard test, and does not 
compress the solid material of the matrix in one dimension only.
Changes in the permeability of cartilage during consolidation 
have not been investigated directly.

The permeability of cartilage to solutes has 
been reviewed briefly by Sokoloff (1969). Maroudas and her 
co-workers (1968) measured the rates of diffusion of various 
low molecular weight solutes into articular cartilage. They 
concluded that most ions diffuse through cartilage at a rate some 
40% lower than through water and that the diffusion rate of 
glucose was slightly lower. Larger molecules such as iron
dextran (M.W. = 7,000) are excluded from the matrix (Ball et al., 1964). The charge of the ions may influence their rate of diffusion. Cationic dyes (M.W. = 200-900) diffuse more readily into cartilage both in vivo and in vitro than anionic dyes of similar molecular weight (Kantor & Schubert, 1957, Brower et al., 1962). The permeability of cartilage appears to decrease with age (Stockwell & Barnett, 1964). All these experiments were, however, performed with normal, uncompressed articular cartilage. The extent to which the diffusion of solutes through cartilage is affected during compression is largely unknown.

The Present Investigation

It would appear from the preceding discussion that although both mechanical and metabolic changes seem to initiate osteo-arthritic changes in articular cartilage, little is known about the interrelationship of these factors. The many excellent investigations of the effects of compression on animal joints were able to reproduce some of the symptoms of osteo-arthritis. They did not, however, distinguish between the effects of loading and compression. It is also difficult, in such experiments, to distinguish between changes initiated in the bone, the synovium or the articular cartilage. Most of the signs of degeneration, furthermore, were not evident until several days had elapsed. It would be of interest to know the immediate effects of loading on the metabolism of articular cartilage, since in vivo most mechanical loading is short lived. It might also be useful to measure changes in the articular cartilage caused by compression alone rather than, for example,
changes in the composition of the synovial fluid, or damage to
the subchondral bone.

Initial changes in the cartilage matrix
may be caused either by a decrease in the rate of its synthesis
or by an increase in the rate of its destruction or by both.
The present investigation was designed to investigate the former
possibility.

The Edwards standard test apparatus seemed
to be the most appropriate of the various methods of applying
pressure to the cartilage. The method has the advantage of
using samples of cartilage uncontaminated by any other tissue.
It also provides a means of applying a load fairly reproducibly
to the whole area of a cartilage specimen. The apparatus was
redesigned for the purposes of the present investigation.
CHAPTER 4
THE EFFECTS OF MECHANICAL LOADING ON GLYCOSAMINOGLYCAN SYNTHESIS
IN ARTICULAR CARTILAGE

The Compression Apparatus

The apparatus used for compressing the cartilage was a modification of the one designed by Edwards (1967, 1970). The modified apparatus was designed so that all parts of the apparatus which came in contact with the specimen could be sterilised and easily cleansed of radioactive compounds. The modified design also permitted the simultaneous compression of four separate discs. The apparatus enabled the specimen dishes to be gassed during incubation, thereby allowing bicarbonate buffered tissue culture media to be used. (Fig. 1-l, Plate I-l)

The cup, which contained the cartilage specimen was formed by a steel ring 2.2 cm. in diameter and 0.3 cm. thick, with a hole, 0.66 cm. in diameter in the centre. This ring was attached to the base of a stainless steel dish, 3.3 cm. in diameter and 0.8 cm. deep, by four stainless steel countersunk screws. The joint between the steel ring and the dish was sealed by a rubber 'O' ring located in a groove cut in the base of the dish. The porous metal discs were machined from porous metal sheeting, 1.14 mm. thick with a pore size of 2.5 and a permeability coefficient of $2.53 \times 10^{-6}$ cm. dyne$^{-1}$ sec$^{-1}$ (Type RH 2½ B, Sintered Products Ltd., Sutton in Ashfield, Notts.). The sintered metal discs were made 0.025 mm. smaller in diameter than the holes in the centre of the stainless steel ring. (Plate IV-2b)
The diagram shows a unit for compressing a single disc of cartilage. The apparatus is assembled with the cartilage in position.

**KEY:**

- **A** = Pneumatic Supply ($N_2$ or Compressed Air).
- **B** = Pneumatic Membrane Transducer.
- **C** = L.D.V.T.
- **D** = Spring.
- **E** = Rubber Seal.
- **F** = 'Florosint' P.T.F.E. Bearing.
- **G** = Glass Cylinder.
- **H** = Glass Lid.
- **I** = Medium; (Ringer's solution or T.C.199).
- **J** = Porous Stainless Steel Disc.
- **K** = Cartilage.
- **L** = Rubber 'O' Ring.
- **M** = 5% CO$_2$, 20% O$_2$, 75% N$_2$, Supply.
- **N** = "" "" "" Exhaust.
FIG. IV-1: COMPRESSION UNIT.
PLATE IV-1

Compression apparatus, showing unit for compressing one disc of cartilage.
The plunger which acts on the porous metal disc was also made from stainless steel. The lower end, which came into contact with the disc had four grooves cut in it and this end could be detached from the main shaft. The plunger was mounted in a self-lubricating polytetrafluoroethylene (P.T.F.E.) bearing ('Florosint', supplied by Polypenco, Welwyn Garden City, Herts.). This bearing was fitted to a stainless steel platform attached by three stainless steel rods to the stainless steel base. A circular depression was machined in the base to enable the specimen dish to be accurately located under the plunger. All these steel parts were manufactured from stainless steel type EN 58 J. (Plate IV-2a)

The specimen dish was covered by a glass lid with a hole in the centre to admit the plunger. The whole of the bottom part of the apparatus was encased in a glass cylinder sealed at the top by a rubber washer and at the bottom by its lightly greased rim in contact with the base. The glass cylinder was fitted with two connections which enabled tubes to be fitted leading to a cylinder containing 5% carbon dioxide, 20% oxygen and 75% nitrogen (B.O.C. Special Gases, Deer Park Rd., London).

The linear differential variable transformer (L.D.V.T.) (type 060S-I, supplied by Electro-Mechanisms Ltd, 220, Bedford Ave., Slough, Bucks.) was mounted on a block attached to the upper surface of the stainless steel and its iron core fixed to a side arm projecting from the upper part of the plunger. The position of the iron core could be adjusted by two screws
PLATE IV-2

a) Base of compression unit, with glass cover removed, showing the stainless steel dish with the plunger in position.

b) Stainless steel dish dismantled, showing the dish, the steel ring, the rubber 'O' ring, the four screws and the porous stainless steel disc.
one of which secured the side arm to the plunger and the other, the L.D.V.T. core to the side arm. Electrical connections to the L.D.V.T. units were made through four of the pins of five pin sockets (DIN, line mounting type). This arrangement allowed the compression units to be disconnected and removed from the incubator. The L.D.V.T.s were powered by a signal generator set at 2.0 kHz. The secondary coils were connected in opposition and their output rectified by a two phase silicon diode rectifier. A 200 μF capacitor and a 3.0 Kohm resistor were connected in parallel, across the output from each rectifier. The D.C. signals were fed into a multichannel recorder Type B-341 supplied by T.E.M. sales, Gatwick Rd., Crawley, Sx. (Rikadenki, Kyogo, Japan). The damping controls on the recorder were set to the minimum necessary to prevent instability in the recorder.

The weight of the plunger was counterbalanced by a steel spring, adjusted so that the weight of the plunger was exactly balanced when its lower end was level with the bottom of the specimen dish.

Pressure was applied to the plunger by means of a pneumatic membrane transducer. The membrane unit was supported by an aluminium frame attached to an aluminium base. The whole compression apparatus could be slid in and out of this framework without dismantling either component. Two stops were fixed to the aluminium base, and a loose steel sleeve fitted over the plunger to enable the compression apparatus to be positioned accurately. (Fig. IV-1)
Four of these units were mounted inside an incubator (L.E.C. type P3) modified by the addition of two holes each 1" in diameter in one side. Pneumatic connections were made through 0.25" diameter high pressure nylon tubing. The pressure in the tube was measured by a pressure gauge (0-50 p.s.i.). For experiments with constant loads, the compression units were connected directly to a cylinder of nitrogen through the normal pressure reduction valve. The pressure in the tube was then adjusted with the valve. Fluctuating loads were produced by an electric current to pneumatic pressure transducer (Type 69 TA 1, style B, 170 ohms, 0-50 Ma, 0-27 p.s.i. supplied by Foxboro-Yoxall Ltd., Redhill, Surrey) connected in series with the pneumatic supply. The current-pressure transducer was controlled by a function generator (Type TWG 501, 0.01-10^6 Hz, 0-20 Hz, supplied by Feedback Ltd.). (Plate IV-3)

The Articular Cartilage Samples

The articular cartilage used in the compression experiments was taken from the humeral heads of young adult goats of both sexes. The goats were put down with a humane killer. In one or two experiments it was necessary to sedate the goats first with a sublethal dose of Nembutal. Both humeral heads were dissected out within two hours of the death of the animal and each wrapped immediately in aluminium foil. One humeral head was used immediately, and the other was stored at 4°C for 24 h. The discs of cartilage were cut with a specially designed stainless steel borer and carefully dissected away from the
PLATE IV-3

Complete compression apparatus, showing the incubator with four compression units.

A = Pressure gauge.
B = Function generator.
C = Current / Pressure transducer.
D = Chart recorder.
E = Signal generator for powering the four L.D.V.T units.
underlying bone with a small scalpel. They were immediately placed into a petri dish containing either Locke's mammalian heart Ringer's solution or the chemically defined tissue culture medium 199 (Morgan et al, 1950).

Experiments Using Static Loads

The samples of cartilage were blotted dry, weighed, and their average thickness measured with a micrometer. They were then placed either in the centre cup of the stainless steel dish or in the outer trough. The dish was then filled with either Ringer's solution or medium 199 (Morgan et al, 1950) without added isotope and containing unlabelled sulphate. The porous metal disc was placed on top of the specimen in the centre well and the compression apparatus fitted into the incubator (set at 38°C). Steady pressure was applied to the cartilage until its thickness became constant. The dish was then rapidly removed from the apparatus and both specimens weighed and their thickness measured. The unlabelled medium was replaced with similar medium containing 2.0 - 5.0 μCi/ml. $^{35}$S$_4$O$_4$ (Specific activity 40-60 mCi/mmol, Radiochemical Centre, Amersham). Unlabelled sulphate was omitted from the Ringer's solution but not from the 199. Fresh ascorbic acid and glutamine (0.2 mg./ml. of each) were added to the 199 immediately before use. The samples were replaced in the compression apparatus and the same pressure re-applied as rapidly as possible. The samples were incubated for varying periods of time, then removed from the apparatus, weighed and their thickness measured. Samples for the
biochemical studies were frozen in liquid nitrogen and samples for histology and autoradiography were washed in three changes of 0.9% sodium chloride and then fixed in neutral buffered normal saline at +4°C for at least 48 h. In some experiments, the discs were cut in half. One half was then processed for autoradiography and the biochemical studies performed on the other half. The portions of the divided samples used for the biochemical studies were re-weighed.

Experiments Using Fluctuating Loads

A similar procedure was followed in the experiments with fluctuating loads, except that the samples were equilibrated without applied pressure. They were then set up with radioactive medium in the apparatus. The input pressure to the current-pressure transducer was set to 5 p.s.i. higher than the maximum output pressure. The function generator was adjusted to a frequency between 0.01 and 0.3 cycles/sec. and either a square or a sine wave output.

Frozen Controls

In all experiments, a control disc was twice frozen in liquid nitrogen and thawed. This disc was incubated in radioactive medium for as long as the longest test incubation. These discs were weighed and measured at the end of the incubation and subsequently treated in the same way as the other discs.
Preparation of Samples for Chemical Analysis and Liquid Scintillation Counting

The frozen specimens were freeze dried to constant weight (48 h.) at -40°C on a Pearse Speedivac freeze dryer (mark II). The dried samples were weighed and then digested in 2.0 ml. (whole discs) or 1.0 ml. (half discs) of a 0.2 M borate buffer, pH 9.2 containing 0.02 M calcium chloride and 4 mg./ml. protease (Sigma, Type IV, 0.7 - 1.0 units/mg.) at 60°C. The samples were cooled when digestion was complete, and an equal volume of 4% (w/v) cetylpyridinium chloride in the borate/calcium chloride buffer added. The tubes were allowed to stand for at least 30 min. at room temperature and then centrifuged at 1,400 g. for 15 min. After centrifugation, 0.5 ml. of the supernatant from each tube were pipetted into scintillation vials containing 10 ml. of the scintillation mixture; 0.1% Butyl PBD in toluene: Triton X-100, 2:1. The remainder of the supernatant was removed and the precipitate washed at least three times in distilled water. The precipitate was then dissolved in 2.0 ml. (whole discs) or 1.0 ml. (half discs) of 6N hydrochloric acid and 0.2 ml. of this solution together with 0.3 ml. water added to liquid scintillation vials containing 10 ml. of the Triton/Butyl PBD-toluene cocktail. All liquid scintillation vials were shaken until a clear emulsion was formed and counted on a 'Tracerlab' or Packard 'Tri-Carb' liquid scintillation counter adjusted for 14C counting. Counting efficiencies were determined with mock standards of 14C hexadecane (Calibrated
standard, supplied by the Radiochemical Centre, Amersham).

In some experiments, 1.0 ml. of the supernatant from the cetylpyridinium chloride precipitate was removed, made acid with 0.5 ml. 1.0 N hydrochloric acid and 3.0 ml. absolute alcohol added. These specimens were kept for at least 15 h. at +4°C and centrifuged at 2,000 g. for 15 min. The precipitate was washed at least three times in 70% ethanol and dissolved in 6M hydrochloric acid. This solution was treated subsequently in the same manner as the solution of the cetylpyridinium precipitate.

Chemical Analysis

0.5 ml. samples of the hydrochloric acid solutions were scaled in ground glass stoppered test tubes, hydrolysed for 4 h. at 95°C, evaporated to dryness over phosphorous pentoxide and sodium hydroxide in vacuo and the residues each dissolved in 5.0 ml. distilled water. Three 1.0 ml. aliquots of each of those solutions were assayed for hexosamine using a micro-modification of the Eleon-Horgan reaction (Kraan & Muir, 1957). Results were expressed as disintegrations/min. (D.P.M.)/mg. hexosamine or as μM SO₄²⁻/mL hexosamine. In some experiments, 0.1 ml. aliquots of the unhydrolysed solution were assayed for uronic acid using the method of Bitter & Muir (1962).
Statistical Treatment of Results

Results are expressed as \( \pm \) one standard deviation from the mean and these values are also entered on the graph. Significant differences between groups of results were assessed by Student's 't' test for small samples where:

\[
t = \frac{\bar{x} - \bar{y}}{\frac{1}{n_1} + \frac{1}{n_2}}
\]

and

\[
s = \frac{(x - \bar{x})^2 + (y - \bar{y})^2}{n_1 + n_2 - 2}
\]

\( \bar{x} \) and \( \bar{y} \) are the means of two different groups of results consisting of \( n_1 \) observations of \( x \) and \( n_2 \) observations of \( y \) respectively.

The regression lines of the graphs were calculated from the formula;

\[
b = \frac{(x - \bar{x})(y - \bar{y})}{(x - \bar{x})^2}
\]

where \( b \) is the slope of the line \( \frac{\bar{x}}{y} \). The displacement of the line from the origin was then calculated from;

\[
a = \bar{y} - bx
\]

The standard deviation of \( y \) from the regression line is calculated from;

\[
s = \frac{1}{n - 2} \left( (y - \bar{y})^2 - (x - \bar{x})(y - \bar{y})^2 \right) \frac{1}{(x - \bar{x})} \]

and the standard deviation of the slope of the line from;
where \( s \) is calculated from (5). The correlation coefficient, \( r \), was given by:

\[
 r = \frac{(x - \bar{x})(y - \bar{y})}{(x - \bar{x})^2 (y - \bar{y})^2} \]

and the significance was assessed by the 't' test;

\[
 t = r \frac{n-2}{1 - r^2} \]

Regression coefficients, \( b_1 \) and \( b_2 \) were also compared by the 't' test;

\[
 t = \frac{b_1 - b_2}{s \frac{1}{(x - \bar{x}_1)^2} + \frac{1}{(x - \bar{x}_2)^2}} \]

where \( x_1 \) and \( x_2 \) are derived from the two different sets of data and \( \bar{x}_1 \) and \( \bar{x}_2 \) are their respective means and

\[
 s = \frac{(n_1 - 2)s_1^2 + (n_2 - 2)s_2^2}{n_1 + n_2 - 4} \]

Probabilities were assessed for 't' in equations (1) and (8) by reading to \( n - 2 \) degrees of freedom and for 't' in equation (10) by reading to \( n - 4 \) degrees of freedom.

RESULTS

a) Mechanical Behaviour of the Discs

Static Loading Experiments
The discs subjected to static loads decreased in thickness until equilibrium was reached. This process of consolidation was normally completed within 90 minutes. The thickness and weight of the specimens at equilibrium decreased with increasing pressure. Some characteristic consolidation curves are shown in Fig. IV-2 and the relationship between the thickness and weight at equilibrium is shown in Table IV-1.

The final thickness to which the cartilage could be compressed reached a constant value of around 0.55 of the original thickness at pressures over 10 kg./cm$^2$. The weight of the specimens showed a similar tendency. The time taken to remove the cartilage from its container and to weigh and measure it was less than three minutes. The amount by which cartilage can recover in this time if it is allowed to swell without applied pressure is less than 2% of the original thickness. Evaporation during weighing was less than 1%. After the cartilage had been replaced in its dish and the pressure re-applied, equilibrium was regained within 20 minutes.

Experiments with Fluctuating Loads

The cartilage decreased in thickness while pressure was applied and recovered measurably when the pressure was removed. The size of each fluctuation, and the average thickness of the cartilage rapidly decreased with time. A steady state was eventually reached. At this stage, the compression
VARIOUS SIZES

3.2 kg.cm⁻²

6.0 kg.cm⁻²

10.3 kg.cm⁻²

60.0 kg.cm⁻²

TIME (Hours)
### TABLE IV-1  PHYSICAL RESPONSE OF CARTILAGE TO STATIC LOADING

<table>
<thead>
<tr>
<th>Load (kg.cm$^{-2})$</th>
<th>Final Thickness</th>
<th>Final Weight</th>
<th>Time to reach Equilibrium (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Thickness</td>
<td>Initial Weight</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>0.88 ± 0.02</td>
<td>0.90 ± 0.01</td>
<td>65 ± 15</td>
</tr>
<tr>
<td>5.9</td>
<td>0.75 ± 0.03</td>
<td>0.70 ± 0.03</td>
<td>105 ± 15</td>
</tr>
<tr>
<td>10.0</td>
<td>0.67 ± 0.04</td>
<td>0.69 ± 0.02</td>
<td>105 ± 15</td>
</tr>
<tr>
<td>16.9</td>
<td>0.67 ± 0.04</td>
<td>0.65 ± 0.04</td>
<td>95 ± 15</td>
</tr>
<tr>
<td>61.6</td>
<td>0.54 ± 0.01</td>
<td>0.55 ± 0.07</td>
<td>92 ± 15</td>
</tr>
</tbody>
</table>

Each result is the mean of four observations ± standard deviation.
during loading was exactly equalled by the expansion when the
load was removed. The size of the fluctuations in thickness
at this stage, were proportional to the log. of the frequency
with which the load was applied. (Fig. IV-3). Characteristic
recorder traces are reproduced in figures IV-4 & IV-5. The
steady state was reached more rapidly than in samples subjected
to constant loads. No correlation could be established between
frequency and the time taken to reach equilibrium.

The final thicknesses and weights of the
discs were higher than those recorded for discs subjected to the
same loads applied continuously. A pressure of $50 \text{ kg. cm.}^{-2}$, if
applied steadily compressed the discs of cartilage to 0.55 of
their original thicknesses. If the same pressure were applied
intermittently the equilibrium thicknesses of the specimens lay
between 0.67 and 0.82 of the original thickness. The equilibrium
thicknesses and the weights of the specimens did not appear to
be influenced by the frequency of the loading within the range
studied. These data are summarised in Table IV-2.

b) Incorporation of $^{35}$S Sulphate

Incorporation into the Cetylpyridinium Chloride Precipitate (PI)

All the uronic acid and approximately 0.75 of
the hexosamine was precipitated by the 2% cetylpyridinium chloride
solution. The molar quantities, expressed as μg. mg. dry weight
of the original cartilage for each precipitate are shown in Table IV-3.
The ratio of hexosamine to uronic acid was approximately 0.81:1.00
in the cetylpyridinium precipitate, and greater than 14:1 in the
FIG. IV-3: RELATIONSHIP BETWEEN FREQUENCY OF LOADING AND VARIATION IN THE THICKNESS OF CARTILAGE AT EQUILIBRIUM.

(Load equals 50 kg/cm² in each case)
FIG. IV-4: MECHANICAL RESPONSE OF CARTILAGE TO A LOAD OF 50 kg·cm⁻²

APPLIED WITH A SQUARE WAVE FREQUENCY OF 0.05 c/s.

THICKNESS OF DISC: mm. or \( \frac{\text{Final Thickness}}{\text{Initial Thickness}} \)

TIME (Minutes)
FIG. IV-5: MECHANICAL RESPONSE OF CARTILAGE TO A LOAD OF 50 kg.cm⁻²

APPLIED WITH A SQUARE WAVE FREQUENCY OF 0.2 c/s.
The rate of incorporation of the radioactive sulphate into PI was approximately linear for periods of up to 4 h. The rate of sulphate incorporation varied markedly between samples of cartilage from different goats, and was decreased by up to 30% by overnight storage at +4°C. Little difference was noted, however, between samples taken from different sites on the same humeral head or from both humeral heads in the same animal. Fig. IV-6 shows some typical results. Rates of sulphate incorporation varied between 500 and 1,800 pM sulphate/M hexosamine over the 4 h. period.

**Incorporation into the Alcohol Precipitate (PII)**

Small amounts of activity were associated with the alcohol precipitate. It was not possible however, to show a steady increase with time.

**Uptake into the Supernatant**

The bulk of the radioactivity extracted from each labelled disc remained in the supernatant and could not be precipitated either by 2% cetylpyridinium chloride or by 66% ethanol. In most experiments, the supernatant from the cetylpyridinium precipitation was counted. There was no significant decrease in the amount of radioactivity recovered in the supernatant after removal of the alcohol precipitate. Radioactivity was expressed as D.P.M./mg. dry weight of the tissue before digestion. The radioactivity in the supernatant
TABLE IV-2  PHYSICAL RESPONSE OF CARTILAGE TO FLUCTUATING LOADS.

All the specimens were subjected to an intermittent load of 50 kg cm⁻².

<table>
<thead>
<tr>
<th>Frequency cycles/sec. (Square wave)</th>
<th>Final Thickness Initial Thickness</th>
<th>Final Wt. Initial Wt.</th>
<th>Variation in Thickness at Equilibrium</th>
<th>Time to reach equilibrium (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.71 ± 0.05</td>
<td>0.75 ± 0.04</td>
<td>0.0089 ± 0.0016</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>0.05</td>
<td>0.77 ± 0.06</td>
<td>0.82 ± 0.05</td>
<td>0.0070 ± 0.0020</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>0.10</td>
<td>0.77 ± 0.05</td>
<td>0.79 ± 0.05</td>
<td>0.0049 ± 0.0010</td>
<td>55 ± 15</td>
</tr>
<tr>
<td>0.20</td>
<td>0.75 ± 0.04</td>
<td>0.80 ± 0.05</td>
<td>0.0027 ± 0.0008</td>
<td>19 ± 5</td>
</tr>
</tbody>
</table>

*Expressed as proportions of the initial thickness.

Each result is the mean of eight observations ± standard deviation.
TABLE IV-3:
THE COMPOSITION OF THE GLYCOSAMINOGLYCAN PRECIPITATES.

<table>
<thead>
<tr>
<th></th>
<th>Uronic Acid *</th>
<th>Hexosamine *</th>
<th>Hexosamine/ Uronic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>P I (C.P.C.)</td>
<td>0.118 ± 0.019 (6)</td>
<td>0.096 ± 0.020 (16)</td>
<td>0.81</td>
</tr>
<tr>
<td>P II (EtOH)</td>
<td>&lt;0.0025 (6)</td>
<td>0.036 ± 0.012 (16)</td>
<td>&gt;14</td>
</tr>
</tbody>
</table>

* Expressed as µM Hexosamine or Uronic Acid/mg. Dry wt.

+ Molar ratio.
FIG. IV-6: INCORPORATION OF SULPHATE INTO THE CETYLPYRIDINIUM PRECIPITATE BY A SERIES OF UNLOADED SPECIMENS OF ARTICULAR CARTILAGE.
rose to a constant level during the first hour of exposure to
the labelled medium. No significant change with time could
be detected thereafter (Fig. IV-7).

The Effects of Freezing

Discs of cartilage which had been frozen
before they were incubated in the presence of $^{35}$SO$_4$— incorporated
less than 0.05 as much sulphate into PI, as unfrozen specimens
over a period of 4 h. The amount of sulphate in the supernatant
however, was unaffected by freezing the disc before incubation.

The Effects of Static Load on the Incorporation of Sulphate

The incorporation of sulphate into the
cetylpyridinium precipitate was markedly reduced by static
loading. The uptake of sulphate into this fraction decreased
rapidly with increasing load and in cartilage compressed by
loads greater than 15 kg. cm.$^{-2}$ was almost entirely inhibited.
The porous metal disc did not significantly decrease the rate of
sulphate incorporation in the absence of applied pressure.
Results were expressed as ratios between the 'specific activities'
of the PI fractions of compressed and uncompressed specimens
(Fig. IV-8). The inhibition of sulphate incorporation into PI
was related to the amount by which the cartilage had been compressed.
If compression were expressed as a ratio between the initial
and equilibrium weights of the disc, and the inhibition ratios
were plotted on a logarithmic axis, the relationship could be
shown to be exponential (Fig. IV-9). The correlation coefficient,
r, for this relationship was 0.93 and the probability, p, of its
FIG. IV-7: UPTAKE OF SULPHATE INTO THE CETYLPYRIDINIUM SUPERNATANT BY A SERIES OF UNCOMPRESSED SPECIMENS OF ARTICULAR CARTILAGE.
FIG. IV-8: THE EFFECTS OF STATIC LOADING ON THE INCORPORATION OF SULPHATE INTO THE CETYLPYRIDINIUM PRECIPITATE BY ARTICULAR CARTILAGE.
being a random distribution less than 0.001. The formula for the regression line is:

$$\log_{10} I = (5.10 \pm 0.3)T - 1.19$$

Activity of compressed specimens

where $I = \text{Activity of control specimens}$

Initial weights of specimens

and $T = \text{Weight at equilibrium}$

**Uptake into Supernatant**

The supernatant fraction from specimens which had been compressed also contained less radioactivity than the supernatant from uncompressed controls. The decrease in radioactivity was much less, however, than the corresponding decrease in the activity of PI. In specimens for example, compressed to 0.60 of their original thickness by a pressure of 17 kg.cm$^{-2}$ the activity of the supernatant was 0.4 that of uncompressed controls. The activity of the cetylpyridinium precipitates from these specimens, however, showed only 0.05 of the activity of the same fraction from uncompressed specimens. (Fig. IV-10) shows the results of a typical experiment in which the rates of uptake into the cetylpyridinium precipitates and supernatants of a series of specimens were studied. Uptake into the supernatant from the compressed specimens appeared to be complete within 30 min.

The activity in the supernatant at equilibrium was directly proportional to the amount by which the disc had been
FIG. IV-9: THE INHIBITION OF SULPHATE INCORPORATION INTO
THE CETYLPYRIDINIUM PRECIPITATE EXTRACTED FROM
SPECIMENS COMPRESSED BY CONTINUOUS LOADS.

- Best straight line
- Standard deviation

SO^4^- UPTAKE : Compressed Disc / Uncompressed Disc

COMPRESSION : \[
\frac{\text{Final Weight}}{\text{Initial Weight}}
\]
INCORPORATION OF SULPHATE BY ARTICULAR CARTILAGE.

Supernatant; Unloaded Discs

CPC Precipitate; Unloaded

Loaded

Loaded

TIME (Hours)
compressed (Fig. IV-11). The correlation coefficient $r$, was 0.85 and the probability, $p$, of a random distribution less than 0.001. If the calculated regression line is extrapolated to the point at which \[ \frac{\text{Activity of compressed specimens}}{\text{Activity of control specimens}} \] is equal to zero, a value $0.265 \pm 0.06$ is obtained on the compression axis. This figure is within the range of values of 

\[ \frac{\text{Dry weight}}{\text{Fresh weight before compression}} = 0.235 \pm 0.05, \] calculated for the same series of specimens.

The Effects of Fluctuating Loads

Fluctuating loads also caused a decrease in the uptake of sulphate, both into the cetylpyridinium precipitate and into its supernatant. The incorporation of sulphate into the cetylpyridinium precipitate from discs subjected to a particular load applied intermittently was greater than if the same load was applied continuously. Fig. IV-12 shows the results obtained from a series of specimens subjected to a load of 50 kg. cm$^{-2}$ applied with a square wave frequency of 0.1 cycles sec$^{-1}$.

The frequency with which the load was applied had little effect on the sulphate uptake, provided that the maximum load was the same (Table IV-4). Loads applied with half-sinusoidal fluctuations inhibited sulphate uptake by an even smaller extent. In these experiments, the incorporation of sulphate into the cetylpyridinium precipitate was only slightly below the control values.
Fig. IV-11: The effects of compression on the uptake of sulphate into the cetylpyridinium supernatant.

- Best straight line
- Standard deviation

Compression: $\frac{\text{Final Weight}}{\text{Initial Weight}}$
FIG. IV-12: THE EFFECTS OF AN INTERMITTENT LOAD OF 50 kg.cm⁻² APPLIED WITH A FREQUENCY OF 0.1 c.s⁻¹ ON THE INCORPORATION OF SULPHATE INTO THE CETYLPYRIDINIUM PRECIPITATE.

Unloaded Specimens

Loaded Specimens

TIME (Hours)
TABLE IV-4:

THE EFFECTS OF FREQUENCY OF LOADING ON THE UPTAKE OF SULPHATE INTO THE CETYLPYRIDINIUM PRECIPITATE.

<table>
<thead>
<tr>
<th>Frequency (c/s)</th>
<th>$\text{SO}_4^-$ Uptake Compressed Disc</th>
<th>Uncompressed Disc</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 *</td>
<td>$0.25 \pm 0.05$ (6)</td>
<td></td>
</tr>
<tr>
<td>0.02 *</td>
<td>$0.38 \pm 0.21$ (6)</td>
<td></td>
</tr>
<tr>
<td>0.10 *</td>
<td>$0.30 \pm 0.09$ (6)</td>
<td></td>
</tr>
<tr>
<td>0.10 +</td>
<td>$0.76 \pm 0.18$ (6)</td>
<td></td>
</tr>
</tbody>
</table>

* Square Wave  + Half Sine Wave
The amounts by which the specimens were compressed by the fluctuating loads were less than by the same loads applied continuously. Inhibition of uptake into the cetylpyridinium precipitate bore a similar logarithmic relationship to the amount by which the specimen had been compressed (Fig. IV-13). The correlation coefficient of this relationship was 0.55 and the probability of its being a random distribution, between 0.05 and 0.02. The slope of the regression line for the intermittently loaded specimens was over twice the coefficient calculated for the continuously loaded ones (p 0.001). The formula of this regression line is:

\[ \log_{10} I = (6.27 \pm 0.31) T - 3.65 \ldots (2) \]

where \( I \) and \( T \) have the same meaning as in (1).

**Uptake into the Supernatant**

The uptake into the supernatant from the cetylpyridinium precipitate showed a similar relationship to compression as uptake into the supernatant from the continuously loaded cartilage. The activity in the supernatant at equilibrium was directly proportional to the amount by which the cartilage had been compressed. There was no significant difference in this relationship between specimens which had been continuously or intermittently loaded. The results are shown on the same graph (Fig. IV-11). The value of the correlation coefficient, \( r \), was 0.85 and the probability, \( p \), of its being a random distribution less than 0.001. The formula of the regression line is

\[ I = (1.4 \pm 0.20) - 0.33. \]
Uptake into the Ethanol Precipitate

No significant difference between loaded and the unloaded specimens could be demonstrated.

Recovery from Compression

In some experiments, specimens of articular cartilage were compressed continuously by a load of 15 kg.cm$^{-2}$ for different lengths of time and then transferred to the radioactive medium for two hours. The uptake into the cetylpyridinium precipitate during this period was slightly under half that of specimens which had not been compressed (Fig. IV-14). There was no significant difference between the uptakes of specimens which were compressed for different periods of up to two hours. After four hours compression the specimens incorporated 0.3(± 0.08) of the activity incorporated by uncompressed specimens. This result was significantly different to the uptakes of the other compressed specimens at the 5% level. The label acquired during the recovery period was at least ten time greater than the labelling of specimens compressed at 15 kg.cm.$^{-2}$ for the same periods of time.
SULPHATE INTO THE CETYLPYRIDINIUM PRECIPITATE BY ARTICULAR CARTILAGE.

- Best straight line
- Standard deviation

**Compression** : \( \frac{\text{Final Weight}}{\text{Initial Weight}} \)
FIG. IV-14: INCORPORATION OF SULPHATE BY CARTILAGE WHICH HAD BEEN COMPRESSED FOR VARIOUS PERIODS OF TIME BY A CONTINUOUS LOAD OF 15 kg cm^-2

TIME OF COMPRESSION (Hours)
DISCUSSION

Mechanical Results

The mechanical response of the cartilage discs to continuous loading resembled the behaviour observed by Edwards (1967, 1970), although the period taken for the cartilage to reach equilibrium was rather longer. The experiment of Edwards, however, used adult dog cartilage and these data may represent a species difference. The loss in weight of the cartilage, during consolidation was accounted for almost entirely by the liquid expelled from it. The proportional decreases in weight and thickness of individual discs agree within ± 2%.

The response of the cartilage to intermittent loading is similar to the behaviour expected from the theory concerning the behaviour of continuously loaded cartilage. The times taken for continuously compressed cartilage to reach a constant thickness, and the time taken for cartilage which had been compressed to recover its original thickness are both over 60 minutes. It is hardly surprising, therefore, that cartilage when subjected to intermittent loads at frequencies greater than 0.01 cycles sec.\(^{-1}\) showed fluctuations of less than 10% of the thickness before compression.

The much smaller amounts of consolidation caused by loads being applied intermittently rather than continuously is undoubtedly due to the tendency of the cartilage to swell while the load was removed. The equilibrium thickness and weight are difficult to measure accurately as the cartilage
begins to swell as soon as the load is removed. The maximum
time between removal from the apparatus and weighing and measuring
was however less than three minutes. The cartilage swells by less
than 5%, during this time. The differences observed between
weights of specimens subjected to continuous and intermittent
loads, and between most of the different continuous loads were
significant. There was no significant difference in equilibrium
weight or thickness, between specimens which had been subjected
to fluctuating loads of the various frequencies studied.

The frequencies of the loading cycles chosen
lay between 0.01 and 0.5 cycles/sec. These probably represent
the lower limits of the physiological range in the live animal.
They would correspond to an animal moving around slowly or
standing in one position, shifting most of its weight from one
leg to another. This type of movement would probably be made by
a grazing animal. Experiments with higher frequencies were
precluded by the frequency response of the current-pressure
transducer.

The maximum load used (50 kg.cm⁻²) probably
represents the upper physiological limit of a large goat taking
most of its weight on one leg. A large animal weighs about
70 kg. and probably has a contact area in the humeral head of
approximately 1 cm².

Characterisation of the Glycosaminoglycan Fractions

The hexosamine/uronic acid ratio of 0.31 ± 0.2
for the cetylpyridinium chloride precipitate suggests that
chondroitin sulphate accounted for most of the hexosamine in it. The ratio of 0.81 ± 0.2 is not significantly different from 1.00 which is the molar ratio expected if chondroitin sulphates were the sole glycosaminoglycans. This result agrees with the observations of Scott, 1960, 1961 that an excess of cetylpyridinium chloride did not precipitate keratan sulphate. Although keratan sulphate can be precipitated by specific concentrations of cetylpyridinium chloride and high ionic strengths, the procedure is not reproducible (Scott, 1960, 1961). Attempts were made to precipitate the keratan sulphate with 66% ethanol and a fraction was obtained which contained hexosamine, but no detectable uronic acid (Table IV-3). It accounted for approximately 35% of the total hexosamine. The radioactivity incorporated into this fraction was small and no significant comparisons could be made between discs, subjected to the different treatments. No detectable hexosamine remained in the supernatant, after the ethanol and cetylpyridinium precipitates had been removed.

Sulphate uptake into the ethanol precipitate was not measured in most experiments, and this fraction was not prepared. Of the remaining two fractions, the supernatant from the cetylpyridinium precipitate contained most of the activity. The amount of this activity attributable to unprecipitated keratan sulphate was probably negligible. The linear relationship with the amount by which the cartilage had been compressed suggested that the bulk of this activity was accounted for by unbound sulphate. This suggestion was corroborated by the observation
that the extrapolated regression line gave a compression value not significantly different from the dry weight at the point at which sulphate uptake was equal to zero. The rate at which the supernatant became labelled suggests a process of diffusion into the cartilage. The rate of diffusion did not appear to be slowed appreciably in the compressed specimens. The process of diffusion into both compressed and uncompressed specimens appeared to be complete within one hour. The rate of uptake into the supernatant was not affected by freezing and thawing. Freezing and thawing in the absence of protective agents destroys the cell (Smith, 1961). These data suggest therefore, that no active cellular transport or synthetic process was involved.

In contrast, the uptake of sulphate into the cetylpyridinium precipitate was reduced almost to zero by freezing. These data imply that sulphate incorporation into the chondroitin sulphate was dependent on living cells. The logarithmic relationship between sulphate incorporation into chondroitin sulphate and the amount to which the specimen was compressed is of particular interest. Chondroitin sulphate synthesis does not appear to be dependent on the rate of diffusion of sulphate into the cartilage. There seems to be much more unbound sulphate than bound sulphate in the compressed cartilage at all times during the four hour period. The reduction in sulphate incorporation is also much greater than the reduction in unbound sulphate and it bears a different relationship to the amount of compression. Cell death seems unlikely to provide an
explanation as the cartilage is able to recover a considerable part of its ability to label chondroitin sulphate even after four hours continuous compression by loads sufficient to stop synthesis almost completely. These data agree with the findings of Edwards and Smith (1966) that 95% of the chondrocytes isolated from cartilage which had been compressed in a similar apparatus were alive.

The inhibition of sulphate incorporation by compression of cartilage was shown in the presence of both Ringer's solution and medium 199. Medium 199 contains all the amino acids and most of the other low molecular weight compounds present in serum. It seems unlikely therefore that the diffusion or synthesis of amino acids or glucose is the limiting factor. The lack of a significant difference between sulphate incorporation of uncompressed discs incubated in Ringer's solution or medium 199 suggests that under the conditions of the experiment there are probably enough of the low molecular weight precursors within the cell or the matrix to last for four hours. The possibility that oxygen tension was the limiting factor could not however be excluded.

The compression of the cartilage results in an effective increase in the chemical concentration of the matrix and it is conceivable that the chondroitin sulphate synthesis may be inhibited by some feedback effect. Alternatively the freshly synthesised protein-polysaccharides may be less able to diffuse away from the cell and the resultant local increase
in their concentration may inhibit synthesis. The difference in the regression coefficients between the continuously loaded and intermittently loaded cartilage may be important in this respect.

Autoradiographs of the loaded cartilage were prepared to confirm these data, and to see whether sulphate incorporation was inhibited in more or less different zones of the cartilage. Samples for autoradiography were taken in most experiments, from the same discs as were used for the biochemical studies. Sections from the same joints as those used in the biochemical studies were also examined histologically. This study was undertaken to ensure that the cartilage showed no signs of disease and to assess the maturity of the animals by seeing how much growth plate remained.
CHAPTER 5

HISTOLOGY AND AUTORADIOGRAPHY OF COMPRESSED CARTILAGE

Method

Discs of cartilage were cut from the humeral heads of recently killed adult goats. They were then incubated at +36°C in Locke's mammalian heart Ringer solution modified by the omission of non-radioactive sulphate, but containing 5 μCi/ml. $^{35}$S sodium sulphate (Specific activity 40 - 60 mCi/mmol, Radiochemical Centre, Amersham). Some of the discs were simultaneously subjected to various types of compressive treatment in the radioactive medium. The apparatus and methods used are described in the previous chapter. Some discs were frozen and thawed several times before incubation. The cartilage discs were halved in some experiments, and one piece used for the biochemical measurements and the other for autoradiography.

The samples for autoradiography were washed briefly in three changes of 0.9% w/v sodium chloride solution and fixed for at least 48 h. in 5 ml. of 10% neutral buffered formal saline (9 g. NaCl, 4 g. NaH$_2$PO$_4$.H$_2$O, 6.5 g. Na$_2$HPO$_4$, 100 ml. 40% formaldehyde per litre pH 7.2). The fixed specimens were dehydrated in increasing concentrations of ethanol in water, cleared in chloroform and embedded under vacuum in fibrowax at +60°C. The wax blocks were mounted, trimmed and cooled to +4°C. At least fifteen transverse sections were then cut to a nominal thickness of 5μ using a Jung rotary microtome. The sections were floated out on a water bath at +60°C and mounted
onto alcohol washed slides, frosted at one end. The slides were heated, with the sections downward, on a hotplate set to +65°C until the sections had completely flattened out. They were washed in three changes of xylene and one of absolute alcohol and then brought to water. The rehydrated sections were washed for at least 30 minutes in running tap water and in two changes of distilled water. The sections were then coated with Ilford K5 nuclear emulsion (Ilford Ltd., Ilford, Essex), melted at +45°C and diluted 1:1 v/v with distilled water which had been heated to the same temperature. The slides were coated with the emulsion in a dark room illuminated only by red light (Wratten No. 1, filter, Kodak Ltd., Hemel Hempstead, Herts.). The slides were allowed to dry in a vertical position at +45°C and packed into light tight boxes. The autoradiograms were exposed for 3 - 4 weeks at +4°C.

After exposure, the autoradiographs were developed for 12 minutes in Ilford 'Phen-X' developer diluted 1:1 (v/v) at 18-20°C. They were washed in running tap water for 30 sec. and then fixed for 5 minutes in Ilford 'Hypan' fixer diluted 1:4 (v/v). This entire sequence of operations was performed in the darkroom illuminated by red light and the slides only exposed to white light when photographic fixation was complete.

The sections were washed in running tap water for at least 30 minutes. They were then stained in Cole's haematoxylin and counterstained in 1% w/v aqueous eosin (C.I.45380),
dehydrated in alcohol and mounted in D.P.X. mounting medium.
The sections were examined, after the mounting medium had dried, and photographed with a Photomicroscope II (Carl Zeiss, Oberkochen, W. Germany).

Histology of Bone and Cartilage

Vertical wedges were cut from the humeral heads. The base of the wedges was about 1 cm. x 1 cm. and the sides were approximately 1 cm. These samples were cut to include, where possible, areas of intact cartilage, areas of cartilage from which discs had been cut, the underlying bone and any growth plate. The wedges were fixed in neutral buffered formal saline at +4°C for at least four days. They were then decalcified in a solution containing 2% formic acid (v/v) and 20% sodium citrate (v/v) for 10-12 days. The decalcified tissue was dehydrated, embedded in fibrowax and sectioned by the method already described. The sections were mounted on plain slides and brought to water. Some sections were stained with Coel's haematoxylin and counterstained with eosin. Other sections were stained with alcian blue (C.I. 74240) in 10% acetic acid, pH 1-2 and counterstained with neutral red (C.I. 50040) (Bancroft, 1967). Sections were also stained by the periodic acid-Schiff technique and counterstained with Coel's haematoxylin (Bancroft, 1967). The stained sections were dehydrated, cleared, and mounted in D.P.X.
RESULTS

Morphology

e) Sections of Humeral Head

The articular cartilage in the sections of humeral head appeared normal. The articular surface was smooth in all the specimens studied, and showed no signs of degenerative joint disease. The sections stained with haematoxylin and eosin showed few pyknotic nuclei, even if the specimen had been stored overnight at +4°C before fixation. Most of the cells in the lower regions of the cartilage were arranged in vertical columns. In these decalcified specimens, stained with haematoxylin and eosin, the calcified layer was indistinct and difficult to identify. In some areas, the articular cartilage appeared to be continuous with the matrix of the underlying bone. In other places, calcified cartilage appeared to have been adjacent to the marrow cavities (Plate V-1b). The chondrocytes of the superficial layer were mostly flattened and arranged parallel to the articular surface (Plate V-1a).

The matrix stained well with both alcian blue (Fig. V-2a) and with P.A.S. (Fig. V-2b). The alcian blue staining of the matrix was most intense in the regions of cartilage slightly below the articular surface whereas the P.A.S. staining was more intense in the deeper zones. Some chondrocytes in the calcified layer appeared to be immediately surrounded by areas of intense P.A.S. staining. There were similar zones of intense alcian blue staining around some chondrocytes in the intermediate
layer. The cytoplasm of some chondrocytes appeared to be P.A.S. positive. These cells were more prevalent and their staining more intense in the calcified and basal layers.

Remnants of epiphyseal growth plate were noted in most of the specimens of humeral head examined. Fig. V-3 shows a low power view of a typical section of humeral head from a female goat, judged to be sexually mature from the appearances of the udder and vagina.

b) Sections of Cartilage Discs

Examination of sections of the cartilage discs revealed that the cartilage had been cut slightly above the calcified layer. In the sections from the majority of superficial, intermediate, and most of the basal zone appeared to be undamaged. The uncompressed discs retained the histological appearances of intact cartilage. Few pyknotic nuclei were seen, even in sections of specimens which had been stored overnight, and then incubated for four hours in Ringer's solution at 37°C before fixation.

c) Compressed Discs

The columns of chondrocytes, which were seen in the uncompressed discs, were less obvious after the cartilage had been subjected to fluctuating loads. The articular surfaces of some discs had become wrinkled, although the surfaces of nearly all the discs examined, remained intact after loading. These changes were less apparent in discs which had been loaded
PLATE V-1

a) Top; Superficial and intermediate zones of goat humeral head articular cartilage. Haematoxylin & Eosin, x 340.

b) Bottom; Calcified zone of articular cartilage bordering a marrow cavity. H. & E. phase contrast, x 340.
Plate: V-1
PLATE V-2

Goat humeral head articular cartilage;

a) Top; Alcian blue X 105

b) Bottom; P.A.S. X 105
Plate V-2
Vertical section through part of a goat humeral head. P.A.S. x 27. In addition to the articular cartilage and cancellous bone, some growth plate is present. The growth plate stains strongly with P.A.S.
continuously. Pyknotic nuclei were rare, even after large static loads had been applied to the cartilage for four hours.

**Autoradiography**

Discs which had been incubated for thirty minutes without compression showed the first signs of uptake of the isotope (Plate V-4a). The labelling at first appeared to be mainly associated with the cells. Within one hour however, it had spread to the matrix immediately around the cells (Plate V-6a). Many silver grains could be seen over both the cells and the matrix in autoradiographs of cartilage which had been exposed to the isotope for two hours (Plate V-5, V-7).

The cells in the basal and intermediate layers were the first to show sulphate uptake and these cells remained the most heavily labelled even after four hour's incubation (Plate V-10a). Few cells in the superficial layer showed appreciable sulphate incorporation if the disc had been incubated for less than two hours.

**The Effects of Static Loads**

Autoradiographs from discs which had been compressed by a static load of .5 kg./cm.² showed little sulphate uptake. Even after two hours, labelling was restricted to a few cells of the basal and intermediate zones. Hardly any silver grains appeared over the cells or matrix of the superficial zone. Plates V-4, V-6, V-7 compare autoradiographs prepared from discs which had been incubated for various periods of time.
Autoradiographs of articular cartilage incubated for 30 minutes in the presence of $^{35}$S$_4$O$_4$ (5 μCi/ml. in Ringer's solution), H.& E. X 265.

a) Top; Uncompressed

b) Bottom; Compressed by a static load of 5 kg.cm$^{-2}$
Autoradiographs of articular cartilage; H.& E. X 1,7C
Chondrocytes of the basal zone in a disc which had been incubated for 2 h. in 5μCi/ml. $^{35}$SO$_4^{--}$ without compression.
The two illustrations are of the same area with the microscope focused in two different planes to reveal the outlines of the cells (Top) or most of the grains in the emulsion (Bottom).
Autoradiographs of articular cartilage incubated for 1 h. in the presence of \( {^{35}}\text{SO}_4^{--} \) (5 \( \mu \text{Ci/ml.} \) in Ringer's solution), H. & E X 265.

a) Top; Uncompressed.

b) Bottom; Compressed by a static load of 5 kg.cm\(^{-2}\).
PLATE V-7

Autoradiographs of articular cartilage incubated for 2 h. in the presence of $^{35}$SO$_4^{-}$ (5 µCi/ml. in Ringer solution), H.& E. X 265.

a) Top; Uncompressed.

b) Bottom; Compressed by a static load of 5 kg. cm.$^{-2}$
Plate: V-7
with or without static loading.

The Effects of Fluctuating Loads

Discs subjected to fluctuating loads of 50 kg/cm² also showed decreased sulphate uptake as compared with their uncompressed controls. Appreciable activity had, however, become associated with the cells of the intermediate and basal zones after one hour. The labelling became more intense if the incubation was further prolonged, and after four hours, even the chondrocytes of the superficial layer showed some signs of having taken up sulphate. Much more sulphate appeared to have been incorporated by these intermittently loaded discs than by samples which had been subjected to the far smaller static load for the same periods of time. Plates V-8, V-9, V-10 illustrate autoradiographs prepared from discs which had been intermittently loaded for various periods. They are compared with appropriate, uncompressed controls. The samples illustrated were taken from discs used in the series of experiments summarised in Fig. IV-12. These autoradiographs should therefore be directly comparable with the biochemical data.

Some of the cells, even in the basal and superficial zones of discs which had been compressed in either manner remained unlabelled after 4 h. By contrast, nearly all the cells in the uncompressed discs showed uptake after this time.
PLATE V-8

Autoradiographs of articular cartilage incubated for 1 h. in the presence of $^{35}$SO$_4^-$ (5 µCi/ml. in Ringer's solution), H. & E. X 265.

a) Top; Uncompressed.

b) Bottom; Compressed by a fluctuating load of 50 kg. or applied with a frequency of one cycle every 10 sec. (Square waveform).
Plate: V-8
PLATE V-9

Autoradiographs of articular cartilage incubated for 2 h. in the presence of $^{35}\text{SO}_4^{--}$ (5 µCi/ml. in Ringer solution) H. & E. X 265.

a) Top; Uncompressed.

b) Bottom; Compressed by a fluctuating load of 50 kg, applied with a frequency of one cycle every 10 sec (Square waveform).
PLATE V-10

Autoradiographs of articular cartilage incubated for 4 h. in the presence of $^{35}$SO$_4$$^-$ (5 μCi/ml. in Ringer solution), H.& E. X 265.

a) Top; Uncompressed.

b) Bottom; Compressed by a fluctuating load of 50 k applied with a frequency of one cycle every 10 sec (Square waveform).
Frozen Discs

Most cells appeared degenerate in the discs which had been frozen before incubation. There was no significant labelling in the autoradiographs. Even those from specimens which had been incubated for 4 h. remained unlabelled. (Fig. V-11).

DISCUSSION

Morphology

a) Sections of Humeral Head

The smooth articular surface, and the ability of the matrix to stain with alcian blue, even in the superficial layers indicates that the joints used in these experiments were not affected by degenerative joint disease. The almost total absence of degenerative cells, even in the specimens stored at +4°C suggests that the cells were alive up to the moment of fixation. This finding tallies well with the biochemical finding that sulphate incorporation was only slightly reduced after overnight storage at +4°C.

The location of most of the alcian blue staining in the upper part of the cartilage, and of most of the P.A.S. staining in the calcified layer is of interest. It may reflect the differences in biochemical composition reported by Maroudas et al (1969). The higher intensity of P.A.S. and alcian blue staining in the vicinity of cells may suggest local concentrations of mucopolysaccharide in these areas. The intra-
PLATE V-11

Autoradiographs of cartilage discs which had been frozen and thawed twice before incubation, without compression, for 4 h. in the presence of $^{35}$S$_4$ (5 μCi./ml. in Ringer's solution), H.& E. X 265.
Plate V-11
cellular P.A.S. positive material may be mucopolysaccharide or glycogen. The streaming artefacts associated with these granules suggest that glycogen may have been present (Pearse, 1968). Glycogen granules have been detected by electron microscopy (Chadially & Roy, 1969). They are most common in the chondrocytes of the basal and calcified layers.

b) The Disc

The structural organisation of the matrix and the chondrocytes did not appear to be altered much by dissecting it away from its bony backing. Compression did, however seem to disrupt the columns of chondrocytes which were present in the untreated cartilage. Intermittent loading caused far more alteration than static loading. It would be of interest to know whether the attachment to the bone would prevent this disruption. The infrequency of degenerate cells suggests that cartilage can survive even prolonged loading.

e) The Autoradiographs

The results of autoradiography appear to confirm the biochemical findings of reduced sulphate incorporation in compressed cartilage. The cartilage discs were rinsed with aqueous solutions both before and during histological fixation, and the sections were well washed before they were coated with photographic emulsion. Nearly all the soluble sulphate should be removed by these processes and therefore the observed labelling is probably all associated with insoluble compounds. The lack
of labelling, and the obvious signs of degeneration in the specimens which had been frozen, suggests that incorporation of sulphate is dependent on living cells.

The cells in the lower levels of the cartilage became labelled first, even in the specimens which had been compressed in the steel dish. Diffusion through the bottom and round the sides of the cartilage is probably inhibited under those conditions. It would appear therefore that the differences in the apparent metabolic activity between the cells of the different zones is not dependent on the availability of sulphate.

The rate of incorporation by the cells in the basal and intermediate zones appeared to be decreased in the compressed discs. However, few cells in the intermittently loaded discs ceased incorporating sulphate altogether. The cells which did accumulate sulphate appeared to become more heavily labelled after longer periods of incubation. Some of the cells in the superficial zone became labelled if the incubation time was sufficiently prolonged. It seems unlikely, therefore that the decrease in sulphate incorporation of the cartilage was accounted for by cell death.

Both the biochemical and autoradiographic results suggest that protein-polysaccharide synthesis is inhibited by mechanical loading. The biochemical data indicate that the chondroitin sulphate protein-polysaccharides are principally affected. The autoradiograms support the impression that sulphate
incorporation is not limited by the rate at which sulphate
can diffuse through the cartilage. Experiments were conducted
with cell cultures of isolated chondrocytes to see whether
chondroitin sulphate could inhibit protein-polysaccharide
synthesis. These are described in the next section.
CHAPTER 6
THE ISOLATION AND CULTIVATION OF ARTICULAR CHONDROCYTES

Methods

Intact elbow or knee joints were removed aseptically from adult goats which had been put down with a humane killer. The joints were removed within three hours of the death of the animal and either used immediately or wrapped in aluminium foil and stored at +4°C for not more than 48 h.

The joints were opened in a laminar air flow cabinet which had been sterilised by ultraviolet light. The hyaline cartilage was dissected carefully away from all the articular surfaces of each joint. Care was taken to ensure that no blood or fragments of synovium or subchondral bone were included in the cartilage samples. The freshly dissected cartilage was transferred immediately to a sterile plastic petri dish (5 cm., Sterilin) containing 2 - 3 ml. of the tissue culture medium T.C. 199 (Wellcome Reagents Ltd., Beckenham, Kent). The cartilage was chopped into fragments approximately 2 mm.³

The chondrocytes were freed of their matrix by a minor modification of the method of Smith (1965). All the cartilage collected from each of the joints was placed in a 25 ml. conical 'Quickfit' flask containing 9.0 ml. T.C. 199, 0.2 ml. of a solution containing 2 mg./ml. cysteine, buffered at neutrality with sodium bicarbonate was added. This was followed by 1.0 ml. of a solution of papain (0.1 g./ml., E.C. 3.44.10,
Sigma, type II, 1.6 - 2.8 B.A.E.E.U./mg). Each flask was gassed with a gas mixture consisting of 20% Oxygen, 5% Carbon Dioxide, 75% Nitrogen. The flasks were then incubated for 5 h. at 38°C in a shaking water bath. At the end of this time, the tissue culture medium containing the papain was carefully decanted, and the partially digested fragments of cartilage washed three times in T.C. 199. Finally 10 ml. fresh T.C. 199, containing 2 mg. collagenase (Sigma, type II, 125 - 200 u/mg.) were added to each flask.

The flasks were re-gassed, sealed, and incubated for a further 15 h. After the incubation in collagenase, 0.2 mg. protease (Sigma, Grade IV, 0.7 - 1.0 u/mg.) were added to each flask. The flasks were then gassed and incubated for a further three hours.

At the end of this time the medium from each flask, containing the cell suspension was decanted into a 10ml. conical centrifuge tube. The tubes were closed with silicone rubber stoppers and centrifuged for 10 minutes at 750 g. The cell pellets were resuspended and washed in two changes of T.C. 199 and then combined into one centrifuge tube. The cells were suspended in a total of 4 ml. of modified Bigger's B.G.J. medium (Fitton-Jackson, personal communication, prepared by Wellcome Reagents Ltd.). The ascorbic acid and glutamine were dissolved freshly and the medium was added to the cells through a 25 mm. millipore filter, (0.22μ, GS, white, plain) which had been prewashed with 20 ml. hot distilled water and 10 ml. cold B.G.J. (Cahn, 1967). 5 mg./l. ferrous sulphate were also added
to the medium. All these operations were carried out with full aseptic precautions.

Samples of the cell suspension were mounted on agar coated slides, stained with Euchrysine 3R (Gurr, 1 in 50,000 in T.C. 199) and viewed by transmitted fluorescent illumination using a dark ground condenser. Other samples were stained with methylene blue and the cells counted on a haemocytometer. A total of approximately 0.5 ml. of the cell suspension was used in these two operations. The remainder of the suspension was used to set up tissue cultures.

**Tissue Cultures**

0.5 ml. samples of the cell suspension, containing 1.0-2.5 x 10^6 cells, were spread evenly over the bottoms of 3 cm. plastic petri dishes (Sterilin, pre-sterilised). Two of these 3 cm. dishes were placed inside a 9 cm. plastic petri dish. Alternatively, 0.5 ml. samples of the cell suspension were spread over glass coverslips in Leighton tubes. A further 0.5 ml. of the modified B.C.J. containing 20 mg./l. Nystatin (Sigma, 4,600 USP units/mg.) were added to all the cultures.

The petri dishes were then placed in a McIntosh-Fildes jar together with a 9 cm. petri dish containing distilled water. The McIntosh-Fildes jar was sealed and gassed with the gas mixture (20% Oxygen, 5% Carbon Dioxide, 75% Nitrogen) for 5 - 6 minutes at 2 - 3 l./min. The Leighton tubes were each gassed with the gas mixture for 30 seconds at 100 ml./min. and sealed with silicone rubber bungs. All the cultures were
incubated at 38°C. The medium was changed at least every 40 h. The cells were counted at various times during cultivation, using an inverted microscope fitted with an eyepiece graticule.

**Sulphate Incorporation**

$^{35}$S labelled sodium sulphate, 5 mCi, 40 - 60 mCi./mmol. was obtained as a freeze dried solid from the Radiochemical Centre, Amersham. The whole of this quantity was made up in 100 ml. distilled water and aliquots containing 25 μCi (0.5 ml.) were measured into 5 ml. glass ampoules. The solution was evaporated to dryness in vacuo over phosphorous pentoxide. Each ampoule was sealed and the contents sterilised by heating at 140°C for 90 minutes.

The radioactive sodium sulphate was added to the cultures when they were set up. The contents of each ampoule were dissolved 2.5 ml. of the B.C.J. medium, modified by the addition of 20 mg. Nystatin/l. 0.5 ml. of this radioactive medium was added to 0.5 ml. of the cell suspension in each culture chamber. A sample of the medium was retained for liquid scintillation counting. Cultures were grown in the presence of the radioactive sulphate for four days with one intermediate medium change. The medium removed after two days was stored at +4°C and combined with the medium removed at the end of the culture period.
Chondroitin Sulphate

Chondroitin sulphate was obtained from Sigma (Grade III, extracted from shark and whale cartilage). 100 mg/ml. of this preparation were dissolved in the B.C.J. together with the fresh ascorbic acid and glutamine. In these experiments, the washed cell pellet was suspended in the medium containing the nystatin and radioactive sulphate. The medium containing the freshly dissolved ascorbic acid, glutamine and chondroitin sulphate was then added to the cultures through the prewashed millipore filter.

Measurement of Sulphate Incorporation

The medium was removed from the petri dishes at the end of the period of culture, and the cells were washed from the bottoms of the dishes with several changes of distilled water. The medium and the washings were normally combined with the fluid removed at the intermediate change. In some experiments the supernatant was filtered and the two samples of medium, and the cell washings processed separately. Carrier sodium sulphate (approximately 0.5 g.) was added to each sample and the solutions dialysed for at least 15 h. in running tap water, followed by three, two litre changes of distilled water. Each dialysate was made up to 20 ml. and transferred quantitatively to a 50 ml. centrifuge tube. Approximately 50 mg. carrier chondroitin sulphate were added to the dialysates from the cultures which had not been grown in the presence of chondroitin sulphate. 20 ml. of a solution containing 4% (w/v) cetylepyridinium chloride,
0.15 M sodium borate, and 0.02 M calcium chloride, pH 9.2, were added to each centrifuge tube. The samples were allowed to stand for at least 30 minutes at room temperature. They were then centrifuged at approximately 2,000 x g for 10 minutes and the precipitate washed 5 times in a solution of 0.2% (w/v) cetylpyridinium chloride in distilled water. The precipitate was dissolved in 2.0 ml. 6N hydrochloric acid and duplicate 0.2 ml. samples added together with 0.3 ml. distilled water to liquid scintillation vials containing 10 ml. of the triton, toluene and butyl PBD mixture. The samples were counted on a 'Tracerlab' or Packard 'Tri-Carb' liquid scintillation counter adjusted for 14C. Counting efficiencies were determined using internal mock standards of 14C hexadecane.

Incorporation and Hydroxylation of Proline

L-Proline, uniformly labelled with 14C (50 μCi, 37 mCi/mmol.) was obtained from the Radiochemical Centre (Amersham) as a freeze dried solid. The modified B.G.J.b was prepared by Wellcome Reagents Ltd. (Beckenham, Kent) without proline, and the cultures set up in 5 cm. plastic petri dishes as already described, except that 5μ Ci of the 14C proline, dissolved in the medium was added with the Nystatin.

The cultures were grown in the presence of the 14C proline for four days with one intermediate medium change. At the end of this period the radioactive medium was removed, the cells scraped from the base of the petri dish and the petri dish washed several times with distilled water. The washings and cell
debris were combined with the medium removed both at the end of culture, and at the intermediate medium change. This mixture was homogenised with a hand homogeniser and approximately 10 mg. carrier L-proline (Sigma) added to it. The homogenate was dialysed for at least 15 h. against running tap water, followed by three two litre changes of distilled water. The volume of the dialysate was reduced to not more than three ml. by boiling. An equal volume of concentrated hydrochloric acid was then added. The mixture was sealed in glass ampoules and hydrolysed for 48 h. at 110°C. After hydrolysis, the ampoules were opened and their contents evaporated to dryness at 95°C. The hydrolysate was made up in 2-3 ml. distilled water and re-evaporated to dryness three times to remove the last traces of acid. The hydrolysate was finally made up in 0.1 m. 10% (v/v) propan-1-ol in water.

The amino acids were separated by two dimensional chromatography. 10 µl. aliquots were spotted onto 20 x 20 cm. plates coated with 0.25 mm. (wet thickness) silica gel 'C' (Woelm, supplied by Koch-Light Laboratories Ltd.).

The solvent for the first dimension was butan-1-ol:acetic acid:water, 4:1:1 (by volume) and for the second, phenol:water, 3:1, (v/v) (Brenner & Niederwieser, 1960). Standard plates spotted with proline, hydroxyproline, glutamine, glucosamine and galactosamine (5 µl. aliquots of a 2 mg./mL solution) were run with each set of samples. Duplicate plates were run for each hydrolysate.

The amino acids were indentified by spraying the plates with a polychromatic ninhydrin spray (Moffat & Lytle, 1959).
The spots corresponding to proline and hydroxyproline were scraped carefully into liquid scintillation vials. 0.4 g. Cab-O-Sil were added to each vial followed by 10 ml. of toluene, containing 5 g./l. 2,5-diphenyloxazole (PPO) and 0.5 g./l. p-bis-2-(4-methyl-5-phenyloxazolyl-benzene) (POPOP). The vials were shaken to obtain a uniform suspension and counted in the manner previously described. Counting efficiencies were determined with the standard $^{14}$C hexadecane.

**Scanning Electron Microscopy**

Some cell cultures were grown in medium without radioactive compounds for varying periods of time. At the end of the period of culture, the medium was removed without disturbing the cells, and the cultures were fixed in neutral buffered formal saline at $+4^\circ$C for at least 48 h. After fixation, the fixative was removed and the cells washed carefully with distilled water at least ten times. The washed cultures, either on a Leighton tube coverslip, or on the base of a plastic petri dish, were air dried in a dessicator over fresh phosphorous pentoxide. The last traces of moisture were removed by applying a light vacuum.

The Leighton tube coverslips were broken into three or four pieces, or the petri dish was cut into several pieces with a hot scalpel. The fragments of coverslip or petri dish were mounted on metal stubs using an adhesive. Each sample was coated in gold-palladium and viewed in a Cambridge 'Stereoscan Mark II'.
RESULTS

Freshly Isolated Cells

Appearances

The freshly isolated cells after the pronase digestion were completely separated and free of matrix. They were rounded in appearance and the nucleus was displaced slightly to one side. Viewed by phase contrast microscopy, the cytoplasm was seen to contain dark granules. When the cells were stained with Euchrycine 3H and viewed by transmitted ultraviolet light the nucleus fluoresced a brilliant green. The cytoplasm contained many granules of different sizes, which fluoresced red (Plate VI-1). The red fluorescence of the granules faded after prolonged exposure to the ultraviolet light. Nearly all the cells isolated had this characteristic appearance.

Occasionally, cells were seen which fluoresced less brilliantly, without red granules and with large cytoplasmic vacuoles. These cells were judged to be either dead or dying and were frequently seen to disintegrate in the ultraviolet light. The proportion of these cells was less than 10% in the cell preparations which were used for tissue culture.

Cell Counts

The numbers of cells isolated from the pooled cartilage of two elbows and one knee joint of the goat were between 8 and 15 million. The final cell yield depended mainly on the size of the animal.
PLATE VI-I

Living Chondrocytes isolated from adult articular cartilage, stained with Euchrysine 3R and viewed by transmitted illumination, X960.
Cultures

Behaviour of the Cells in Culture

The cells became rapidly attached to the base of the culture vessel and only very few remained free at the end of the first 48 h. These cells were removed with the first medium change. The population of cells remained constant or increased slightly during the next 14-16 days (Fig. VI-1). During the first 4-5 days of cultivation, the cells appeared to be randomly distributed (Fig. VI-2a). As the cultures aged, however, the cells became arranged in clumps. After 12 days, the clumping of the cells was most apparent (Fig. VI-2b). In places, the cells appeared to overlap and to be surrounded by amorphous material. Small areas of the petri dish or coverslip were completely bare. The cells showed the same behaviour when they were grown on glass as they did when they were grown on plastic.

The aggregation of the cells could be seen in greater detail by scanning electron microscopy. The chondrocytes, which are spherical or ovoid after isolation, had acquired small processes within 48 h. (Fig. VI-3a). These processes emerged from the base of each cell and extended away from it. Some of the processes met those from neighbouring cells. In twelve day cultures, most of the cells appeared to be in contact with their neighbours, and some cells seemed slightly elongated (Figs. VI-3b, VI-4a). In some instances, the cells appeared to be aligned along slight scratches on the bottom of the petri dish (Fig. VI-4b). The cell processes were longer than
FIG. VI-1: CHANGES IN SOME TYPICAL CHONDROCYTE POPULATIONS DURING CULTIVATION.
PLATE VI-2

Cultures of isolated chondrocytes.

a) Top; 4 day culture on Leighton tube coverslip, photographed in situ. X 420.

Bottom;

b) 12 day culture on Leighton tube coverslip, photographed in situ. X 420., phase contrast.
Scanning electron micrographs of cultures of isolated chondrocytes.

a) Top; 4 day culture on plastic, X 2,200.

b) Bottom; 12 day culture on plastic, X 2,200.
Scanning electron micrographs of cultures of isolated chondrocytes.

a) Top; 12 day culture on plastic, X 1,100.

b) Bottom; 12 day culture on plastic, X 2,200.
in the shorter term cultures.

**Chondrocytes in the Presence of Chondroitin Sulphate**

The medium containing the chondroitin sulphate was much more viscous than the medium from which it had been omitted. Slightly fewer cells had adhered to the base of the petri dish after 48 h. The cells which had adhered to the bottom of the container behaved in a similar manner to the cells which had been grown in medium without chondroitin sulphate.

**Sulphate Incorporation**

The chondrocytes incorporated sulphate into the fraction which could be precipitated by cetylpyridinium chloride. 60-75% of this activity was associated with the medium in the cultures which had been grown both with or without chondroitin sulphate. The rest of the activity which could be precipitated by cetylpyridinium chloride was associated with the cells.

The total activity incorporated by the chondrocytes appeared to be unaffected by chondroitin sulphate concentration of 50 μg./ml. These results are summarised in Table VI-1.

**Proline Incorporation**

The combined cell homogenate and medium retained large amounts of radioactivity after dialysis. The thin layer chromatograms separated proline and hydroxyproline from one another and from the other amino acids and amino sugars.
Up to sixteen amino acids could be resolved by this technique. Most of the radioactivity was associated with the proline. Smaller, but significant quantities of activity were associated with the hydroxyproline. The results are summarised in Table VI-2.

DISCUSSION

The apparent motility of the chondrocytes in culture, and their ability to incorporate sulphate and proline suggests that the cells are alive and actively metabolising. The slow rate of increase of the cell population suggests that the rate of cell division was low. The chemically defined medium, however, was unsupplemented with serum. This type of medium is designed to maintain cells rather than to promote division. The low rate of cell division of chondrocytes grown without serum has been noted by previous workers (Sokoloff et, 1973).

The development of small processes by the chondrocytes was of interest. Boyde et al (1972) and Bard et al (1974b) have observed similar processes extending from fibroblasts and bone cells respectively. These processes also only appeared after the cells had been grown on flat surfaces.

Although a few of the cells in the older cultures appeared elongated, they retained their plump appearance and did not resemble fibroblasts which had been grown in cell culture. These appearances are in marked contrast to the appearances of adult and embryonic chondrocytes which have been...
**TABLE VI-1**

**SULPHATE INCORPORATION BY CULTURES OF ISOLATED CHONDROCYTES.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>D.P.M./10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.G.J₂ + 50 mg./ml. Chondroitin Sulphate</td>
<td>9,570 ± 2,500</td>
</tr>
<tr>
<td>B.G.J₂ without Chondroitin Sulphate</td>
<td>10,740 ± 1,800</td>
</tr>
</tbody>
</table>

**TABLE VI-2**

**PROLINE METABOLISM BY CULTURES OF ISOLATED CHONDROCYTES.**

<table>
<thead>
<tr>
<th>D.P.M./10⁶ Cells</th>
<th>Hydroxyproline Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>7,500 ± 1,200</td>
<td>173 ± 24</td>
</tr>
</tbody>
</table>

cultivated in media which contain serum. In the presence of serum, many of the chondrocytes become flattened and extend large processes, which are visible by phase contrast microscopy (Sokoloff et al, 1970, Takeichi, 1973). These large processes differ from the small processes observed by scanning electron microscopy, which are not easily visible by phase contrast microscopy. Both types of process may, however, extend from one cell (Boyd et al, 1972, Bard et al, 1974b). The rounded appearances of the cells, even after many days of cultivation suggests that the cultures were not contaminated by significant quantities of fibroblasts.

The chondrocytes which had been cultivated for more than ten days appeared to be surrounded by quantities of amorphous debris. This may represent the products of synthesis and degradation. During the first four days, before the cells had clumped, however, most of the labelled products were identified in the medium.

**Sulphate Incorporation**

The ability of chondrocytes to manufacture glycosaminoglycans has been demonstrated by several previous authors, both histochemically, in cultures of cell pellets (Manning & Bonner, 1967) and biochemically, in monolayer culture (Sokoloff et al, 1970, Lavietes, 1970, 1971, Malecud & Sokoloff, 1971, Sokoloff et al, 1973).

In the sulphate incorporation experiments reported here, neither the medium nor the cell homogenate was
subjected to proteolytic digestion. The cetylpyridinium chloride precipitate might therefore contain some protein and keratan sulphate if the main product of the cultures is undegraded protein-polysaccharide. Some hyaluronic acid might also, if present, be precipitated by cetylpyridinium chloride. Small quantities of heparan sulphate which is produced by some other types of connective tissue cells (Bates and Levene, 1971) might also be produced by chondrocytes and be precipitated by cetylpyridinium chloride. Sokoloff et al (1975) has however, noted that the predominant glycosaminoglycans produced by articular chondrocytes in monolayer culture are the chondroitin sulphates. Only small quantities of hyaluronate is produced.

The concentration of chondroitin sulphate added to the cell cultures in the experiments reported here was approximately three times the concentration in normal, uncompressed articular cartilage. This value is one and a half times greater than the concentration in the most highly compressed specimens. It appears, therefore, unlikely that sulphate incorporation into the chondroitin sulphate of the compressed specimens is inhibited by extracellular chondroitin sulphate concentration alone. The commercial preparation of chondroitin sulphate used in these experiments was derived from whale and shark cartilage and contained small quantities of chondroitin sulphate B. Chondroitins 4 and 6 sulphate predominated however, and the preparation was effectively protein-free. The possibility that chondroitin sulphate synthesis is inhibited by the intact protein-
polysaccharide has not therefore been eliminated.

**Proline Incorporation and Hydroxylation**

The ability of the cells to incorporate proline and to hydroxylate some of it, suggests that the cells were able to manufacture collagen precursors. The low values of the hydroxyproline/proline ratios suggests however, that collagen was not a major product of chondrocytes under these conditions. Some of the proline may have been incorporated into the protein portions of protein-polysaccharide. Ascorbic acid and glutamine were added freshly to the medium, and the other necessary cofactors and activators, ferrous iron and lactate were also present (calcium lactate, 200 mg/l.), although the absolute concentration of proline was low. The possibility that proline hydroxylation was partially inhibited under these conditions has not however been excluded. Chondrocytes in adult articular cartilage synthesise little collagen in situ, and there is a lag of eight to ten days after the injection of \(^{14}C\) proline before significant quantities of labelled hydroxyproline appear in the cartilage (Hepp & Mitchell, 1971). However, chondrocytes from adult rabbits synthesise quite large quantities of collagen when grown in the presence of media supplemented with serum (Layman et al, 1972). The collagen produced by chondrocytes under these conditions, however, resembles the type produced by fibroblasts more closely than the collagen of intact cartilage (Layman et al, 1972).
Isolated chondrocytes may resemble explants of some other types of connective tissue in producing neutral collagenases (Vaes, 1971, Werb et al, 1973) or cathepsin B1 during cultivation. If this were the case, any collagen synthesised might be degraded to low molecular weight products, which would have been lost during dialysis.
CHAPTER 7
THE ISOLATION AND CULTIVATION OF CELLS FROM CANCELLOUS BONE

Introduction

The structure of cancellous bone differs markedly from that of articular cartilage. It consists of a mesh of interconnecting trabeculae. The spaces between the trabeculae are, in normal cancellous bone, filled with marrow. The matrix of the bone contains large quantities of collagen, and this protein accounts for about 90% of the total organic material in the matrix. Mineral constitutes approximately 50% of the fresh weight of bone (Pouxman & Boyer, 1968, Vaughan, 1970). This mineral has been described as hydroxy-apatite, but its exact nature is still uncertain. It contains the ions Ca\(^{2+}\), H\(^{+}\), PO\(_4\)\(^{2-}\), OH\(^{-}\), in the approximate molar ratios of 9:2:6:2 respectively. Small quantities of magnesium, carbonate, citrate, fluoride, sodium, potassium, and strontium are also present. Between 50% and 60% of the inorganic material in cancellous bone appears to be crystalline, as judged by X-ray diffraction. The exact relationship between these mineral salts and the collagen is a subject of continuing speculation. There appears, however, to be a definite relationship between the banding pattern of the collagen fibres and the arrangement of the hydroxy-apatite crystals, (Fitton-Jackson, 1957, N. J. Dickens, personal communication).

Cancellous bone also contains some protein-polysaccharide and glycoprotein. Herring, (1966) has isolated
three fractions containing chondroitin sulphate. Two of these fractions appear to contain chondroitin sulphate-protein complexes, and one to consist almost entirely of pure chondroitin sulphate. The protein-polysaccharides obtained from bone differ from those isolated from cartilage by the same technique. The protein-polysaccharides of bone, unlike those of articular cartilage, contain large quantities of sialic acid.

The glycoprotein, sialoprotein, is also present in bone. This compound contains a peptide chain of about 90 amino acids and a single, highly branched, carbohydrate group, containing sialic acid (Williams & Peacocke, 1965, 1967).

The Cytology of Cancellous Bone

The types of cell associated with cancellous bone exhibit a greater diversity than the cells of articular cartilage. The osteocytes, which are surrounded by matrix, have long, branching processes, which extend into the matrix through fine canaliculi. Transmission electron microscopy reveals that the cytoplasm of osteocytes contains a well developed rough endoplasmic reticulum, a prominent Golgi area and numerous mitochondria (Baud, 1966, 1968, Hancox, 1972b). Mitochondria have been identified in the cell processes (Bard et al, 1974b). Some of the mitochondria may contain very dense granules. These granules are thought to consist of the salts of divalent cations (Baud, 1966). Centrioles and microtubules have been identified, and lysosome-like bodies have also been seen in the cytoplasm of osteocytes of partially decalcified bone prepared and sectioned by
H. J. Dickens (Bard et al, 1974b).

The osteocytes further away from the surface of the bone tend to be larger than the ones near it (Belanger et al, 1963). The osteocytes near the surface of the bone resemble closely the osteoblasts from which they are derived. Osteocytes, unlike chondrocytes, are linked to a blood supply by their processes. The canaliculi form a continuous network throughout the calcified matrix and connect with the marrow cavities.

The osteoblasts are situated on the surfaces of the trabeculae. They have shorter processes which are frequently seen to be in contact with the processes of other osteoblasts. Other processes extend towards the bony surfaces and into the matrix (Dudley & Spiro, 1961). The nucleus is frequently found at the end of the cell furthest from the bony surface. The osteoblast has an extensive rough endoplasmic reticulum with many ribosomes (Dudley & Spiro, 1961). The Golgi area is, however, less prominent than it normally is in osteocytes. Mitochondria are numerous in osteoblasts. Dense granules, probably glycogen, have been seen in the cytoplasm (Scott, 1967).

The electron microscopic appearances of osteoblasts suggest that most of them are engaged in synthesis, and a main function of the osteoblast seems to be the manufacture of bone matrix. Amino acids, especially glycine and proline, are rapidly taken up by osteoblasts, and subsequently appear in the matrix (Carniero & Leblond, 1959, Owen, 1963, Owen & Macpherson,
1963, Young, 1964). Most of these amino acids are converted into collagen (Dienes et al, 1962, Vaes & Nichola, 1962). Osteoblasts in growing bones also synthesise large quantities of RNA (Owen, 1965).

Less is known about the metabolic functions of the osteocyte. In spite of the extensive rough endoplasmic reticulum and Golgi area, little collagen appears to be synthesised by the osteocytes of mature bone (Owen, 1965).

The third type of cell associated with bone, is the osteoclast, which is primarily concerned with bone resorption. Typical osteoclasts are large cells with many nuclei. Some of these nuclei may appear degenerate (Hancox, 1972a,b). Osteoclasts vary considerably in size and cells with over a hundred nuclei have been observed (Hancox, 1972a, b). The majority of osteoclasts have fewer nuclei and some osteoclasts may only have two or three. Cells with single nuclei which are capable of resorbing bone have been described by some authors (Tonna, 1960, Walker, 1961). Under some conditions, osteocytes may also be able to remove matrix (Delange, 1965, 1968). The cytoplasm of osteoclasts contains little rough endoplasmic reticulum. The Golgi area is, however, prominent (Scott, 1967). Many mitochondria and unattached ribosomes may also be seen. The cytoplasm contains many vacuoles (Vaughan, 1970, Hancox, 1972b).

Osteoclasts are frequently found in small depressions on the surfaces of bone which are being resorbed. These depressions are termed Howship's lacunae. Osteoclasts may
also be found free in the marrow cavities. When the cells are in contact with bone, the surfaces nearest the bone form brush borders. The brush border can be seen by electron microscopy, to consist of many folds in the cytoplasmic membrane, giving rise to channels which run towards the interior of the cell. Some of these channels may connect with vacuoles within the cytoplasm (Baud, 1966). The brush borders of osteoclasts are revealed by time-lapse cine photomicrography to be in continuous motion. Osteoclasts give strong histochemical staining reactions for acid phosphatase, succinate dehydrogenase, and aminopeptidase activity. Alkaline phosphatase has not been detected in osteoclasts (de Voogd van der Straaten, 1965, Vaes, 1969).

In addition to the osteoblasts and osteoclasts, a number of spindle shaped cells are found on the surfaces of the bony spicules. Some of these cells may show acid phosphatase activity. Others appear to contain alkaline phosphatase (Owen, 1970, Vaughan, 1970). These cells have been named 'osteoprogenitor cells' and it is possible that they may be capable of developing into osteoblasts or osteoclasts (Vaughan, 1970). Fibroblasts are also found on the surfaces of bone and some of these may also be capable of becoming osteogenic if they are influenced by appropriate stimuli.

The heavily calcified matrix has, in the past, hindered the examination of the cells of mature bone. The osteocytes are particularly inaccessible. The isolation of bone cells from adult bone and their cultivation might enable their metabolism to be studied in greater detail and changes
caused by disease to be examined. A comparison with articular chondrocytes cultivated under the same conditions might also be instructive.

Cells have been isolated from embryonic bones by Peck and his co-workers (1964) by digesting the partially calcified matrix with collagenase. Fully calcified bone is, however, unaffected by the enzyme alone (Bard et al, 1972). In devising an isolation technique, therefore, it is necessary to remove most of the mineral in order to allow the enzymic digestion to proceed.

Methods

Normal cancellous bone was obtained from the femoral condyles of goats which had been freshly killed with a humane killer. Specimens of osteoarthritic bone were cut from severely arthritic human femoral heads which had been surgically removed and replaced by a prosthesis. The human bone was wrapped in moistened gauze and transferred to a sterile jar immediately after removal from the patient. The specimen was stored overnight at +4°C.

Intact knee joints were removed from the goat under aseptic conditions and opened in a laminar air flow cabinet. The two condyles were sawn away from the end of the femur and transferred to a universal container. T.C. 199 was then added. One of the condyles was attached to the stage of a Metals Research Macrotome II by a low melting point wax. The condyle was positioned so that the cut surface was against the stage.
Wedges were cut from the human femoral heads with a sterile hacksaw, and divided into two. One of these specimens was attached to the microtome stage with the low melting point wax and the other kept immersed in T.C. 199 until needed.

Up to 40 vertical sections, each approximately 100 thick, were cut with the rotating sintered diamond blade. At no time was the rate of rotation of the blade allowed to exceed 200 r.p.m. The cutting edge was continuously lubricated with T.C. 199, squirted onto the blade with a hypodermic syringe. Both the preparation of the bone specimens, and the cutting of the sections were carried out in a laminar air flow cabinet.

The 100 thick sections of cancellous bone were suspended in 10 ml. T.C. 199 in a universal container. The technique of sectioning has been described in detail elsewhere (Bard et al., 1974c).

The bone slices were shaken in six changes of T.C. 199 at 37°C to remove as much marrow as possible. The sections were then rinsed for 2 s. in distilled water to lyse any remaining marrow cells and transferred immediately to 25 ml. conical quickfit flasks containing 10 ml. T.C. 199 containing 1.5% (w/v) dipotassium E.D.T.A. This medium consisted of 1 ml. T.C. 199 10 x concentrate (Wellcome Reagents Ltd. Wellcome Research Laboratories, Beckenham, Kent), 2.5 ml. 4.4% (w/v) sodium bicarbonate (Wellcome) and 0.15 g. dipotassium E.D.T.A. dihydrate (Koch-Light Laboratories, pharmaceutical grade) made up to a total volume of 10 ml. with distilled water. Two of the
bone slices were put into each flask.

The flasks were gassed with the mixture of Oxygen (20%), carbon dioxide (5%) and nitrogen (75%) and sealed. They were then shaken overnight for about 15 h. in a shaking water bath set at +38°C. At the end of the overnight incubation, the medium, together with the chelated calcium, was decanted away and the partially decalcified slices rinsed three times in 5 ml. T.C. 199. 10 ml. T.C. 199 containing 0.2 mg./ml. collagenase (Sigma, grade II approx. 125 units/mg.) were then added to each flask. The flasks were again gassed and sealed. They were then incubated for a further 3 h. at +38°C in the shaking water bath.

At the end of this time, the contents of each flask were decanted into a 10 ml. conical centrifuge tube and centrifuged for 5-10 minutes at 750 g. The resulting pellet was washed twice in T.C. 199 and resuspended in 0.2 ml. of the medium. Samples of this suspension were mounted on agar coated slides, stained with Duchsrysin 3R and examined by transmitted fluorescent illumination using a dark ground condenser. Some preparations were also viewed by phase contrast microscopy, and some were used for Eosin dye-exclusion viability tests or cell counts. Only two or three of the twenty centrifuge tubes from each experiment were used for these morphological and viability studies. The cells from the remaining tubes were combined, and used for transmission electron microscopy or tissue culture (Bard et al, 1974b). The isolation procedure, up to the microscopical examination of the cells was performed under aseptic
conditions.

In some experiments, intact slices, both before and after treatment with E.D.T.A., were stained with Buchryline and viewed by incident fluorescence microscopy. In other experiments fresh slices and slices which had been incubated in the presence of E.D.T.A. were analysed for calcium concentration. The results of these experiments have been reported elsewhere (Bard et al., 1974a).

Cell Cultures

The cell deposits from 12-16 centrifuge tubes were combined to give a total cell count of at least one million cells. These cells were suspended in 0.5 ml. of the modified B.G.J. from which proline had been omitted, but which contained 0.02 mg. Kystatin/ml. (Sigma, 4,800 USP units/mg.).

The cells were spread either over a coverslip in a Leighton tube or over the bottom of a 3 cm. plastic petri dish. The plastic petri dishes were divided into two unequal segments with a 27 mm. long glass rod. The cells were added to the smaller of these two segments. A further 0.5 ml. B.G.J. containing freshly dissolved ascorbic acid (0.4 mg./ml.), glutamine (0.4 mg./ml.) and proline (1.6 mg./ml.). These compounds were dissolved in 10 ml. and delivered into the culture vessel by a 10 ml. hypodermic syringe fitted with a 25 mm. millipore filter (0.22 µm, white, plain). The millipore filter was washed with 20 ml. hot distilled water, followed by 10 ml. of the B.G.J. b.
The Leighton tubes were gassed with the oxygen, carbon dioxide, nitrogen mixture and sealed with silicone rubber stoppers. The 3 cm. plastic petri dishes were enclosed in 9 cm. plastic petri dishes and placed in a McIntosh-Fildes jar. The McIntosh-Fildes jar was gassed and sealed. Both the Leighton tubes and the petri dishes were incubated for 48 h. and the medium changed daily for the rest of the culture period. Ascorbic acid and glutamine were dissolved freshly before each medium change.

Proline Incorporation Experiments

5 μCi/ml. uniformly labelled $^{14}\text{C}$ proline was added to the cultures in the manner described in the previous chapter (p. 175). The cells were grown in the presence of the radioactive proline for four days with one intermediate medium change. The treatment of the specimens, the identification of the amino acids and the measurement of their radioactivity were exactly the same as those already described (p. 175 et seq.).

RESULTS

Freshly Isolated Cells

The originally brittle slices of cancellous bone became limp after treatment with E.D.T.A. These limp slices disintegrated entirely after incubation in collagenase and preparations of cells free from debris could be obtained.

Most of the cells in the centrifuged pellet had many processes, some of which were branched. Plate VII-1 shows a typical example of this type of cell viewed by phase contrast microscopy. Another example is shown in the Appendix.
When they were stained with Euchrysine 5R and viewed by transmitted fluorescent illumination, the nuclei of these cells fluoresced a brilliant green. The cytoplasm contained small red granules which faded very rapidly in the ultraviolet light. These granules were smaller, less numerous, and faded more rapidly than most of the granules in the cytoplasm of chondrocytes.

In addition to the cells with processes, a few, large cells with several separate nuclei were seen. Some of the nuclei appeared to fluoresce more brilliantly than others. These cells were rather more prevalent in preparations of cells from arthritic human, rather than normal goat bone. A typical example is illustrated in the Appendix (Fig. 2, Bard et al, 1972).

Spindle shaped cells with two, long, unbranched processes were seen in some of the preparations. These were seen in some of the preparations. They were found most frequently amongst the cells isolated from the normal goat bone.

**Cell Counts and Viability**

The number of cells with branched processes isolated from pairs of slices of normal goat cancellous bone varied between 72,000 and 805,000. 80-90% of these cells were alive as judged by the eosin dye exclusion test in the preparations from normal goat bone. 70-75% of the cells with processes isolated from arthritic human bone excluded eosin. A few rounded cells were isolated from arthritic human bone. These were not included in the cell counts. 90-95% of the rounded cells failed
PLATE VII-1

Cell with several processes, isolated from goat cancellous bone, phase contrast, X 2,000.
Plate: VII-1
to exclude eosin. The large, multinucleated cells were not sufficiently numerous to count accurately.

Cell Culture

Most of the cells had become firmly attached to the base of the culture vessel within 48 h. Those which had not adhered were removed at the first change of medium. The cell population subsequently remained constant for at least 11 days (Fig. VII-1 shows a typical series of counts).

The cells with processes retained their distinctive appearances in the chemically defined medium. When the cultures were first set up, the cells were spread uniformly over the bottom of the culture chamber. They became aggregated into clumps after 5-10 days of cultivation. Some of the cells became piled on top of others, leaving some areas completely bare. Reliable counts could not be made on cultures of longer than twelve days duration as a consequence of the clumping.

The large multinucleated cells were identified in the cultures after four weeks of cultivation. Plate VII-2 shows a preparation of cells from arthritic human bone, which had been cultivated for four weeks in the chemically defined B.C.J. medium on a Leighton tube coverslip. The cells have been fixed in situ and stained with haematoxylin and eosin. A seven day culture in T.C. 199, fortified by the addition of 10% heated calf serum (Wellcome Reagents Ltd.), 500 mg./l. glycine, 200 mg./l. proline and 100 mg./l. glutamine is illustrated in the Appendix (Fig. 3, Bard et al, 1972).
FIG. VII-1: CHANGES IN SOME TYPICAL BONE CELL POPULATIONS DURING CULTIVATION.
Incorporation and Hydroxylation of Proline

The chromatographic technique clearly separated proline and hydroxyproline both from one another and from the other amino acids. Most of the non-dialysable radioactivity was associated with the proline. Hydroxyproline showed smaller, but significant, amounts of radioactivity. The results are summarised in Table VII-1.

DISCUSSION

The appearances of the freshly isolated cells, the ability of the majority of them to exclude eosin, and their survival for long periods in culture suggest that most of the cells were alive. The ability of the cells to incorporate proline and to hydroxylate small quantities of it in culture confirmed this conclusion.

The cell population again remained constant, and this was probably attributable to the chemically defined medium. The clumping of the bone cells, however, differs from the aggregation of the chondrocytes. The most striking difference was the tendency of the bone cells to become piled on top of one another. They did not appear to show contact inhibition. In the chondrocyte cultures nearly all the cells remained in contact with the bottom of the culture vessel even after they had been cultivated for twelve days.

The large, multinucleated cells had the appearances of osteoclasts. Their presence amongst cells which had been cultivated for four weeks is of interest. Hancock (1965)
PLATE VII-2

4 week culture of cells isolated from human cancellous bone in B.G.J.\textsubscript{b}, on a Leighton tube coverslip. Fixed and stained with H.& E. X 105.
TABLE VII-1:

**PROLINE METABOLISM BY CULTURES OF ISOLATED BONE CELLS.**

<table>
<thead>
<tr>
<th>Origin of Cells</th>
<th>D.P.M./10^6 Cells</th>
<th>Hydroxyproline Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat (3)</td>
<td>7,000 ± 2,500</td>
<td>106 ± 23</td>
</tr>
<tr>
<td>Osteoarthritic Human (5)</td>
<td>1,800 ± 750</td>
<td>57 ± 14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Hydroxyproline Proline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.015</td>
</tr>
</tbody>
</table>
has maintained osteoclasts in culture for eight days. The possibility that the osteoclasts observed in these long term cultures arose from other types of cell during cultivation has not been excluded.

The identification of the cells with processes is less certain. In addition to osteocytes, osteoblasts and some pre-osteoblasts or osteoprogenitor cells from the surfaces of the bony spicules might be present. The identification of bipolar cells in the freshly isolated preparations suggests that osteoprogenitor cells could have been present. The preliminary rinse of the bone slices in distilled water might have been expected to lyse most of the cells on the bone surface. The presence of osteoclasts amongst the isolated cells suggests however that this process was not complete.

Proline Metabolism

The large quantities of proline incorporated into the non-dialysable fraction of the cell homogenate suggests that the cells were actively metabolising, and engaged in protein synthesis. This conclusion was supported by the ultrastructural appearances of the isolated cells (Bard et al, 1974b). The small proportion of proline converted to hydroxyproline suggests that under these conditions of cultivation, collagen or its precursors were not synthesised in large quantities. The presence of osteoclasts may, however, partially account for the low quantities of hydroxyproline remaining after dialysis. The production of collagen by the osteocyte in intact adult bone \textit{in vivo} appears to
be small (Owen, 1970) under normal conditions. The larger quantities of hydroxyproline produced may be of significance. Species differences, however, preclude direct comparison. The presence of alkaline phosphatase in some of the cells with processes, and their ability to concentrate calcium in culture (Bard et al, 1974b) suggest that these cells might have been originally concerned with calcium metabolism.
GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The Effects of Mechanical Loading on Protein-Polysaccharide Synthesis by Articular Cartilage

The finding that mechanical pressure in vitro can reversibly inhibit chondroitin sulphate synthesis suggests a link between abnormal loading and the first biochemical indications of osteo-arthritis. It may also imply that the reduction in protein-polysaccharide content of the matrix can precede, rather than be a result of mechanical disruption of the cells and matrix. The continuous application of a load resulted in less synthesis than for greater loads applied intermittently. These data support the observations of previous workers that continuous compression causes a rapid degeneration of cartilage in vivo and is more injurious than interrupted loading (Salter & Field, 1960, Thompson & Bassett, 1970). Cartilage unloaded by any method, however appeared to synthesise most protein-polysaccharide. The degenerative changes found in cartilage kept without compression in vivo, for example the resected rabbit joints of Thompson and Bassett, (1970) might possibly have some other origin than decreased protein-polysaccharide synthesis.
The autoradiographs suggest that synthesis of protein-polysaccharide in the superficial zones of the cartilage is more readily reduced by compression than synthesis in the basal and intermediate zones. The rate of synthesis of matrix by the superficial chondrocytes in uncompressed cartilage is much less than by the chondrocytes of the basal and intermediate layers and compression probably does not affect the superficial zone specifically. It is of interest, in this context, that the first decrease of protein-polysaccharide during arthritis is in the cartilage nearest to the articular surface.

These studies shed little light on whether, the reduction in protein-polysaccharide synthesis is general, or whether some fractions are affected more than others. Some data suggest that under normal conditions, the lowest molecular weight fractions are synthesised most rapidly (Gross et al, 1960, Dowsness, 1961, Kleine et al, 1971). Other data imply that some of the low molecular weight protein-polysaccharides catalyse the aggregation of the higher molecular weight ones (Hascall & Sajdera, 1969, Sajdera et al, 1970). A selective decrease in the synthesis of this low molecular weight fraction might therefore hasten the loss of protein-polysaccharides from the matrix by
reducing their mutual binding. More detailed characterisation of
the protein-polsaccharides produced by the cartilage during
these experiments might therefore be instructive.

The decrease in protein-polsaccharide
synthesis as a result of mechanical stress may be accompanied by
an increase in its rate of breakdown. This possibility could be
examined histochemically by staining reactions for cathepsins or
non-specific esterases. The presence or absence of extra-
cellular acid phosphatase might also indicate whether the
contents of the lysosomes were being discharged.

Changes in the ultrastructure of the
chondrocytes could also shed light on this problem. It would be
of interest to know whether the decrease in protein-polsaccharide
synthesis was accompanied by a decrease in the extent of the
rough endoplasmic reticulum or the Golgi area, or whether there
was an increase in the number of lysosomes as a result of
mechanical loading. Any cellular damage would also be revealed
more clearly.
Cathepsins may alternatively be released in abnormal quantities only after the matrix has become partially depleted of protein-polysaccharide. This possibility might be examined by in vivo studies on animals in which glycosaminoglycan synthesis was inhibited by glutamine analogues. Removal of protein-polysaccharide of rabbit joints by enzymic digestion in vivo leads rapidly to the development of symptoms and histological changes resembling osteo-arthritis (Bentley, 1971, 1972). It would be of interest to know whether a temporary reduction in protein-polysaccharide synthesis could have similar effects.

The inhibition of sulphate incorporation is strongly correlated to the amount by which the cartilage is compressed. Cartilage in which the hexosamine concentration is lowered compresses more rapidly and to a greater extent than normal cartilage when a probe is forced into it (Keppen et al., 1970). If the relationship between the amount of compression and the decrease in protein-polysaccharide synthesis also applies to osteo-arthritic cartilage, these data may imply that loading can impair the metabolism to an even greater extent once extensive loss of glycosaminoglycans has occurred.
The mechanism by which chondroitin sulphate synthesis is reduced by mechanical stress remains obscure. The ability of the chondrocytes to resume synthesis after periods of compression suggests that the effect cannot be largely accounted for by cellular damage. It also seems unlikely that the concentrations of sulphate or of the amino acids is limiting.

Compression represents only one type of mechanical stress to which cartilage may be subjected. The histological appearances of osteo-arthritic cartilage suggest that abrasion might also be important in the destruction of the matrix. It might be instructive to examine the effects of abrasion as well as compression on the metabolism and histology of cartilage in vitro.

Cell Cultures

The behaviour of the cell cultures in the presence of chondroitin sulphate indicates that under these conditions at least, the free glycosaminoglycans cannot exert inhibition. It is conceivable that intact protein-polysaccharides might reduce synthesis. This possibility could be investigated by cultivating chondrocytes in medium in which various fractions of extracted and purified cartilage protein-polysaccharide had
been dissolved.

The effects of oxygen tension could also be examined by cultivating cells or performing compression experiments while the apparatus was gassed with gas mixtures containing different proportions of oxygen.

Neither the isolated chondrocytes nor the bone cells appeared to synthesise large quantities of collagen. The production of collagen by both cell types in situ appears, however, to be small. Production of collagen by the isolated cells might be enhanced by serum and the hydroxylation of proline stimulated by higher oxygen tension. The observations of Hayman and his co-workers (1972) suggest that when collagen is produced by isolated chondrocytes it resembles more closely the collagen produced by fibroblasts than the collagen of intact cartilage. The conditions under which cells manufacture collagen and the factors which determine type produced still require full elucidation. The possibility that collagen was produced during cultivation and degraded by enzymes present in the cells or medium has not been eliminated.

Many other aspects of bone cell metabolism could be studied in cell culture. In particular their ability to concentrate calcium (Bard et al, 1974b) might be studied in greater
detail. The effects of hormones and vitamins on this and other metabolic functions would be of particular interest.

Both bone cells and chondrocytes appear to aggregate in cell culture, although the appearances of the clumps formed by the different types of cell differ. The behaviour of adult bone cells and chondrocytes in the presence of one another might be instructive. The cells might also be grown on three dimensional matrices, and the capacity of the different cell types for forming organised structures examined. The capacity of the different cell types for influencing the metabolism of the others could also be studied. Any differences in this respect between the cells isolated from normal and those from osteo-arthritis bone would be most instructive.

The complexity of the interactions which may lead to osteo-arthritis, and the improbability of this disease being attributable to a single cause, have been commented on by Sokoloff (1969). It is hoped that this study may have helped to demonstrate in vitro a few of the interactions between pressure and metabolism that have been suspected from clinical observations and animal experiments.
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Isolation of Living Cells from Mature Mammalian Bone

Living cells have been isolated from articular cartilage of adult mammals by successive digestion with appropriate proteolytic enzymes to remove the matrix. Viability was checked by phase contrast microscopy; the chondrocytes were

Fig. 1 A typical cell isolated from a slice of human cancellous bone, mounted on an agar coated slide and compressed slightly. Phase contrast (×1,500).
motile at 37° C. Eosin dye-exclusion tests were compared with
uptake of the acridine orange dye, ‘Euchrysine 3R’ as seen by
fluorescence microscopy. The conclusion was that the vivid
green fluorescence of nuclei and the red fluorescence of cyto-
plasmic granules in intact cells gave an excellent indication
of the viability of the chondrocytes. It tallies well with other
preliminary tests of viability, and was quicker and easier to
perform. Fluorescence microscopy using ‘Euchrysine 3R’
had previously given a good indication of the viability of other
mammalian cells. Other evidence that the chondrocytes were
alive after isolation by digestion of the matrix was provided
by allografting and by cultivation in vitro. Isolated living
chondrocytes have also been used for immunological typing.

Recently techniques have been developed at the Institute of
Orthopaedics for allografting articular cartilage with varying
amounts of underlying bone. Members of our own group
have undertaken certain aspects of this work. Our first task
was to develop viability tests for adult compact and cancellous
bone. We decided to attempt the isolation of living osteocytes
from adult bone.

Living cells have been isolated from the calvaria and long
bones of foetal mammals by digestion with collagenase; at
this stage of development the bones are not fully calcified.

Fig. 2 A multinucleated cell isolated from a slice of human can-
cellous bone, mounted on agar and stained with ‘Euchrysin 3R’.
Transmitted fluorescent lighting (× 640).
Digestion of mature bone is complicated by the presence of calcium salts. The evidence suggests that they are situated partly outside and surrounding the individual collagen fibrils and we therefore decided to remove these mineral salts first.

Slices of bone were cut from vertical segments of femoral heads or condyles of goat, pig, cow, dog, horse and man, using the Metals Research macrotome II fitted with a sintered diamond blade. Specimens from goats and pigs were sectioned within 2-3 h of death, while other bones were received 24 h after death. The human femoral heads had been removed in surgical operations 24 h previously. During cutting, the blade was lubricated and cooled by continuous irrigation with physiological medium. The slices were rigid and approximately 1.5 cm² and 100 μm thick, weighing 15-20 mg. The rim of articular cartilage was excised. The slices were then dipped into distilled water for 2 s to disrupt the haematopoietic and other cells in the marrow cavities without damaging the cells within the bony spicules. Vertical slices were also cut from segments of compact bone from femoral shafts. The periosteal surface was excised but the distilled water rinse was unnecessary because of the absence of marrow. Samples from some slices were stained with 'Euchrysin 3R' and examined by fluorescence microscopy using incident illumination. Osteocytes could be clearly seen within lacunae and fluoresced green. The slices were transferred to conical flasks with 10 ml of culture medium (TC 199) containing the dipotassium salt of ethylenediaminetetraacetic acid (EDTA) in a concentration of 1.5% for cancellous bone and 3.0% for compact bone. The pH was adjusted to 6.8, and the flasks were gassed with 5% CO₂ in air, then stoppered, sealed and shaken in a water bath at 38°C for about 14 h. The limp, decalcified slices were washed three times and then resuspended in 10 ml of TC 199 containing 2.5 mg of collagenase (Sigma Type I, 125-200 U/mg). They were shaken for 2-3 h at 38°C until they had completely disintegrated. The turbid medium was centrifuged at 700-800g for 5 min and the deposit was washed and recentrifuged. The instruments, glassware and solutions used had been sterilized and aseptic precautions were taken throughout these procedures.

Samples of the deposit were mounted on agar coated slides, stained with 'Euchrysin 3R' and examined by phase contrast and fluorescence microscopy, using transmitted illumination. In spite of a certain amount of granular debris, many intact cells could be seen. They varied in size and shape, particularly in the deposits from cancellous bone. Phase contrast microscopy showed that many of the cells had several branched processes in different planes. They were difficult to photograph except after compression (Fig. 1). Cytoplasmic granules also
Fig. 3 A sample from a 7 day culture of cells isolated from goat cancellous bone, fixed and stained with haematoxylin and eosin (×250).
showed up well by phase contrast microscopy and surrounded the nuclei, thereby tending to obscure their outlines. When seen by fluorescence microscopy the nuclei appeared bright green with sharp outlines; in the slender cells they were elongated, but in the plumper cells they were oval. A few cytoplasmic granules which fluoresced red could be seen, but they faded rapidly in ultraviolet light. The cells were similar in size and shape to the cells already seen in the undigested slices by incidental fluorescent illumination. Small numbers of large multinucleated cells, possibly osteoclasts, were also seen in deposits from fresh cancellous bone (Fig. 2). Other deposits were used for eosin exclusion tests: 50–75% of the cells appeared to be alive. Smears were fixed and stained with haematoxylin and eosin. When slices of bone had been killed by boiling or by freezing and thawing, the osteocytes did not fluoresce and only a few fragmented cells were isolated by the technique described. The differences between live and dead bone were striking.

Cultures of cells isolated from goat and human bone were set up in Petri dishes using TC 199, fortified by the addition of 10 ml. heated calf serum (Wellcome Type I) and 50 mg glycine, 20 mg proline and 10 mg glutamine per 100 ml. They were left to settle for 48 h and thereafter the medium was changed daily. After 7 days, collections of cells with long processes were adhering to the base of the dishes. Samples were removed for microscopical studies (Fig. 3). Quantitative experiments and biochemical studies with these cells are now under way.

We thank Mr P. J. Stiles and Dr P. D. Byers for human bone. The dog bone was supplied by Mr J. T. Robson and the goat bone by Mr T. Brightwell. We also thank Drs J. Edwards and Sylvia Fittion-Jackson for advice and encouragement, and Mr C. Tillson for technical help. This work was supported by the Medical Research Council. D. R. B. is in receipt of a University of Surrey research grant.

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APPENDIX

Reprints of published papers.
STUDIES ON SLICES AND ISOLATED CELLS FROM FRESH OSTEOPATHIC HUMAN BONE

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The histological and cytological examination of normal and pathological specimens of adult bone has presented problems mainly related to the high mineral content and consequent hardness of the tissue in which the living cells are embedded. Prolonged fixation and subsequent decalcification seemed to be essential but were inevitably associated with formation of artefacts and distortion of the cells, and they precluded the use of most of the more delicate histochemical techniques. One advance was to cut frozen sections in a cryostat using the Jung K microtome fitted with a profile K knife (Pearse and Gardner 1972). Another approach was to study embryonic or fetal bone before extensive mineralisation had occurred. Admirable as the results of these two lines of research have been, they may have given a slightly distorted picture of the structure and metabolism of mature bone. Recently a technique has been described for slicing live bone without killing the cells. Living cells were isolated from these slices, examined microscopically and cultured (Bard, Dickens, Smith and Zarek 1972).

This technique has been used to study osteoarthritic human femoral heads removed at operation (Bard, Dickens, Edwards and Smith 1972). It might be applicable to various problems in the study of normal and pathological bone. It therefore seemed worthwhile to report our method and findings in greater detail than has hitherto been possible.

APPARATUS, MATERIAL AND METHODS

The Macrotome—The Metals Research Macrotome II (Fig. 1) was used to cut slices of bone 100 μ thick. It is a precision engineering tool which fits conveniently into a SLEE Laminar Air Flow Cabinet with a work area 72 centimetres by 50 centimetres by 63 centimetres. The macrotome has a sintered diamond cutting edge 200 μ thick on a rotating blade fixed to an accurately machined shaft. The blade is easily removed for cleaning and sterilisation by heat. The speed of rotation of the blade is controllable and relatively slow. It can be varied between 50 and 1,200 revolutions per minute, depending on the nature of the material to be cut. A

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speed of approximately 200 revolutions per minute was generally used for bone. The specimen was attached to the adjustable table fixed to the swing arm by means of the universal clamp. The surface of bone to be cut was held lightly against the blade by a dampened counterpoise on the other end of the swing arm. The advantage of this cutting system is that the bone backs away automatically if the blade encounters a harder area or if the edge of the blade has become slightly uneven. We have used our blade for over a year and cut at least 2,500 slices of bone without noticeable deterioration in performance. This cutting system avoids excessive mechanical and thermal stresses.

Bone specimens—Fifty-seven severely osteoarthritic human femoral heads which had been replaced by prostheses were kindly supplied by surgeons at the Royal National Orthopaedic Hospital, Stanmore, with the cooperation of Dr P. D. Byers. The specimens were kept overnight in sterile airtight jars at +4 degrees Celsius and transported to Guildford, on ice, the following morning. They were transferred to the laminar air flow cabinet and cut up into vertical sectors with a sterile hacksaw and with aseptic precautions. The sectors were 2-5 centimetres in thickness and not more than 1-4 centimetres in radius. The angle of the sector was between 45 and 60 degrees. These dimensions were dictated by the diameters of the macrotome stage and the necks of the flasks to which the slices were eventually transferred.

No normal human bone was available for comparison, nor were we able to obtain specimens from arthritic animals. The controls were bones from fifteen normal young adult goats which had been killed with a humane killer. The knee joint was chosen in preference to the hip because the entire joint could be removed intact in the post-mortem room and opened under aseptic conditions in an animal operating theatre within three hours of death. The femoral condyles were sawn apart in the laminar air flow cabinet.

The portion of human arthritic bone or the normal goat condyle was attached to the specimen table of the macrotome using the special, hard, low melting-point mounting wax (No. 405136) supplied by Metals Research Ltd. The pieces of bone were positioned so that the articular surfaces of the goat condyles were uppermost and the articular surfaces of the wedges of human bone were to the right. The specimens were completely covered with moist sterile swabs. The specimen table was attached to the swing arm with the universal clamp and adjusted so that its surface was at right angles to the blade, to cut the specimen transversely. The blade was positioned 4 millimetres from the end of the piece of bone. The moist swab was discarded and the counterpoise weight on the swing arm was moved until the edge of the bone pressed lightly against the blade.

Method of sectioning—The macrotome was switched on and the speed of rotation of the blade set at approximately 200 r.p.m. The blade and the specimen were continuously cooled and lubricated with sterile medium (T.C.199 Wellcome Reagents Ltd.) from a 10-millilitre syringe. When the cut was within 2-3 millimetres of the base of the bone the specimen table was lowered and the blade moved towards the operator by rotating the micrometer dial so that the next cut would leave a slice 100 μ thick. It is important to bear in mind that the cutting edge is 200 μ thick and that this thickness of bone is wasted between each slice. Another small point is that, with continued use, the micrometer which controls movements of the blade may develop a backlash; this must be allowed for by turning the dial through a complete revolution and returning it to the correct position. After twenty to thirty slices had been cut in this way they were detached from the specimen table together with the residual bony base and separated in a dish of medium. Another sample of human or goat bone was then sectioned to give a total of at least forty-four slices for each experiment.

Two or three slices were used for estimation of calcium (see below) and one or two slices were stained with the acridine orange dye Euchrysine 3R (Young and Smith 1964) and examined by incident fluorescence microscopy.

Removal of marrow and decalcification—Sets of twenty slices were transferred to universal containers and shaken in several changes of medium to remove as much as possible of the
bony debris and marrow. The articular cartilage was excised; pairs of slices were then dipped into distilled water for two seconds in order to lyse any remaining marrow cells. Some slices were re-examined by incident fluorescence microscopy; the remaining slices were then transferred to stoppered 25-millilitre conical flasks containing 10 millilitres of tissue culture medium (T.C. 199) with added EDTA.

In the preliminary experiments various concentrations of the dipotassium salt of EDTA (1 per cent, 1.5 per cent, 3 per cent and 5 per cent w/v) were made up in T.C. 199 and the pH adjusted to 7.2 with sodium bicarbonate. In the majority of experiments 1.5 per cent EDTA was used. The flasks were gassed with 5 per cent CO₂ in air, stoppered, sealed and shaken in a water bath at +38 degrees Celsius overnight. The following morning the EDTA and chelated calcium was decanted and the slices washed three times with T.C. 199. Two pairs of EDTA treated slices were kept for estimation of calcium and for microscopy.

Calcium determination—Two or three of the untreated slices and the same number of EDTA treated slices from each specimen were washed in T.C. 199, blotted and then dried to constant weight in vacuo over P₂O₅. Each slice was dissolved completely in 1 millilitre of concentrated nitric acid. These samples were evaporated to dryness, the residue redissolved in 5 millilitres of distilled water and calcium estimated by the oxalate method (Varley 1967). Alternatively, the acid digests were diluted with ammonia (0.880) until the pH was about 9.0 and calcium estimated by direct titration with 0.0025 M or 0.00125 M disodium EDTA using Eriochrome Black T as indicator. Results obtained were expressed as percentage dry weights of the original slice.

Digestion of collagen—Each of the other eighteen pairs of slices was resuspended in 10 millilitres of T.C. 199 containing 2.5 milligrams of collagenase (Sigma, Type I, 125 units/milligram). They were shaken for 2-3 hours in the water bath at +38 degrees Celsius until the slices had disintegrated. The turbid suspensions were centrifuged individually at low speed (700-800 g) for 5-10 minutes and each of the deposits washed with 10 millilitres of T.C. 199 to remove the collagenase. In a few experiments freshly cut slices were treated with collagenase first and then EDTA in the concentrations and for the times given above.

Histological, histochemical, cytological and scanning electron microscopy studies—Undecalcified slices (100 μ thick) of arthritic human femoral heads and normal goat femoral condyles were mounted on slides and stained with Euchrysine 3R (1 in 50,000 in T.C. 199). They were examined by incident fluorescent illumination (Bard, Dickens, Smith and Zarek 1972). Other undecalcified slices were quenched in liquid nitrogen, freeze-dried at -40 degrees Celsius, using the Pearse Speedivac tissue freeze dryer (Edwards High Vacuum, Mark 2), coated with gold-palladium and examined by scanning electron microscopy, using a Cambridge Stereoscan (Mark II). Slices which had been treated with EDTA were also stained with Euchrysine and examined by incident fluorescent lighting; an excitation filter (Zeiss, B.G.12, 4 millimetres thick) and a barrier filter (Zeiss, No. 53) were included in the path of light. In one experiment, slices were freeze-dried, for scanning electron microscopy. Other slices were fixed in neutral buffered formol saline, processed histologically, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin, van Gieson or von Kossa's stain following the techniques of Bancroft (1967).

Staining and examination of deposits—The deposit from complete digestion of a pair of slices in one flask was resuspended in 0.5 millilitre of T.C. 199 and cell counts carried out in a haemocytometer chamber. Eosin dye exclusion tests were done as described by Hanks and Wallace (1958). Fixed smears were stained with haematoxylin and eosin or by the periodic acid-Schiff reaction following Pearse's modification of McManus's method (Pearse 1968). Unfixed samples of the deposit were mounted on agar-coated slides, stained with Euchrysine 3R and examined by phase contrast and transmitted fluorescent lighting using a Zeiss photomicroscope II, fitted with a dark ground condenser. Photomicrographs were taken using Kodak High Speed Ektachrome film (daylight).
Fig. 2

100 μ thick section of normal goat femoral condyle stained with Euchrysine 3R, viewed by incident fluorescent lighting. (×150.) The articular cartilage is on the left.

Fig. 3

A single trabeculum in the normal goat cancellous bone, prepared and examined as for specimen shown in Figure 2. (×380.)
RESULTS

Microscopic appearances of slices—When the freshly cut 100 μ slices of normal goat bone were stained with Euchrysine and viewed with incident illumination, the columns of the chondrocytes in the articular cartilage fluoresced vividly but the matrix gave a greenish background fluorescence so that cytological details were obscured (Figs. 2 and 3). The osteocytes in lacunae in the underlying spicules of cancellous bone emitted a bright yellowish green fluorescence against the dark non-fluorescent calcified matrix. The marrow also fluoresced but individual cell outlines overlapped and cell types could not be distinguished.

In contrast, some of the fresh slices of arthritic human bone (Fig. 4) had a ragged rim of residual cartilage matrix containing a few chondrocytes; others were edged with burnished bone on the articular surface. Near the articular surface the cancellous bone was abnormal; some areas were acellular but the matrix gave faint yellow fluorescence under the burnished areas. Trabeculae were replaced by a considerable thickness of dense but relatively acellular bone. Elsewhere there were curious whorl-like structures outlined by faint lines which fluoresced pale orange (Fig. 5). The cells in the matrix surrounding these whorls were rounded and fluoresced brightly; they were intermediate in size between chondrocytes and osteocytes. In other areas there were well marked channels, heavily outlined by greenish fluorescence leading from marrow cavities towards the articular surface. Some of these channels connected with cysts of varying size containing amorphous debris. The marrow cavities near the surface of the arthritic bone contained faintly defined fat globules and a few fluorescing cells. Three to 5 millimetres away from the arthritic surface the trabeculae were apparently normal and contained osteocytes which fluoresced vividly as in the goat bone (Fig. 6). In this area, the marrow cavities contained much fat and many haematopoietic cells which fluoresced but were difficult to identify. Photomicrographs of the fluorescing slices were subsequently compared.
Another specimen of arthritic human bone, prepared and examined as for specimen shown in Figure 4. (x150.) The remains of the articular cartilage are on the left.

A single trabeculum in human cancellous bone, about 1 centimetre from the arthritic surface of the femoral head, prepared and examined as in specimens shown in Figures 3 to 5. (x380.)
with stained paraffin sections of fixed decalcified bone from the same specimens of normal goat femoral condyle or arthritic human femoral head. In the arthritic bone the articular cartilage was ragged, almost acellular, or absent altogether and the degenerate underlying bone contained cystic areas; under the burnished surface there were denser areas in which many osteocytic lacunae were empty. Other areas stained deeply with haematoxylin and there were eosinophilic fibrotic areas. The whorls seen by fluorescence were obvious and looked degenerate. The sections were too thin to show up continuous channels. Further from the surface the histological structure was more normal and osteocyte nuclei were clearly seen in the cancellous spicules. The paraffin sections showed nothing which had not previously been identified in the fresh slice viewed by fluorescence. After dipping in distilled water the fresh slices of normal condyles or arthritic human femoral heads still showed brilliant fluorescence of osteocytes in the normal areas of cancellous bone, but the marrow cells had ruptured and nothing but acellular debris including isolated nuclei remained.

After treatment with EDTA the outline of the cancellous trabeculae appeared blurred but brightly fluorescing osteocytes could still be seen against a dull greenish background fluorescence.

When the cut surface of slices of normal goat condyle were viewed by scanning electron microscopy, vascular channels showed up clearly. A pattern of lamellae was clearly seen. It was distinct from straight lines made by the diamond blade, which could be avoided when photographing the surface at higher magnifications. On the surface of the lamellae there were small projecting bodies with processes, some of which appeared to enter the matrix (Figs. 7 and 8). The appearances suggested that these might be bone cells, seen in the fresh, unfixed material by fluorescence microscopy.

When the slices had been treated with EDTA, the surface seen by scanning electron microscopy was undulating; lamellae were not clearly visible and contours of cells barely distinguishable.

### TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Number of specimens</th>
<th>Percentage of dry weight</th>
<th>Initial calcium concentration</th>
<th>After EDTA</th>
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<tbody>
<tr>
<td>Normal goat</td>
<td>3</td>
<td>25.9</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Arthritic human</td>
<td>4</td>
<td>19.0</td>
<td>6.8</td>
<td></td>
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</table>

Estimation of calcium in slices—The calcium content of fresh and EDTA treated slices of normal goat bone and arthritic human bone are summarised in Table I. It is interesting that the initial calcium content was lower in the arthritic human bone than in the normal goat bone. The residual calcium after treating the slices with EDTA was, however, higher in the arthritic specimens than in the normal ones.

The optimum EDTA concentration—One per cent EDTA was insufficient to decalcify the slices of bone, which remained hard and did not disintegrate after treatment with collagenase. Similarly, when slices were treated with collagenase before EDTA in any concentration, the slices remained intact. When 5 per cent EDTA was used, and followed by collagenase, the slices disintegrated completely, but no recognisable cells were found in the deposit. The optimum concentration was found to be 1.5 per cent. Three per cent could probably be used if the incubation time were reduced, but after eighteen hours few living cells were recovered.

Cell count—The total number of cells with processes in the deposits obtained from a pair of
Fig. 7
Scanning electron micrograph of a microtome slice (100 μ thick) of normal goat bone. (×450.)

Fig. 8
Same specimen as shown in Figure 7. (×960.)
slices from the normal goat condyles varied between 107,000 and 428,000. Occasionally cells, lacking processes, were seen but not counted. The total cell count from pairs of slices of arthritic human bone ranged from 72,000 to 805,000. Average figures are shown in Table II. The eosin dye exclusion tests indicated that 80-90 per cent of the cells with processes were alive in the deposits from digested goat bone slices, whereas only 70-75 per cent were alive in the deposits from human bone slices (Table II).

**TABLE II**

<table>
<thead>
<tr>
<th></th>
<th>Number of specimens</th>
<th>Total number of cells with processes</th>
<th>Percentage live</th>
<th>Percentage dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal goat</td>
<td>5</td>
<td>3.11 x 10^7</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>Arthritic human</td>
<td>9</td>
<td>5.05 x 10^7</td>
<td>74</td>
<td>26</td>
</tr>
</tbody>
</table>

Microscopical appearances of the isolated cells—When the deposit of goat cells was viewed by fluorescence microscopy with transmitted light, large numbers of slender cells with multiple delicate processes were seen. The nuclei, some of which appeared to be spiral or twisted, fluoresced a brilliant green; there were minute cytoplasmic granules which fluoresced red at first, but faded quickly to yellow (Fig. 9). Phase contrast microscopy showed up the granular cytoplasm particularly well; the processes were also clearly delineated and unmistakably branched. A few bipolar and rounded cells were also seen by phase and fluorescence. In some preparations, there were many large multinucleated cells with five to twenty nuclei, some of which flattened down to form flat sheets of cells on the substratum (Fig. 12). This histological appearance was not characteristic of the arthritic human bone slices.
which fluoresced more vividly than others. These cells, which might have been osteoclasts, had a wavy outline (Fig. 10). The entire cytoplasm fluoresced a faint yellow and discrete granules could only be distinguished by phase contrast.

In the deposit from the human arthritic bone slices, large numbers of the multinucleated osteoclast-like cells were also seen. Slender cells with multiple processes were present, together with many plumper cells with elongated oval nuclei and tapering cytoplasmic processes. The nuclei of these cells fluoresced bright green and conspicuous cytoplasmic granules which faded from red to orange and yellow were seen. These granules were difficult to photograph (Fig. 11). By phase-contrast, the granularity of the cytoplasm and the branching of the processes were most apparent. In addition, the deposit from the slices of arthritic human femoral heads contained a wide variety of rounded cells which resembled chondrocytes from articular or fibrocartilage. There were also bipolar cells with long unbranched processes which were more like fibroblasts or, perhaps, mesenchymal cells in various stages of differentiation. Some of these cells might have been osteoblasts. Other rounded and oval cells were difficult to classify (Fig. 12).

**DISCUSSION**

We feel that the Metals Research Macrotome is a useful tool for research on normal or pathological calcified tissues. The use of incident fluorescent illumination on slices stained with an acridine orange type of dye might be valuable in studying biopsy specimens of pathological bone.

In addition, it is easy to distinguish live and dead areas of bone by incident fluorescent illumination of stained slices. This technique has already been used in studies on mammalian femoral condyles in which the bone had been killed by freezing with a cryosurgical probe while the cartilage was kept alive by irrigation with warm tissue culture medium (Smith 1971, 1972). More recently the chemical and physical damage to living cells, caused by polymerisation of methacrylate cement in cancellous and compact bone, has been demonstrated by cutting macrotome slices and examining them by incident fluorescence after staining with Euchrysine 3R (Külbel 1972).

Comparison of histological preparations of arthritic bone from the same specimens as the macrotome slices suggested that we had not missed any important features of the specimens although the individual marrow cells, which were readily seen in the 7 μ thick sections, could not be identified by incident fluorescence, using the 16 x objective. However, the greater thickness of the 100 μ thick slices enabled us to follow the course of the channels connecting marrow cavities with the surface of arthritic bone.

The appearance of the isolated cells as seen by fluorescence and phase contrast microscopy suggested that they might be alive, in spite of the fact that the human femoral heads had been kept on ice for twenty-four hours between the time of surgical removal and cutting the macrotome slices. The eosin dye exclusion test confirmed that the majority of cells were alive and suggested that it would be worth while to culture the isolated cells in vitro and to study their metabolism.

It was interesting that the concentration of calcium in the human arthritic bone was so much lower than in the normal goat bone. This result explained why some areas of the arthritic femoral heads cut like cheese. It was even more interesting that after treatment with...
**Fig. 11**
Cells isolated from human cancellous bone prepared as for specimens shown in Figures 9 and 10. (×960.)

**Fig. 12**
Cells isolated from arthritic human femoral head, prepared as for specimens shown in Figures 9 to 11. (×960.)
EDTA for eighteen hours the slices still contained appreciable concentrations of calcium. The EDTA had apparently removed a higher proportion of the calcium from the normal goat bone than from the human arthritic bone. The lowest residual calcium concentrations were in decalcified slices of goat bone; they were at least 100 times greater than the concentration of calcium in mammalian liver or muscle (Eichelberger and McLean 1942). The cells in the bone had not, therefore, been completely depleted of calcium. It was significant that no cells were recovered from slices which had been treated with 5 per cent EDTA. Another interesting point was that the slices did not disintegrate when they were exposed to collagenase before EDTA and no isolated cells were recovered. This suggested that there might be sufficient mineral surrounding the collagen bundles to prevent access of the enzyme. It was quite clear that we should examine the ultrastructure of the cells and matrix of EDTA treated slices by transmission electron microscopy. We were also curious to see electron micrographs of sectioned isolated cells.

SUMMARY

1. The use of the Metals Research Macrotome for cutting 100 μ thick sections of fresh, unfixed specimens of arthritic human femoral heads and normal goat condyles is described.
2. A technique for isolating living cells from these slices by decalcification followed by enzymic digestion is reported.
3. The microscopic appearances of the fresh slices, the decalcified slices and the isolated cells as seen by incident or transmitted fluorescent lighting, by phase-contrast microscopy, by scanning electron microscopy and by histological and cytological techniques are illustrated.
4. These techniques might be applicable to the examination of biopsy specimens of pathological bone or to basic research on bone cells.

We are deeply grateful to Dame Janet Vaughan who kindly read this manuscript and made valuable suggestions. We are indebted to the Medical Research Council who financed this work. One of us (D. R. Bard) was supported by a University of Surrey Research Studentship. We gratefully acknowledge the technical help of Mr C. Tillson. The calcium estimations were carried out by Mr E. P. Morris who was also an expert macrotome operator and helped in other technical matters. Mr P. Scobie-Trumper kindly took care of the goats. The attachment for incident fluorescent lighting was lent by Dr J. Britten of the Metallurgy Department of this University. Dr P. Goodhew allowed one of us (M. J. Dickens) to use his scanning electron microscope and Mrs G. Gibbs demonstrated its use. Figure 1 was generously provided by Dr G. L. Sturgess of Metals Research Ltd.

REFERENCES


Vol. 56 B, No. 2, May 1974
ULTRA-STRUCTURE, IN VITRO CULTIVATION AND METABOLISM OF
CELLS ISOLATED FROM ARTHRITIC HUMAN BONE

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Biomedical Engineering Group, Mechanical Engineering Department, University of Surrey

Methods have been described for examining 100 µ thick sections of fresh cancellous bone from arthritic human femoral heads. After removal of most of the marrow cells and the inorganic and organic matrix, a deposit containing cells with many branched processes was recovered (Bard, Dickens, Smith and Zarek 1972). When examined by the eosin dye exclusion test or by phase contrast or fluorescence microscopy a high proportion of these cells appeared to be alive (Bard, Dickens, Edwards and Smith 1972). In order to verify their viability we cultured them in vitro and tested a single aspect of their metabolism—their ability to take up labelled proline and form hydroxyproline. We have also examined the ultrastructure of freshly isolated cells by transmission electron microscopy for comparison with the various excellent pictures of osteocytes, osteoblasts and osteoclasts already published in recent years by Fitton-Jackson (1957), Gonzales (1961), Cameron (1963), Hancox and Boothroyd (1964), Baud (1968), Hancox (1972) and others.

METHODS

The techniques for cutting the sections of arthritic human bone or normal goat bone have already been described; the method for isolating bone cells by decalcifying the 100 µ thick slices and then digesting them in collagenase was also given in detail (Bard, Dickens, Edwards and Smith 1974).

Cell cultures—The deposits from sixteen flasks containing the digests of either arthritic human bone or normal goat bone slices were pooled. The cells were spread either on the surface of a 30-millimetre diameter Nunclon petri dish (Sterilin), divided into two unequal segments with a glass rod 27 millimetres long, or on a coverslip in a Leighton tube. The cells were cultured in the chemically defined Fitton-Jackson modification of Biggers B.G.J.b. medium, prepared with added ferrous sulphate dihydrate (1 µ gram/millilitre) and freshly dissolved ascorbic acid (400 µ grams/millilitre), 1-glutamine (400 µ grams/millilitre) and 1-proline (1,600 µ grams/millilitre). Half a millilitre of the final medium was delivered into each culture chamber from a 10-millilitre syringe through a millipore filter (G.S. 0.22 µ, 25 millimetres, white, plain) which had been prewashed with 20 millilitres hot distilled water and 10 millilitres medium. Finally, 0.5 millilitre of medium containing Nystatin (96 units/millilitre, Sigma) was added to each culture from a 2-millilitre syringe. A rough cell count was performed on the petri dishes using an inverted microscope with an eyepiece graticule. The petri dishes were then put into a Mackintosh-Fildes jar and gassed with 5 per cent CO₂ in air. The Leighton tubes were gassed individually and stoppered with silicone rubber bungs. The cultures were incubated at +37 degrees Celsius. The medium was changed after forty-eight hours and daily thereafter. Sometimes samples of the medium were tested for acid and for alkaline phosphatase activity by the p-nitrophenol method (Linhardt and Walter 1963).

Proline metabolism—When the incorporation of C¹⁴ proline was to be studied, the cells were set up in petri dishes in the complete medium and cultured for four, six or eight days. At the next change of medium unlabelled proline was omitted and 5 µ Ci 1-proline uniformly labelled with C¹⁴ (87 µ Ci/milligram, Radiochemical Centre, Amersham) added with the Nystatin. The cultures were incubated for four days with one medium change after two days. The cells were washed from the bottom of the petri dish, homogenised and combined with all the radioactive...
medium which had been removed previously. Two milligrams carrier 1-proline were added to this homogenate and it was dialysed against running tap water for at least eighteen hours and then against 3 x 2 litre changes of distilled water. The dialysate was evaporated to 2-3 millilitres and transferred to a glass ampoule. An equal volume of concentrated hydrochloric acid was added. The ampoules were sealed and the contents hydrolysed at 115 degrees Celsius for forty-eight hours. The hydrolysate was evaporated to dryness at 95 degrees Celsius made up in 3 millilitres distilled water and re-evaporated three times to remove the last traces of hydrochloric acid. The residue was finally made up in 0.1 millilitre of 10 per cent Propan-1-ol (n-propanol) in water and duplicate 20 μ litre aliquots spotted on to 20 x 20 centimetres thin-layer plates (Silica Gel G, 0.25 millimetre thick). These together with standard plates were developed in two dimensions. The solvent for the first dimension was Butan-1-ol/glacial acetic acid/water (4 : 1 : 1 v/v) and the solvent for the second dimension, phenol/water (3 : 1 w/w) (Brenner and Niederwieser 1960). The spots were detected by a polychromatic ninhydrin reaction (Moffat and Lytle 1959) and the areas corresponding to 1-proline, 1-hydroxyproline and 1-glutamine were scraped carefully into liquid scintillation vials; 0.4 gram Cab-O-Sil and 10 millilitres toluene containing 5 gram/litre of the scintillator, 25-diphenyloxazole (PPO) and 0.3 gram/litre, p-bis-2-(4-methyl-5-phenyloxazolyl-benzene)(POPOP) were added to each vial and the samples were counted on a Tracerlab liquid scintillation counter. Background counts were made for each plate on areas of silica gel taken from between the proline and hydroxyproline and equal in size to the larger of these two. Counting efficiencies were determined with C14 hexadecane internal standards (Radiochemical Centre, Amersham).

Transmission electron microscopy—Pieces approximately 4 millimetres² from EDTA treated slices of arthritic human femoral heads were fixed at 4 degrees Celsius for four hours in 5 per cent glutaraldehyde buffered with phosphate; they were post-fixed in 1 per cent osmium tetroxide (Sabatini, Bensch and Barnett 1963) dehydrated in alcohol and embedded in araldite (Glauert, Rogers and Glauert 1956). These blocks were cut with an LKB ultratome using 45-degree glass knives, made on the LKB knife-making machine. The sections were stained with uranyl acetate and examined in a J.E.M. Model 100B transmission electron microscope. In some experiments ten deposits of isolated cells were pooled, fixed, dehydrated and embedded in araldite, sectioned and stained as above for transmission electron microscopy.

Cytology and histochemistry of cultures—Fresh coverslip cultures of cells from Leighton tubes were examined at intervals varying from twenty-four hours to fourteen days. They were mounted on agar coated slides, stained with Euchrysine 3R and viewed both by phase contrast and fluorescence microscopy. Other coverslip cultures were fixed in neutral buffered 10 per cent formol saline and stained by haematoxylin and eosin, or van Gieson. Some specimens were examined histochemically for the presence of alkaline phosphatase by Bancroft's Naphthol AS-BI method or for calcium by Bancroft's modification of von Kossa's method (Bancroft 1967). Some fixed cultures were dehydrated, coated with gold palladium and examined by scanning electron microscopy using a Cambridge Stereoscan (Mark II).
processes. In older cultures the cells sometimes appeared to be embedded in an eosinophilic matrix. Mitotic figures were seldom seen. A few large, multinucleated osteoclast-like cells were identified in the cultures up to the eleventh day. Coverslip cultures stained with Euchrysine and viewed by transmitted fluorescent lighting showed cells with nuclei fluorescing vividly green and many cytoplasmic granules which faded rapidly from red to orange.

![Cells from human cancellous bone at seventh day of culture, stained by von Kossa's method for calcium. (x180.)](image)

When coverslip cultures four or more days old were stained by von Kossa's method, large amounts of calcium were seen in association with clumps of cells. Most of this mineral appeared to be extracellular, and not obviously crystalline. These calcium deposits were associated only with cells. There were also minute granules of intracellular calcium in some cells (Fig. 1).

Other four-day cultures on Leighton tube coverslips were stained for alkaline phosphatase; small red granules probably containing the enzyme were seen within the cytoplasm of some of the cells with processes. No alkaline or acid phosphatase activity could, however, be detected in the medium.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>INCORPORATION AND HYDROXYLATION OF C\textsuperscript{14} PROLINE BY CULTURES OF MATURE BONE CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of specimens</td>
</tr>
<tr>
<td><img src="image" alt="Image" /></td>
<td>Normal goat</td>
</tr>
<tr>
<td><img src="image" alt="Image" /></td>
<td>Arthritic human</td>
</tr>
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</table>

Proline metabolism—The thin layer chromatographs showed a clear separation of proline and hydroxyproline from one another and from other amino acids. Up to sixteen amino acids could be resolved by this technique. Alanine and glycine were particularly prominent. All the radioactivity, however, was limited to proline, hydroxyproline and glutamine. Of these three spots, proline showed the greatest activity, with much smaller, but significant activity associated with the hydroxyproline (Table I) and glutamine. The incorporation of activity...
into hydroxyproline was stimulated by the addition of ferrous iron. The hydroxyproline did not vary noticeably with increasing age of the cultures.

Scanning electron microscopy—Cells which have been cultured for forty-eight hours adhered firmly to the coverslips from Leighton tubes. The majority of cells had long, finely branched processes (Figs. 2 to 4). Some of the cells also had short unbranched projections which jutted out from their bodies as though anchoring them to the coverslip.

Transmission electron microscopy—In electron micrographs of sections of EDTA treated slices from arthritic human femoral heads the osteocytes had well defined cell membranes with many

Fig. 2
Stereoscan electron micrograph on an isolated bone cell after cultivation in vitro for forty-eight hours. (×2,400.)

Fig. 3
The same as in Figure 2. (×4,250.)

Fig. 4

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microvilli, some of which extended into the surrounding matrix. The nuclei also had clearly
delineated membranes. Variable amounts of loosely packed nuclear chromatin were seen;
nucleoli were sometimes present. The cytoplasm of some osteocytes contained mitochondria,
centrioles and dense microbodies. Microtubules were observed in some cells (Fig. 5). These
appearances suggested that the cells had been in a state of high metabolic activity. Each
osteocyte was surrounded by a perilacunar space. The matrix bordering the lacunae was, in
many instances, depleted of calcium and clearly banded collagen fibres could be seen. Farther
away from the margins of the lacunae, electron dense calcium was abundant.
Cell pellets—All the cells freshly isolated from human arthritic bone that were subsequently
sectioned and examined by transmission electron microscopy had intact cell membranes. In

some cells the nucleus was irregular in shape or even in two or more parts, although obviously
not in mitosis; the nuclear membranes were intact. Some of the nuclei were dense and
contained tightly packed chromatin. In the cells with these dense nuclei the perinuclear
cytoplasm was scanty; they all had long processes, but few, if any, microvilli were present;
mitochondria were clearly seen, some close to the nucleus and others situated well down the
processes; other cytoplasmic organelles were not obvious (Fig. 6). By contrast, the cells with
paler nuclei had abundant perinuclear cytoplasm, containing many mitochondria and much
rough endoplasmic reticulum; in most instances distinct Golgi areas were also obvious;
microvilli were numerous but had seldom been sectioned throughout their length (Figs. 7 and 8).

DISCUSSION

We were interested to note that a high proportion of the cells cultured had multiple
processes and that this type of cell persisted for many weeks in the chemically defined B.G.J.b.,
medium modified as described. It is not surprising that the numbers of cells had declined

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after the first change of medium, but it was satisfactory that there was no further decrease for the subsequent two weeks. Neither an increase in cell numbers nor many mitotic figures could have been expected because we were not using a "growth medium". On the other hand the persistence for eleven days of the large multinucleated cells which resembled osteoclasts was gratifying, particularly as Hancox (1960) had already reported that osteoclasts survived for seven days in cultures. Our multinucleated cells might, of course, have been formed from other cells during the period of cultivation.

The incorporation of proline showed that the cells from normal goat bone and from the arthritic human bone were actively metabolising; the synthesis of hydroxyproline indicated that they were possibly manufacturing collagen precursors. The hydroxyproline/proline ratio was very much lower than would be expected if collagen precursors were the sole products of protein synthesis; this low ratio suggests that collagen production was a relatively minor activity of mature bone cells under our conditions of cultivation. The figure might have been modified by the presence of osteoclast-like cells in the cultures.

The deposits of calcium in and around the clumps of cells, and the presence of intracellular alkaline phosphatase suggest that the cells were also concentrating calcium.

The scanning electron micrographs of the cultured cells confirmed the persistence of long, branched processes; the short projections from the cell bodies were similar to those illustrated by Boyde, Weiss and Vesely (1972). The transmission electron micrographs of the slices which had been treated with EDTA suggested that some of the osteocytes were highly active. This tentative conclusion was reached because of the presence in the cytoplasm of numerous mitochondria as well as microtubules, centrioles and dense unidentified microbodies, and because their nuclei were lightly stained. One question which might be raised is whether some of the collagen immediately bordering the lacunae had been freshly laid down or whether this area had been differentially decalcified in vivo or in vitro.

A further question is how many of the cells isolated were osteoblasts, differentiating, or undifferentiated mesenchymal cells. We were inclined to think that most of the cells on the bony surfaces would have been destroyed during our attempts to remove the marrow. Live osteoclasts were, however, identified amongst the isolated cells and some osteoblasts or other
Figure 7—Another isolated cell, as in Figure 6. (x 53,000.) Figure 8—A third isolated cell with dark irregular nucleus in two parts. (x 66,800.)
osteoprogenitor cells may have survived. The bipolar cells with long processes isolated both from the normal goat and arthritic human bone resembled closely some of the cells found on bony surfaces (Vaughan 1972a, 1972b).

The behaviour of the cultures suggests that the proportion of osteoprogenitor cells was probably small. Alkaline phosphatase, which is present in osteoblasts and in most other osteoprogenitor cells in situ (Owen 1970) was identified in only some of the cells after seven days. Furthermore the cells did not manufacture nearly as much hydroxyproline as did foetal osteoblasts maintained under similar conditions (Fitton-Jackson 1965). At no time were there enough osteoclasts present to be counted separately and their total number was probably very small. Hancox has, however, commented on the high rate of bone resorption by small numbers of osteoclasts (Hancox 1972) and it is conceivable that they may have made an appreciable contribution.

Our experiments up to date raise many problems. They indicate that further experiments on cells isolated from normal and pathological bone from adult mammals and man would be of interest. Their behaviour during cultivation in different media, containing varying concentrations of calcium, other mineral salts, calcitonin, parathyroid hormone, corticosteroids, and vitamins A and D might be instructive. Both the proline and calcium metabolism could be studied in greater detail and under different conditions. Changes in ultrastructure during cultivation would be interesting. It might also be instructive to compare the metabolism of embryonic and mature bone cells. Our chief conclusion is that much more work on bone cells is needed.

SUMMARY

1. Methods for culturing cells isolated from slices of arthritic human or normal mammalian cancellous bone are described.
2. The capacity of the cultured cells to take up and hydroxylate labelled proline has been investigated.
3. Sections of the partially decalcified bone and of the isolated cells have been examined by transmission electron microscopy.
4. The possible significance of the results and observations are discussed.

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