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Heberden Oration, 1971

The influence of microscopic technology on knowledge of cartilage surface structure

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Of all th'Inventions none there is Surpasses
The Noble Florentine's‡ Dioptrick-glasses.
For What a better, fitter guift Could bee
In this world's Aged Luciocity
To Helpe our Blindnesse so as to devize
A paire of new & Artificiall eyes,
By whose augmenting power wee now see more
Than all the world Has ever donn Before.

DR. HENRY POWER (1661)

It is a privilege to be invited to give this, the 25th Heberden Oration.

In spite of intensive enquiry, the normal mode of lubrication enjoyed by synovial joints remains unexplained (Fig. 1). Fluid film, boundary (McCutchen, 1966), hydrodynamic, elasto-hydrodynamic, and mixed lubrication systems have been considered (*Lancet*, 1969; Dowson, Wright, and Longfield, 1969) and a further possibility, 'weeping' lubrication, has been proposed (McCutchen, 1959, 1962). More recently, the role of electrostatic forces at articulating surfaces has been investigated (Roberts, 1971). In each of these hypothetical mechanisms the thesis had been accepted that these surfaces are 'strikingly smooth' (Davies, 1969). The demonstration that normal load-bearing surfaces might not, after all, be smooth (Dowson, Longfield, Walker, and Wright, 1968; Gardner and Woodward, 1968; Jones and Walker, 1968; Walker, Dowson, Longfield, and Wright, 1968; Inoue, Kodama, and Fujita, 1969; Walker, Sikorski, Dowson, Longfield, Wright, and Buckley, 1969), dramatically changed the theoretical approach to articulation mechanisms. A suggestion emerged (Fig. 2), supported by substantial data obtained with cartilage *in vitro*, that pools of lubricant, trapped between opposing undulations on the cartilage surfaces, could lose fluid, concentrate hyaluronate-protein, and ensure efficient lubrication by a unique 'boosted' system (Longfield, Dowson, Walker, and Wright, 1969).

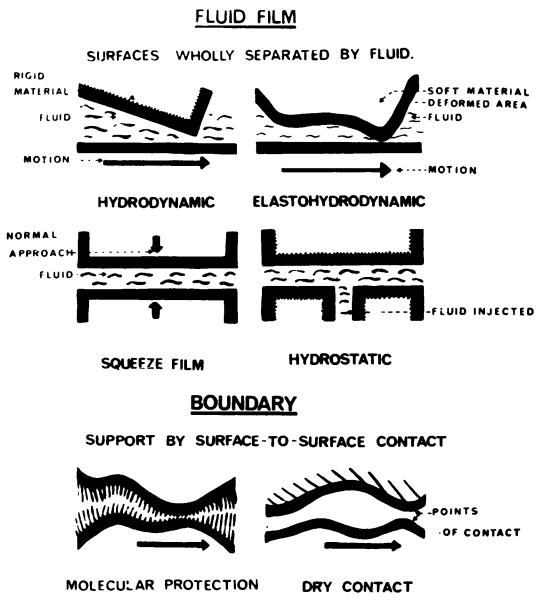

FIG. 1 *Proposed mechanisms of joint lubrication* (Redrawn after Walker and others, 1968)

Much of the support for this proposed mechanism of 'boosted lubrication' would be lost if it could be shown that the gentle surface undulations detected by talysurf tracings and identified by scanning electron microscopy were artefacts caused by the methods used to isolate and manipulate cartilage *in vitro*. During the past 2 years it has therefore been a main interest of this laboratory to examine load-bearing cartilaginous surfaces in detail under conditions that approximate as closely as possible to

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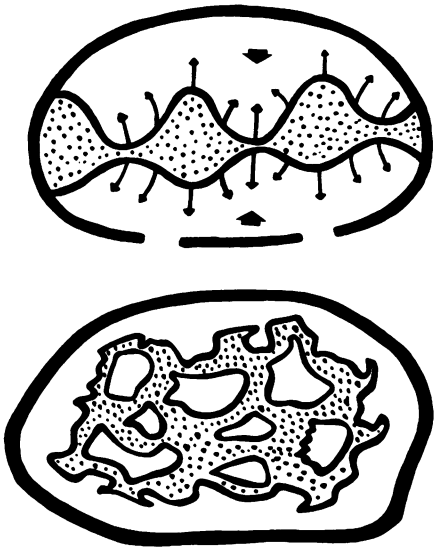


FIG. 2 *Boosted lubrication. Fluid trapped between cartilage surfaces (top). 'Trapped pool' of lubricant seen on surface of cartilage loaded with glass (bottom)* (From Dowson and others, 1969, and Longfield and others, 1969)

those that obtain during life. It has been my concern to contrast the appearance of carefully prepared cartilage surfaces viewed with the scanning electron microscope with similar surfaces, either prepared by a wide variety of non-disruptive biological techniques or seen *in vivo* in synovial joints having an intact blood supply.

The Heberden Oration offers an opportunity of presenting the data that have accumulated during these investigations and of describing previously unpublished results that may help to explain the configuration of loaded articular surfaces *in vivo*. In order to give full consideration to the technical limitations imposed by each method of study employed, I propose to approach my description of articular cartilage surfaces through brief outlines of the various forms of microscopy used. As a device to place advancing knowledge of cartilage surface structure in historical perspective, the descriptions of microscope technology have an historical bias. First, the design and optical pathways of the microscope systems will be described. The preparative techniques that have been used will then be outlined. The extent to which each form of microscope has contributed to present knowledge of cartilage surface structure will be indicated, and finally, on the basis of the most recent observations, a view of cartilage surfaces will be proposed that accords with the new data.

THE EVOLUTION OF MICROSCOPES* APPLICABLE TO THE STUDY OF CARTILAGE SURFACES

(A) **Light microscopy** (Turner, 1967, 1969)

SIMPLE MICROSCOPES

The simple microscope evolved from the spectacle lenses that are said to have been introduced between 1280 and 1311 (Rooseboom, 1956; Rosen, 1956). The single-lens microscope, the forerunner of the modern dissecting instrument, was made by mounting a plano- or biconcave lens. Fleas were more common in society than they are today: they provided convenient objects for the early single-lens microscopists. The early instruments, therefore, were often termed 'flea-glasses' (Bradbury, 1967).

It was established that the magnifying power of a single lens increased in proportion to an increase in the radius of curvature. A diagram from Bradbury (1967) illustrates this point (Fig. 3).

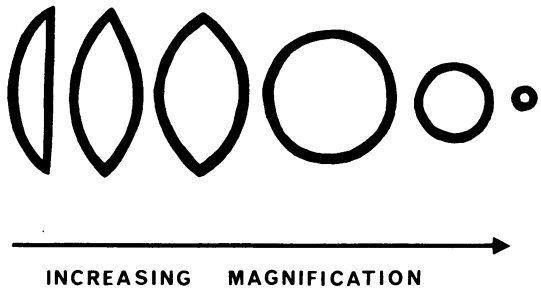


FIG. 3 *Effect of diminishing radius of curvature on magnification. Highest magnification results from smallest lenses*

This principle led to the construction of simple microscopes with progressively smaller glass lenses, which were either 'blown' or ground and polished (Fig. 4). Superior results were obtained with ground lenses: they provided a sharp image over a larger portion of the field of view and were the choice of that master of all early lens-makers, Antonie van Leeuwenhoek. Leeuwenhoek's lenses were very small: one was 1 mm. in diameter and had a radius of curvature of 0·75 mm. Two other examples from the hundreds he made magnified ×79 and ×126 respectively: they provided, in 1660–70, a resolving power of 3·5 μm.

With the simple microscope, Leeuwenhoek (1693–1718) and his successors observed the circulation of the blood, the structure of striated muscle, the life cycle of insects, red blood cells, protozoa, hydra, and (probably) bacteria (Dobell, 1932). Leeuwenhoek's instruments were technically very difficult to use: the

* **Microscope**: an optical instrument, consisting of a lens or a combination of lenses (or, rarely, also of mirrors) by which objects are so magnified that details invisible to the naked eye are clearly revealed (*Shorter Oxford Dictionary*).

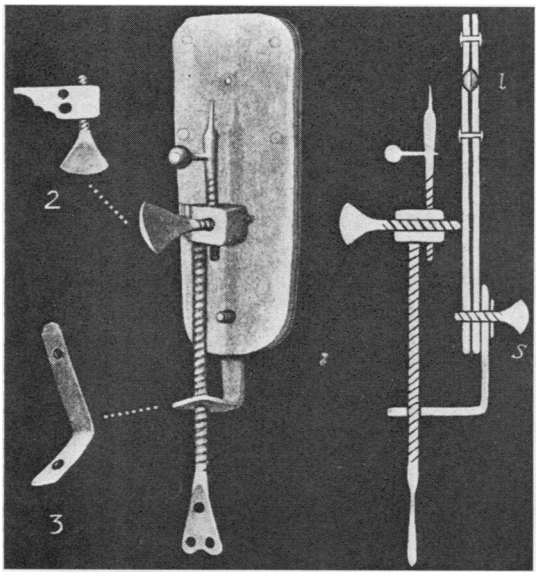


FIG. 4 *One of Leeuwenhoek's microscopes. Note lens (l) (top right)* (From Dobell, 1932, p. 328)

working distance was very short, the field of view small; the observer's eye had to be very close to the microscope and illumination can only have been by daylight or candle. Nevertheless, the lenses were relatively free from chromatic aberration and, fitted with a device for moving a specimen across the field of view, permitted great advances in biological knowledge. In spite of the technical progress made with these instruments, the only suggestion that I have found of their possible use to study cartilage surfaces is contained in the 1742–43 paper of William Hunter. The paper, which is not illustrated, concludes, erroneously, that articular surfaces are smooth and polished.

COMPOUND MICROSCOPES

It is widely accepted that Hans and Zacharias Janssen, and Hans Lippershey, of Middleburg in Holland, played an important part in making compound microscopes during the years 1590–1609. Galileo Galilei (Fig. 5) is thought to have used a (Galilean) telescope as a microscope as early as 1609–10 by extending the length of the tube.* Galileo's instrument, with a concave eye lens, was inconveniently long. The telescope of Kepler (Bradbury, 1967), with a convex eye lens, was shorter and more readily managed: the majority of the compound microscopes of the succeeding three centuries have had convex eye lenses.

* Evidence for this opinion came from John Wedderburn, a Scottish student at Padua, who heard Galileo describe in 1610 how he had distinguished the organs of small animals and an insect by means of his telescope (Fahie, 1903). It is established that Galileo presented a compound microscope to the King of Poland in 1612 and that he helped to explain the mode of action of another instrument to a mystified Cardinal in 1624.

FIG. 5 *Galileo* (From Fahie, 1929)

The early years of exploration with the compound microscope are inextricably associated with the name of Robert Hooke ('Espinasse, 1956), whose wonderfully inspired book 'Micrographia', published by the Royal Society in 1665, is still an enthralling account, not only of the mechanics of microscopy, but also of Hooke's first views of the objects he was required to describe, week by week, to the Society (Figs 6, 7 overleaf).

I have not been able to find any account of bone, tooth, cartilage, or soft connective tissue in the 'Micrographia', but insect eyes, hexagonal connective tissue prisms, were early objects of microscopic interest. The versatility of the pioneer microscopists was considerable: Isaac Newton, in a critique of Hooke's book, commented that insects could be individually anaesthetized before microscopy (Keynes, 1960).

Compound microscopes were much easier to use than simple microscopes. The observer's eye could be some inches from the object and a conveniently long working distance was obtained. Substantial progress with the technical development of compound microscopes was made during the 17th and 18th centuries (Fig. 8): there were mechanical stages, tilting devices, coarse and fine focusing screws, and a variety of forms of focused, external illumination. The fundamental problem remained, however, that lens-makers had not learnt to correct their glasses for the spherical and chromatic aberrations which dreadfully distorted magnified images and diminished resolution. Even the most conscientious observer could be grossly misled by these imperfections. Consequently, the compound microscope was held in contempt as a plaything for amateurs: Bichat (1800, 1802), the

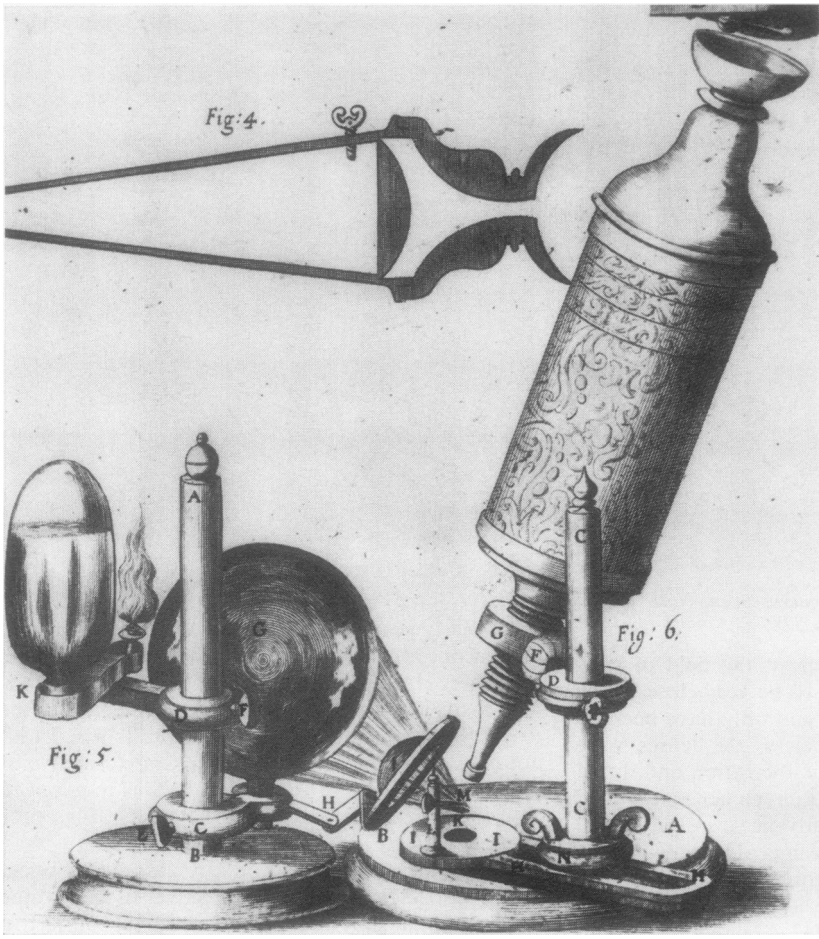


FIG. 6 *Robert Hooke's Microscope*, 1665 (From Hooke, 1665)

FIG. 8 *Culpepper microscope of ca.* 1735 (From Rooseboom, 1956)

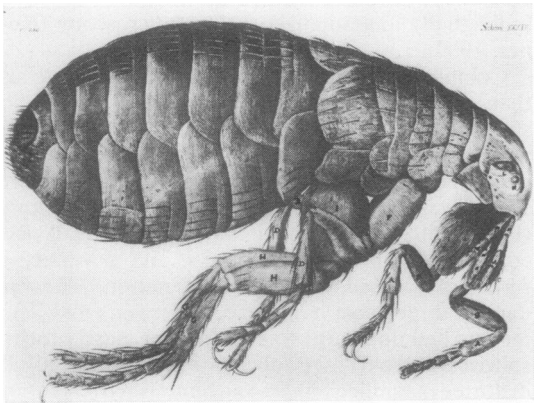


FIG. 7 *Drawing of flea published in Hooke's 'Micrographia' (1665), illustrating the detail resolved by compound microscopes in the 17th century*

'father' of histology, refused to be misled by using a compound microscope: his views on cartilage structure were consequently primitive and superficial.

The advent of the achromatic lens marked a turning point in the microscopic enquiry into cartilage

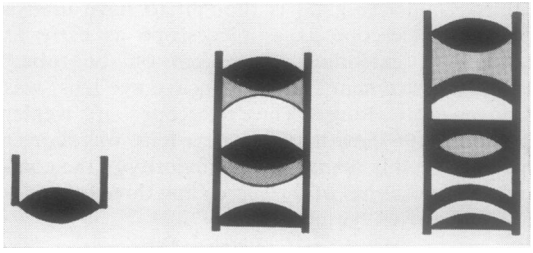


FIG. 9 *Development of achromatic (centre) and apochromatic (right) lenses. Introduction of combination of crown and flint glass correcting spherical and chromatic aberrations of simple lens (left)*

structure (Hughes, 1955, 1956). The advance came gradually. Achromatic lenses (Fig. 9), made by opposing and later cementing together two or more lenses of crown and of flint glass, glasses having different refractive indices, were found to correct chromatic aberration and simultaneously to diminish spherical aberration. Lenses corrected in this way were available by the middle of the 18th century, and were first used in telescopes. The objective lens of Beeldsnijder (1791) was probably the first to be made for microscopy, but the principle was not widely applied in practice until the first decade of the 19th century. Even then, it may be claimed that compound microscopes could contribute very little to the analysis of connective tissue structure until after 1823–24 when Selligue introduced achromatic lenses capable of giving useful resolution at magnifications of up to ×200. This advance, the correction of residual spherical aberration by Amici (1818), and the papers by J. J. Lister (1830, 1913) (Fig. 10) rationalizing the design of fully corrected lenses, were the turning points in modern light microscopy. Henceforth, the instrument could be used with assurance to define the detailed structure of biological materials in general and of the cartilaginous connective tissues in particular.

The consequences were dramatic. It is generally accepted that the work of the botanist Schleiden (1838) and of Schwann (1839), a pupil of Muller, established the cell theory (Cameron, 1952). Armies of workers, with the new achromatic microscopes made in growing numbers in France, Britain, Germany, and Holland, attacked the body tissues systematically. New concepts of the cell as the unit of biological and thus of connective tissue structure, and of the nucleus as the determinant of cell division, soon penetrated the field of pathology. These concepts were first formulated as a systematic explanation for the origin of disease by Goodsir and Goodsir (1845) and by Virchow (1858). Further advances in microscope design, the use of microtomy in preparing thin sections of body tissues (His, 1870), the introduction of condensers, and the design of apochromatic objective lenses (Abbe, 1886) that could be used with homogeneous (oil) immersion systems (Abbe, 1879), led to the identification of bacteria as aetiological agents in diseases and to the recognition of cell organelles such as mitochondria and the Golgi apparatus.

ULTRAVIOLET MICROSCOPY

The light microscope reached this final peak of optical development as early as 1900: the resolving power ($\delta = 0{\cdot}6\lambda/\text{N.A.}$ for dry lenses) of glass lenses had approximated to its theoretical limit. The use of homogeneous immersion lenses of high Numerical Aperture helped to increase resolution. It appeared, however, that the only way to permit further improved resolution would be a reduction in the wave length of the light used for microscopy below that of the visible spectrum. A microscope based on this principle was therefore designed by Kohler (1904) to operate in the ultraviolet spectrum. Theoretically, resolution of the order of twice that obtainable with light of $\lambda = 550$ nm. could be obtained with ultraviolet light of $\lambda = 275$ nm. To focus was difficult: this problem could be overcome, it was thought, by designing a reflecting microscope in which chromatic aberration was inconsequential. Given accurate focusing, a valuable additional tool for surveying the details of connective tissue would be available. However, there is no record that microscopes of this kind have ever been used to define articular cartilaginous structure.

PHASE CONTRAST AND INTERFERENCE MICROSCOPY

A remarkable advance in light microscopy was made by Zernike in 1933, when he showed that differences in optical density of tissue, examined *in vivo* or *in vitro*, could be translated into differences in amplitude of light waves (Fig. 11, overleaf) (Zernike, 1942).

Living unstained preparations could be examined and the detailed structure of preparations

XIII. *On some properties in achromatic object-glasses applicable to the improvement of the microscope. By* Joseph Jackson Lister, *Esq. Communicated by Dr.* Roget, *Secretary.*

Read January 21, 1830.

THE improvement of the achromatic compound miscroscope having been an occasional object of my leisure for several years past, my attention has in consequence been attracted to some properties of object-glasses of short focus and large aperture, which, so far as I am aware, have not been before noticed, and which, I flatter myself, may be applied to increase its powers and the ease of its manufacture.

FIG. 10 *Title of Lister's paper* (1830) *describing theoretical basis of design of achromatic objective lenses*

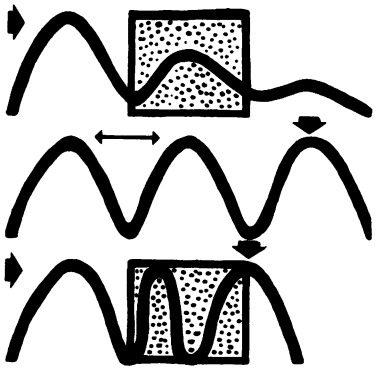


FIG. 11 *Principles of phase contrast microscopy. Normal light wave (at centre) with wave passing through stained tissue (at top) and through non-stained tissue (at bottom). Note diminished amplitude of wave emerging from stained material, retardation of wave emerging from non-stained material*

of living connective tissue cells in culture rendered visible.

Zernike's device took advantage of the approximately ¼ wavelength retardation of phase imposed on a light wave passing through biological material of varying density but uniform appearance. By introducing a further ¼ wavelength retardation on light transmitted through the material, an interference effect was created, resulting in different wave amplitude. Tissue components exerting this effect are seen in sharp amplitude contrast. This method (phase contrast microscopy) permits thin unstained cartilage sections to be examined at high resolution by transmitted light in the living state, after freeze-drying or after fixation.

A further modification of the phase contrast-interference effect, interference microscopy, has been used to measure dry tissue mass as well as to demonstrate differences in refractive index, a function of dry mass, in optically homogeneous tissue. The phase shift caused by the passage of light through the biological material under examination is related to an independent non-retarded reference beam. When the two beams are reunited a change in phase of one is indicated by a difference in amplitude and thus of visible contrast.

Very recently, the principles of interference microscopy, use so successfully in the transmission mode to identify, measure, and weigh biological components such as cell nuclei, have been applied to reflected light microscopy. The reflected light system, adopted for the measurement of surface irregularities by metallurgists, engineers, and crystallographers, offers very considerable scope for the analysis of specialized biological surfaces such as those of articular cartilage. In recent work, I have been able to make qualitative observations of three-dimensional surface structure by the Nomarski system (Fig. 12) (Lang, 1968; Allen, David, and Nomarski, 1969) and measurements of the height of cartilage undulations by the Linnik (1933) system. It may be noted that, under incident interference conditions, an image of a cartilage surface can be obtained, at high resolution, in colour. Contrasting coloured interference fringes represent differences in the path length of the incident light and thus in the distance of the illuminated cartilage surface from the eye or camera. Studies by differential interference contrast microscopy of cartilage surface structure can be used therefore to measure differences in surface smoothness without the difficulties inherent in conventional incident light microscopy of contrast differences caused by the variable absorbance of light at cartilage surfaces. Artefacts that might be introduced by variable drying or a differential distribution of synovial fluid can be avoided.

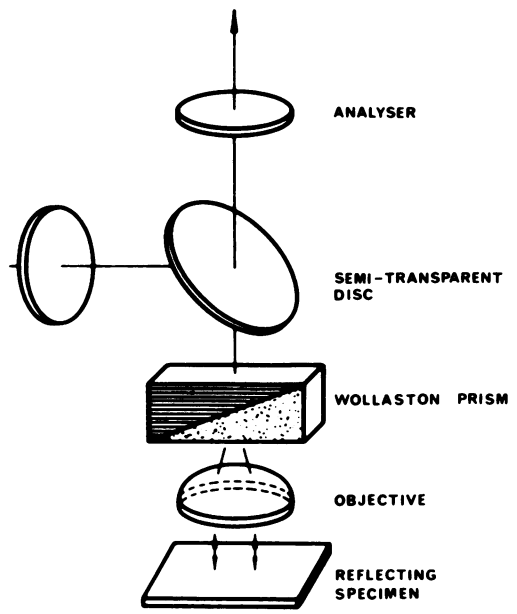


FIG. 12 *Principle of Nomarski interference microscope. Reference beam recombines with beam reflected from specimen to give interference effects*

(B) Acoustic microscopy

Prototypes have been described of an instrument that may prove to be of value in the study of articular surfaces *in vivo*. A high frequency sound wave can be reflected from the cartilage. The reflected wave is transformed into an electric current, which, when amplified, can be displayed on an oscilloscope screen. Resolution is limited but the capacity to work with the living joint must be regarded as of great potential value. The instrument has not yet, to my knowledge, been tested in the study of articular cartilage.

(C) Electron microscopy

TRANSMISSION ELECTRON MICROSCOPY (Fig. 13)

With the propagation of the quantum theory and the introduction of the wave theory of the electron, the possibility of developing electromagnetic lenses for a microscope was explored. Credit for the first patent went to Rüdenberg (1931) but the first instrument used was that of Ruska (Marton, 1968). A prototype was built in 1931 (Knoll and Ruska, 1932). By 1934, sufficient technical progress had been made to enable the first micrographs of biological material (Marton, 1934). The earliest commercially available instrument, built by Siemens, came shortly afterwards (1938). It was not until the 1950s, however, that the connective tissues were first examined by electron microscopy and the earliest account of the structure of thin sections of articular cartilage, as opposed to shadowed preparations of collagen or elastic material, was published in the early 1950s (Mulvey, 1967).

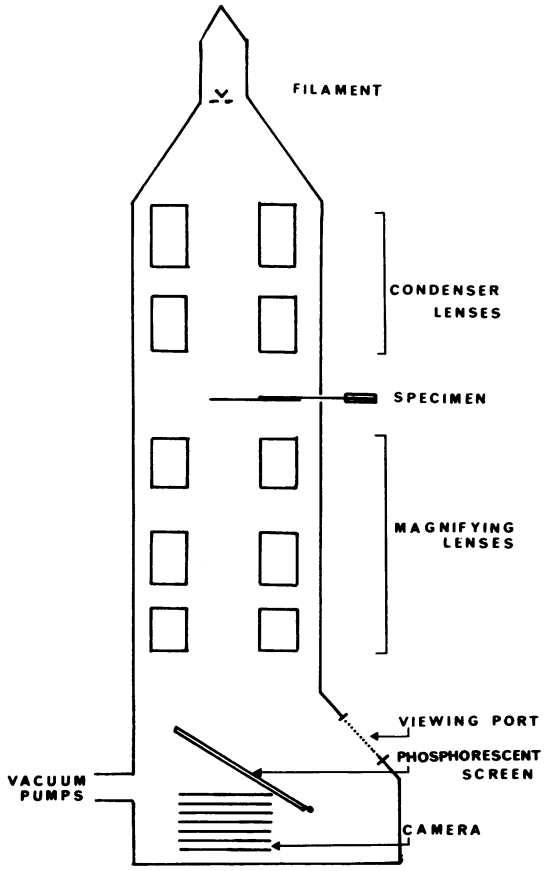


FIG. 13 *Diagram of transmission electron microscope. Electron beam emitted from filament is focused on specimen and yields electron image on phosphorescent screen*

The technical advantages and disadvantages of transmission electron microscopy require only brief mention. The high resolution instrument provides approximately 100 times as much detail as a light microscope but is limited by the need to study dry sections of not more than 40–60 nm. thickness under high vacuum. Contrast is low and staining often essential. Until recently, the fixation of tissue was almost always a necessary preliminary to microscopy. Biological specimens tended therefore to be shrunken and distorted even before they were bombarded by an electron beam.

SCANNING ELECTRON MICROSCOPY (Fig. 14)

In 1965, it became possible to use a new form of electron microscope in which focused electron beams, deflected by fields controlled by a scanning generator, are passed across the surface of a specimen. The beam is conveniently moved in a rectangular form like a television raster, but can also be moved in circular or spiral forms or arranged to give a stepwise incremental scan following a digital input (Nixon, 1971). The primary electrons are scattered at the surface of a specimen exposed to the electron beam, and are detected by an electron collector. In addition to backscattered electrons, the primary electron beam

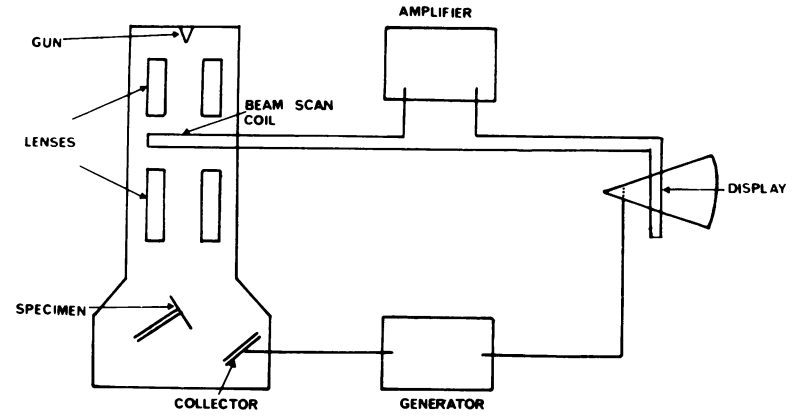


FIG. 14 *Scanning electron microscope. Electrons, scattered from surface of specimen, are collected and displayed on cathode ray tube*

also produces secondary electrons, x rays, and light of various wavelengths.

A dry specimen placed, under high vacuum, in the path of the electron beam of the scanning electron microscope can not only be rotated and tilted but can be heated, cooled, stretched, strained, etched, or probed within the instrument. Electrons, scattered from the specimen surface, which is usually coated with a very thin (*ca.* 20 nm.) layer of protective, electrically conductive metal, provide a signal which is enhanced or processed as required. It is convenient to present the amplified signal as a visible image on a display cathode ray tube, the beam of which is scanned in the same manner as the beam in the main electron optical column. By reducing the area of the specimen surface scanned, an increase is obtained in the viewed magnification (Crewe, 1971). The magnified, viewed image is readily available for photography but the specimen signal may be as easily read on closed circuit television, or recorded in storage tubes, facsimile printers, computer interfaces, or tape recordings.

The instrument offers two immediate advantages to the student of joint surfaces (Echlin, 1971). First, a resolution of >30 nm. can be obtained at magnifications from ×20 to ×50,000, *i.e.* up to 100 times greater than with the incident light microscope. Second, the depth of field at any comparable magnification is several orders of magnitude greater than that obtainable with the best light microscope.

ELECTRON MICROPROBE ANALYSIS (Fig. 15)

When tissue is exposed to an electron beam, as in the scanning electron microscope, not only are electrons backscattered but x rays are emitted. The wave length of these x rays is characteristic of the chemical elements exposed to the beam. A carefully focused electron beam can be used in this way to detect any element in the tissue with an atomic weight >5. The electron beam, focused at the surface of the tissue to a spot 1 μm. or less in diameter, causes the emission of characteristic x rays that are analysed by x-ray spectroscopy. An intermediate diffractor is used to select the x rays with wavelengths characteristic of those element(s) in which the observer is interested, while discriminating against all other x rays. The amount of these selected x rays is then measured by means of an x-ray detector. Quantitative determination of the elements is possible by comparison with known elemental standards (Hall, 1971).

This extremely important technique of electron-probe x-ray microanalysis has begun to be applied to the study of cartilage surface structure. I shall refer later to our early results.

CARTILAGE SURFACE STRUCTURE

I turn now from this brief review of instrumentation to the subject of cartilage surface structure in the study of which I have employed most of the varieties of techniques described.

Little of this revolution in instrument technology had penetrated to the study of the articular connective tissues until very recently: the microscopic structure of articular cartilage, seen in section, was well known by the middle of the 19th century, but investigators were preoccupied with more urgent medical problems than mechanisms of articulation. Consequently it is not surprising to find that articular cartilaginous surfaces were neglected. Indeed, so far as I can determine, the first incident light photomicrograph of such a surface was not published until 1969 (Gardner and Woodward, 1969).

Gardner and McGillivray (1971b) have recently summarized the older literature relevant to the surface structure of articular cartilage. Weichselbaum (1877) and Hammar (1894) were among the few observers who commented on the apparently rough structure of this material. Nevertheless, a widely held opinion that articular surfaces are 'strikingly smooth' persisted until 1968. That opinions on the smoothness of articular cartilage have changed so radically since that date is largely attributable to the commercial availability of the scanning electron microscope. Yet optical instruments with the resolving power needed to prove the uniform presence of patterns of microscopic irregularities and undulations on articular surfaces were freely available before 1900. Indeed, simple microscopes capable of resolving structures as small as chondrocytes were widely employed through-

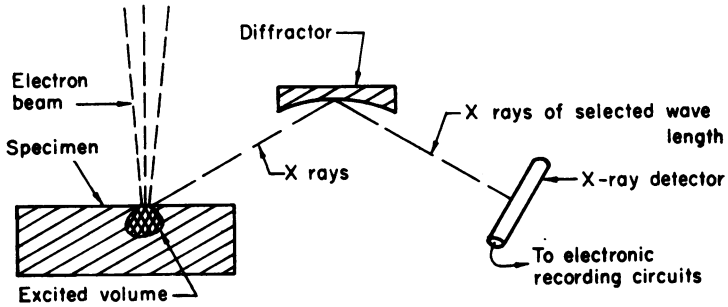


FIG. 15 *Electron microprobe analyser. Electron beam focused to give* 1 μm *zone of bombardment provokes emission of characteristic x rays from approximately* 10 μm^3 *volume of tissue* (From Hall, 1971)

out the 17th century: their use was retarded by difficulties of specimen preparation and of illumination.

In my own observations on cartilage surface structure, my colleagues and I have been closely aware of the hazards of misinterpretation due to preparative or instrumental artefact. The earliest microscopists were also familiar with this problem.*

METHODS OF PREPARING CARTILAGE SURFACES FOR STUDY (Table I)

There is no part of contemporary research in the rheumatic diseases where an appreciation of misleading and artefactual data is more important than in the study of articular surfaces. The usual scientific dilemma must be faced: how is it possible to define the structure and behaviour of a biological material without changing the object under investigation? Compromise is inescapable. During the subsequent parts of this paper the steps we have taken to eliminate artefact will I hope gradually become clear.

In the greater part of the present work, the experimental object has been the inbred (Edinburgh) albino Wistar rat. Human, pig, rabbit, mouse, turkey, and chicken cartilages have been investigated less fully. The largest rat joints studied, the knee and hip, have therefore been less than 1·0 cm. in diameter, the total mass of tissue less than 1 g. The advantages of using so small a species are clear; the disadvantages include the possibility that joint appearances recognized in the rat, an omnivorous quadriped of peculiar form, may differ from those characteristic of other mammals. In favour of the rat is the evidence (Simon, 1970, 1971) that differences in animal size correlate well with different cartilage thicknesses.

Our early studies were made with the whole lower end of the rat femur, dissected out from animals killed either by Nembutal anaesthesia or by trauma. The joint surfaces were neither washed nor touched; they were plunged into cold, buffered 1 per cent. osmic acid, allowed to fix at 4°C. for 24 hrs, and dried in air at room temperature. These preparations, subject to possible distortion and artefact caused by ischaemia, fixation, and slow drying, gave our first indications of mammalian articular surface structure.

However, a variety of other preparative methods have now been compared in terms of incident light and scanning electron microscopy (Table I). Their relevance to the interpretation of articular surface structure will be touched upon in subsequent pages. The particular importance of conserving glycosaminoglycans by avoiding their loss during fixation and dehydration has attracted much comment (Gardner, 1970, 1972). Highton and Donaldson (1970), for example, have confirmed the advantages of using an agent such as ruthenium red in the scanning electron microscopic study of synovial joints. It appears likely that, in future work, rapid freezing/freeze-drying techniques will be preferred to the conventional glutaraldehyde/acetone/air-drying procedure that has been the basis of so much early biological scanning electron microscopy (Fujita, Tokunaga, and Inoue, 1971).

* Their difficulties were clearly expressed by Robert Hooke (1665), who wrote in 'Micrographia' that he constantly endeavoured to 'first discover the true appearance (of a structure), and next to make a plain representation of it. This I mention rather, because of these kinds of objects there is much more difficulty to discover the true shape, than of those visible to the naked eye, the same Object seeming quite different, in one position to the Light, from what it really is, and may be discovered in another'.

Table I *Preparative techniques used in the studies of cartilage surfaces described in this paper*

Living tissue:	Untouched Washed with 0·9 per cent NaCl Covered with polyvinylpyrolidone for immersion microscopy
Fresh, excised tissue:	Surface untouched Snap frozen (liquid nitrogen, or hexane chilled with acetone/CO_2) and freeze-dried
Fixed tissue:	Unbuffered 1 per cent Os 1 per cent Os in B 2·5 per cent Gl in B followed by 1 per cent Os in B 5 per cent Gl in B containing 1 mg./ml. RR (overnight). Then 3 washes in B containing 1 mg./ml. RR. Then 1 per cent Os containing 1 mg./ml. RR (2 hrs). Dehydrate in acetone; air dry. 1·4 per cent Gl in B containing 1·5 mg./ml. RR (5 hrs). Then 3 washes in B containing 1 mg./ml. RR (20 min. each). Then 2 per cent Os in B containing 1·5 mg./ml. RR (2 hrs). Vacuum dry. 1·4 per cent Gl in B containing 1·5 mg./ml. RR (5 hrs). Then 3 washes in B containing 1 mg./ml. RR (20 mins. each). Wash in water. Vacuum dry.

Os: Osmic acid. Gl: Glutaraldehyde.
RR: Ruthenium red. B: 0·1 N cacodylate buffer, pH 7·3.

OBSERVATIONS

I come now to our own observations made with these various techniques.

Incident light microscopy

With the Leitz Ortholux microscope, fitted with ×6·5 and ×11 incident light objectives, high intensity, coaxial illumination is supplemented by an external, oblique light source that can be adjusted to give illumination at a low angle. With this instrument, *osmic acid-fixed, air-dried* articular cartilage is found to have a non-smooth surface in which a pattern of undulations, each approximately the diameter of a chondrocyte, can be recognized (Gardner and McGillivray, 1971a). The surfaces of rapidly-frozen, *freeze-dried* cartilage display corresponding irregularities; they are difficult to photograph because the light microscope has a very limited depth of focus and small cartilages are acutely angled. The fibrocartilaginous surfaces of mammalian menisci bear the same undulations as do the surfaces of hyaline cartilage, but are composed of a coarse meshwork of interlacing fibres on which the undulations are superimposed. Curiously, the surface structure of hyaline cartilage in a young avian species, the turkey, quite closely resembles the surface appearance of mammalian fibrocartilage.

Scanning electron microscopy

Beginning in 1968, four groups of workers took advantage of those features of the scanning electron microscope that I have described to you, to study the surface structure of articular cartilage. McCall (1968a, b) made the first photomicrographs of osteoarthrotic cartilage; Inoue, Kodama, and Fujita (1969) scanned the cartilage surfaces in normal and rheumatoid arthritic joints; Walker and others (1969) examined the surfaces of portions of excised cartilage loaded *in vitro* and quickly frozen; and Gardner and Woodward (1968, 1969) surveyed the femoral and knee joint cartilage of normal guinea-pigs. Valuable additional information has since been added by Clarke (1971a) and by Mital and Millington (1971), using surface stripping techniques.

These early investigators agreed unanimously that normal articular cartilage, astonishingly, is not smooth. So heretical an opinion required immediate critical review and analysis.

The first specimens that we placed in the scanning electron microscope were whole guinea-pig femoral heads and tibial condyles. Even at low magnifications, the cartilaginous surfaces treated in this way appeared conspicuously un-smooth if the term smooth be used to mean 'an absence of perceptible roughness' (*Concise Oxford Dictionary*). Three orders of irregularity were recognized at low magnifications:

(1) Primary anatomical contours;

(2) Secondary irregularities of about 0·4–0·5 mm. diameter;

(3) Finer, tertiary undulations that measured approximately 20–30 μm. in diameter (Gardner and Woodward, 1969).

The irregularities and undulations were so conspicuous, and so different from the 'strikingly smooth surfaces' of classical texts (Barnett, Davies, and MacConaill, 1961), that I immediately suspected that they were artefacts. They can, however, be identified by the scanning electron microscopy of fresh cartilage taken directly from the joint of an anaesthetized animal and placed immediately on the stage of the microscope. By the use of electron beams at no more than 2–3 kV, useful images can be obtained and surface structure thereby confirmed in unfixed, undried, uncoated cartilage (Fig. 16). In addition, I examined the osmium-fixed, dry specimens with a hand lens, with a binocular stereoscopic dissecting microscope, with an incident light microscope, and by interference microscopy (Fig. 17: col. pl.). The secondary irregularities were easily seen with the hand lens, the tertiary undulations with incident light. By the end of 1969 I was reasonably certain, therefore, that mammalian articular cartilage surfaces, at low resolution, were normally not smooth.

More recently, studies with the Linnik interference microscope have allowed the identification of the surface irregularities of excised rat cartilage, the surface of which has been protected with polyvinylpyrrolidone, washed, and dried with alcohol. A

FIG. 16 *Scanning electron micrograph of surface of rat femoral condylar cartilage viewed at 3 kV, fresh, unfixed, and uncoated. Note damage to specimen caused by drying in instrument and by electron beam.* ×630

pattern of interference fringes was indentified and first measurements of these fringes, recorded in green light of wavelength 520 nm., established that the surface undulations varied in height ('wavelength') from approximately 1·0 μm. to approximately 2·6 μm. The interference images (Fig. 18) also confirmed that there was very considerable variation in height, diameter, and contour.

In order to confirm or refute the significance of these observations *in vivo*, further studies were necessary. I therefore extended my surface studies with incident light to freeze-dried, excised cartilage, to freshly excised, non-dried, unfixed cartilage, and to the intact, untouched articular surfaces of living joints opened under anaesthesia, preserving, so far as possible, a normal blood supply (Gardner and McGillivray, 1971a). The observations were repeated in man, and in the mouse, rabbit, guinea-pig, rat, and turkey. Foetal and newborn joints were also investigated. Without exception, the mammalian articular surfaces examined all presented the shallow tertiary undulations described above. Similar but smaller undulations were present on the surface of foetal material.

Occasionally, the surface appearances of cartilage in a freshly-opened joint differed from normal. Thus, when drying was encouraged by raising the ambient temperature or by blowing a current of warm air across the exposed surface of a rat knee-joint, a pattern of highlights,* each of the order of size of a single surface chondrocyte, appeared within 15 to 20 mins. These surface changes were rapidly provoked when the blood supply to the joint was interrupted. It appeared that the normal surface appearance of a rat knee articular cartilage was closely dependent on the surface being moist and on the preservation of a normal blood supply.

* Zones of increased amplitude of reflected white light.

The evidence suggested strongly, therefore, that the surfaces of mammalian, hyaline articular cartilage, observed by incident light or by scanning electron microscopy, were not smooth (Gardner and McGillivray, 1971a). Coarse irregularities were detectable with a simple microscope, finer gentle 20 to 30 μm. undulations with the compound light or scanning electron microscopes. The undulating surface was consistently present: in the living state, in freshly excised or freeze-dried material, and in material fixed in buffered glutaraldehyde and/or osmic acid with or without added ruthenium red; but the height of the undulations varied considerably in different joints. These views are disputed by Highton (1971), but it appears probable that disagreement is more a matter of interpretation than of distinct experimental results: one of Highton's scanning electron micrographs (his Fig. 1), of rabbit synovia and of nearby patellar articular cartilage, shows the very secondary irregularities and tertiary undulations whose existence he denies.

It seemed probable that the validity of these observations could be tested by making replicas of articular surfaces, both from living and from excised

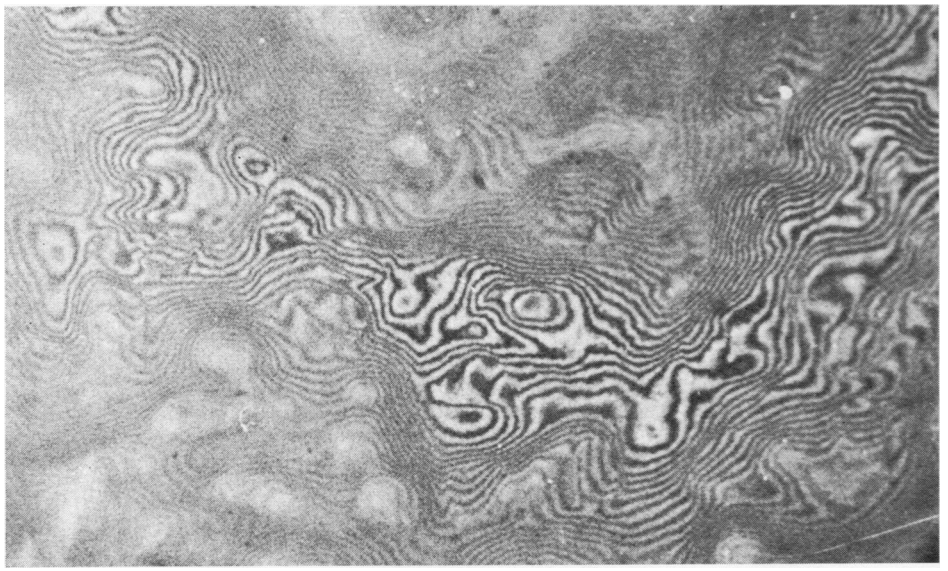


FIG. 18 *Linnik interference photomicrograph of rat femoral condylar surface. Monochromatic (thallium) light of wavelength* 520 *nm. Contours indicate variations in height of surface undulations. Each interference fringe represents a difference in height of* 0·26 μm. *Note irregular areas, and varying heights and different gradients of contours.* × 240

cartilage. Further experiments were therefore made with single and with 2-stage replication techniques. Advantage was taken of the previous experience in this field of Clarke (1971b) and of Walker and his colleagues.

Replica techniques

My colleague D. H. Woodward first suggested that high resolution transmission electron microscopic pictures might be obtained of surface structures by the use of a 2-stage collodion technique (Fig. 19) that he had used for metallurgical studies. The surface is coated with collodion (1) which is then allowed to dry. The collodion is peeled from the joint surface like a 'skin', and coated (2) with carbon under vacuum. The collodion is dissolved away from the carbon, leaving a carbon replica (3), which, placed on a copper grid, can be surveyed by transmission electron microscopy, giving images which have, apparently, a 3-dimensional structure.

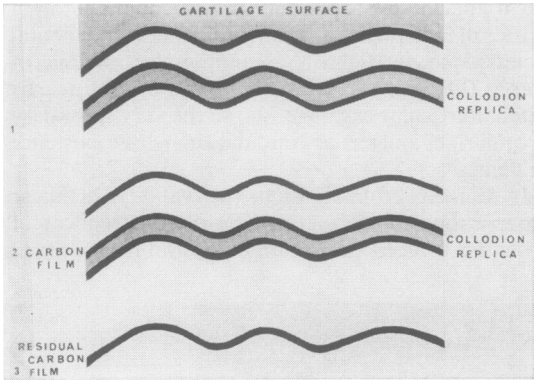


FIG. 19 *Preparation of 2-stage collodion replica from cartilage surface*

The interpretation of 2-stage replicas of cartilage surfaces is not easy. Resolution is of a high order, but the hazards of artefact have constantly to be borne in mind. For studies requiring, at first, resolution of a lower order, I have found that a latex-coating technique, devised by P. Marriott (personal communication), has given worthwhile results (Fig. 20). Replicas of articular surfaces can be readily made by this method. At low resolution, the undulating surface that they reveal can be interpreted by direct comparison with optical or scanning electron microscope images (Clarke, 1971b). At high resolution, the replicated surfaces obtained by the transmission electron microscopy of 2-stage collodion replicas are less easy to analyse.

The data gained from low-resolution surface replication confirmed the presence of the articular cartilage surface irregularities shown by incident light and by scanning electron microscopy.

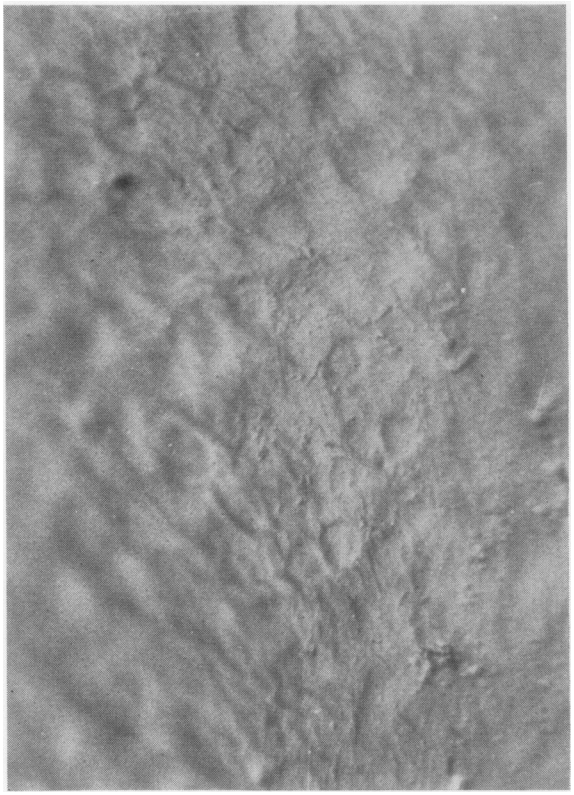


FIG. 20 *Photomicrograph of 1-stage latex replica of femoral surface of rat femur.* ×250

Since it now seemed reasonably certain that conspicuous gentle undulations were a normal feature of hyaline articular cartilaginous surfaces, further questions then arose: namely, 'Of which cartilage components is the surface formed?'; 'What relationship do these component surface structures bear to the surface irregularities detected by low resolution microscopy?'; 'What happens to the living surface structure under load?' In particular an answer to the last question appeared fundamental to the problem of resolving the complex matter of the lubrication mechanism in synovial joints.

I examined cartilage structure first in paraffin and in cryostat section by light microscopy, and secondly in ultrathin araldite section by transmission electron microscopy.

SURFACE STRUCTURE OF CARTILAGE IN SECTION

Light microscopy

10 μm. cryostat sections of undecalcified, unfixed, rapidly frozen rat tarsal joint cartilage were found, by phase-contrast microscopy, to have a conspicuously undulating surface (Fig. 21: col. pl.). The surface

prominences corresponded to the situation of the most superficial chondrocytes. Unfixed sections prepared in this way displayed a narrow surface lamina of dull orange-pink van Gieson-positive material, presumably collagen (Fig. 22: col. pl.). This zone contained little demonstrable metachromatic or Alcian blue-positive matrix. The extensive underlying intermediate cartilage zone yielded a fainter pink colouration with the van Gieson reagents, but contained an abundant metachromatic toluidine and Alcian blue-positive matrix (Fig. 23: col. pl.).

The superficial chondrocytes of the upper half of the articular cartilage differ histochemically from those of the deeper zone. They display no alkaline phosphatase activity; the cells of the deeper half, by contrast, show very high activity. All possess higher succinate, lactate, and glucose-6-phosphate dehydrogenase activities than those of underlying osteocytes. There is detectable 'naphthylamidase' but no β-glucuronidase activity.

Electron microscopy

The most superficial chondrocytes in rat cartilage lie 3 to 6 μm. below the articular surface as measured in fixed dehydrated tissue (Barrand, Gardner, and Bush, 1972a). The chondrocytes are ellipsoidal (Fig. 24) and narrow in the vertical plane; they are elongated laterally, so that mean measurements of 1·5 to 3·0 × 10 to 15 μm. are obtained. It seems unlikely that their shape is not influenced by surface loading. The cells are arranged singly, never in pairs or clusters.

The structure of the superficial chondrocyte of the young rat closely resembles that of similar cells described in other species. Cytoplasmic arms, 40 to 80 × 100 to 150 nm. extend from the lower cell margin into the matrix. A large, ventral, elongated nucleus occupies much of the cell height. There is a moderate amount of rough endoplasmic reticulum, occasional dense and multivesiculate bodies, lipid droplets, and rare single cilia.

Superficial to the surface chondrocyte is an abundant matrix (Fig. 25) in which there are numerous closely-packed fibrils arranged, with few exceptions, parallel to the articular surface. For a depth of 3 to 5 μm. below the surface no perpendicular fibrils are seen. The fibrils have a uniform diameter (20 to 25 nm.); sometimes a recognizable periodic structure can be detected. The articular cartilaginous surface, *i.e.* the most superficial zone of the matrix, is covered by a thin layer of electron dense material that retains ruthenium red (Fig. 26) and is thought to be either a synovial protein-hyaluronate complex with lubricating characteristics or a surface lamina, part of the intercellular matrix, with a proteoglycan structure that is lost during preparation in the absence of ruthenium or cetylpyrridinium chloride (Barrand, Gardner, and Bush, 1972b). Closer to the surface chondrocytes, the collagen fibrils are smaller and lie parallel to the long axis of the cell.

Particularly at the cartilage margins, near the fibrous subsynovial tissue, are occasional patches of thick interlacing collagen fibrils. In other species these fibre aggregates have been misleadingly called 'microscars': they have been recognized near dying

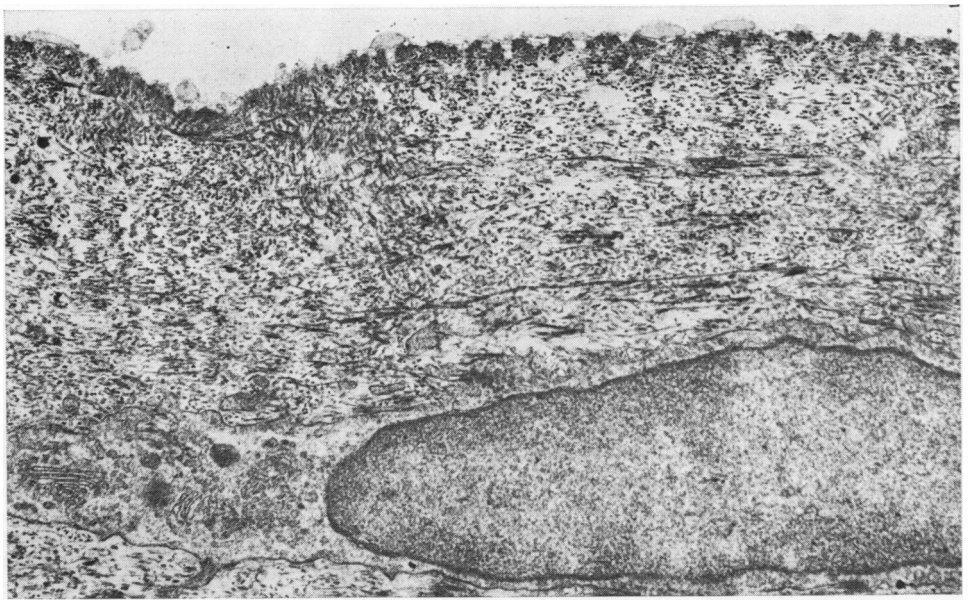


FIG. 24 *Electron micrograph of superficial chondrocyte near cartilage—synovial junction of patellar surface of rat femur. Observe bundles of slender, superficial collagen lying parallel with surface but cut in varying planes.* ×15,300

FIG. 25 *Compare with Fig.* 24. *Deeper cartilage matrix from patellar surface of rat femur. Many thicker collagen fibrils are seen; the arrangement of the matrix collagen is randomly dispersed* (From Barrand and others, 1972a). ×31,000

FIG. 26 *Patellar surface of rat femur, reacted with ruthenium red to show thin lamina of ruthenium-staining material at load-bearing surface*
(From Barrand and others, 1972a). ×79,500

or disintegrating cells. They may be regarded as a non-specific sign of changed fibril-synthetic activity.

The presence of aggregates of membraneous vesicles, of empty vacuoles and of small electron dense particles in the cartilage matrix, especially just beyond the lateral margins of the superficial chondrocytes, has been a feature suggesting cartilage 'partner' cell disintegration *in situ*: this evidence of local superficial cell breakdown is more common in older animals and may be an age change.

A paradox in our first surveys of thin sections by transmission electron microscopy was the apparent absence of recognizable surface undulations corresponding to those detected by all the other techniques used to study cartilage surfaces. Further light was shed on this difficulty when it became clear that the height of the surface undulations, as seen by the light microscopy of cryostat sections or revealed by transmission electron microscopy, varied very considerably in different cartilage areas and in different joints. Thus, cryostat sections of the (less weight-bearing) tarsal joints of the rat show first that the surface chondrocytes are round or ovoid but not flattened and second that the surface over these cells is prominently raised. In comparable sections of the rat ankle-joint, where the cartilage is 6 to 8 cells deep, the surface is much smoother, and the surface undulations much less prominent. This flatter structure is recognized also upon the condylar and central patellar surfaces of the femur, the margins of which tend to display more conspicuous undulations of similar diameter.

In the interpretation of these differences, I kept in mind that blocks cut for transmission electron microscopy might inadvertently extend through the plateaux between surface undulations (Fig. 27, opposite).

It also seemed possible that the speed of sectioning might influence apparent surface structure. We have tested this view at the light microscope level by cutting bone and cartilage sections at different speeds in our bone cryostat (Pearse and Gardner, 1972). No change

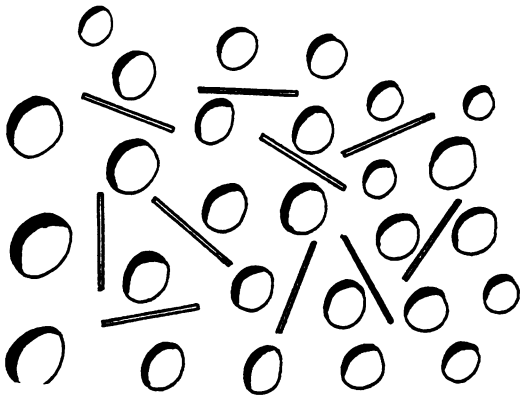


FIG. 27 *Diagram illustrating possibility that electron microscope blocks (black rectangles) could, by chance, be taken from plateaux between cartilage undulations*

in cartilage surface structure was detected when these sections were compared by phase-contrast microscopy, although the incidental observation was made that surface undulations on the weight-bearing surface of the rat ankle-joint were much larger and more conspicuous than those seen on the surface of the rat knee-joint. Deliberate variations in section thickness did not alter the appearance of the surfaces.

Enzymatic digestion (Figs 28–31; 34–37: col. pl., overleaf).

The availability of enzyme preparations capable of degrading cartilage *in vitro* suggested that the use of these preparations might shed light on the structure of cartilage surfaces. In collaboration with Dr. H. Inoue, preliminary investigations of this kind have been made (Table II). Confining our observations to the light microscopy of cryostat sections stained by toluidine blue, Alcian blue, and haematoxylin and eosin, and to the use of the scanning electron microscope at magnifications not exceeding 6000, the following results have been obtained (Table II).

Papain progressively degraded the surface structure with early loss of cartilage intercellular material and the prominence of cartilage cells after 1 hr, and the total disintegration of cartilage structure and the preservation only of the underlying bone after 6 hrs (Figs 28, 29: col. pl.).

Cathepsin D, kindly supplied by Dr. J. T. Dingle, caused the loss of the metachromatic matrix from the whole of the deeper cartilage but spared the superficial, collagen-rich surface lamina (Figs 30, 31: col. pl.).

Scanning electron microscopy revealed total disintegration and loss of articular cartilage from the papain-treated material after 6 hrs (Figs 32, 33, overleaf), but apparent integrity of the cathepsin D-treated surface after 24 hrs.

Electron microprobe analysis

Although the conventional and high-resolution probes have, so far, been used mainly to investigate the phenomena of mineralization in bone formation, considerable amounts of information are beginning to accumulate concerning the mineral content of articular cartilage (Gardner and Hall, 1969). The electron microprobe offers a means not only of de-

Table II *Enzymatic digestion of articular cartilage surfaces* in vitro. *Rat femoral condyles*

Enzyme	*Amount of enzyme*	*Buffer*	*pH*	*Incubation time*	*Results*
Papain	5 mg./ml.	0·2 M PO_4 containing 0·03 M cysteine	6·3	30 min.; 1 hr; 2 hrs; 6 hrs	At 1 hr loss of matrix; at 2 hrs progressive loss; at 6 hrs whole articular cartilage dis-aggregated
	0·5 mg./ml.			2 hrs	
Cathepsin D	600 μ./ml.	0·02 M PO_4	6·0	24 hrs	After 24 hrs cartilage surface intact
	1,200 μ./ml.			24 hrs	Complete loss of metachromatic Alcian blue-positive material from entire deeper cartilage
Muramidase	2 mg./ml.	0·1 M acetate	4·5	2 hrs; 6 hrs	Slight loss of metachromasia
Hyaluronidase	1,500 μ./ml.	0·15 M NaCl 0·005 PO_4	5·0	2 hrs; 6 hrs	Marked loss of metachromasia
Collagenase	1 mg./15 ml.	0·1 M TRIS 0·005 M $CaCl_2$	7·2	1 hr; 2 hrs; 6 hrs	At 6 hrs widespread degradation
Trypsin	1 mg./3 ml.	0·03 M PO_4	7·4	2 hrs; 6 hrs	Slight degradative changes

* Determined by light microscopy of cryostat sections and by the scanning electron microscopy of glutaraldehyde-fixed cartilage surfaces at magnifications of not more than × 6,000. With the exception of Cathepsin D, enzymes were commercial preparations.

FIG. 32 *Scanning electron micrograph of rat femoral condylar surface after 2 hrs' digestion with dilute (1/10) papain solution.* ×700

FIG. 17 *Nomarski differential interference contrast photomicrograph of unfixed femoral cartilage surface of rat. Coloured interference fringes indicate variation in distance from surface undulations to camera.* ×70

FIG. 21 *Phase contrast photomicrograph of unfixed, undemineralized rat tarsal cartilage reacted for lactate dehydrogenase. Observe conspicuously undulating surface of this non-load bearing cartilage and rounded shape of superficial chondrocytes.* ×133

FIG. 22 *Unfixed rapidly frozen undemineralized section of rat tarsal cartilage stained by the von Kossa technique for calcium. Note distribution of cartilage collagen (pink).* ×33

FIG. 23 *Unfixed rapidly frozen undemineralized section of rat tarsal cartilage reacted with toluidine blue and viewed under phase contrast conditions. Note narrow surface lamina of non-metachromatic material, abundant deeper metachromatic matrix.* ×33

FIG. 28 *Section of rat femoral condyle fixed in formalin containing cetylpyrridinium chloride and embedded in paraffin. Note abundant intercellular metachromatic matrix. Toluidine blue.* ×80

FIG. 29 *For comparison with Fig. 28. Paraffin section of rat femoral condyle after 2 hrs' digestion by papain. Note complete loss of superficial articular cartilage. Toluidine blue.* ×80

FIG. 30 *Section of rat femoral condyle after 24 hrs' digestion by Cathepsin D. Note loss of intercellular metachromatic matrix but retention of faint lamina of surface material. Toluidine blue.* ×80

FIG. 31 *For comparison with Fig. 30. Corresponding section stained to show retention of surface collagen. van Gieson.* ×80

FIG. 34 *Rat femoral condylar surface after 1 hrs' digestion with collagenase at pH 7·2. Note diminished quantity of metachromatic cartilage matrix. Toluidine blue.* ×80

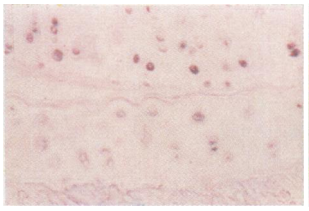
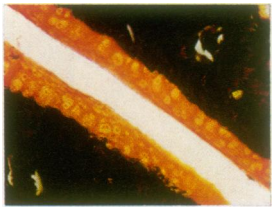
FIG. 35 *For comparison with Fig. 34. After 6 hrs' digestion with collagenase, there is widespread degradation not only of cartilage matrix but of cells and of underlying bone. Toluidine blue.* ×80

FIG. 36 *Digestion with muramidase at pH 4·5 for 2 hrs causes slight loss of overall cartilage metachromatic staining. Toluidine blue.* ×80

FIG. 37 *A commercial preparation of testicular hyaluronidase causes gross loss of cartilage matrix after 2 hrs' digestion at pH 5·0. Toluidine blue.* ×80

FIG. 40 *View of patellar surface of femur in anaesthetized rat, showing mode of application of glass slide*

FIG. 41 *For comparison with Fig. 40. Zone of loading of rat femoral cartilage (centre). Note presence, in zone of loading, of numerous very fine pin-point patterns of reflected light. These are believed to be caused by persistence of tertiary cartilage undulations under load.* × 40

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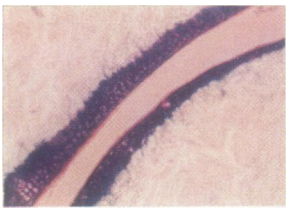
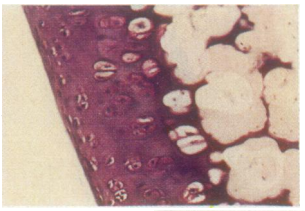
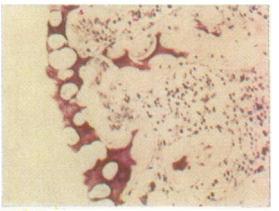

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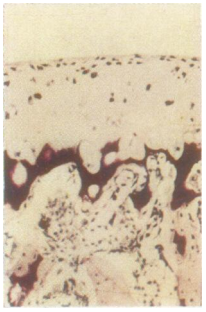
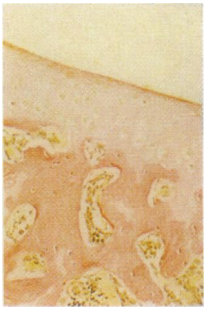
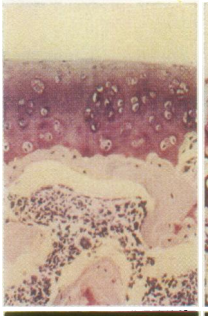
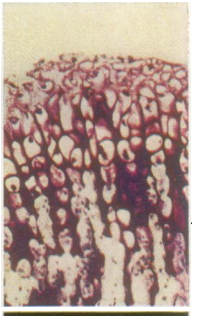

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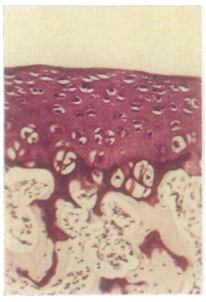
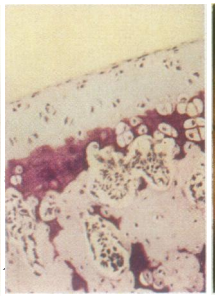
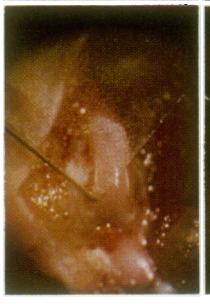
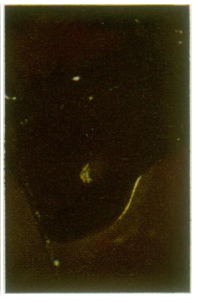

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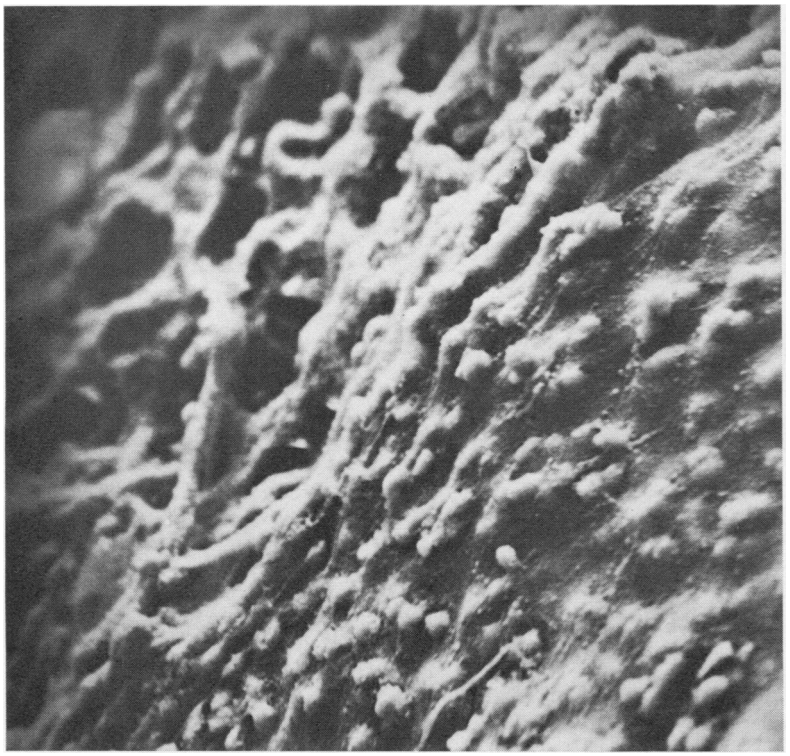

FIG. 33 *Rat femoral condylar surface after 2 hrs' digestion with undiluted papain.* ×595

tecting and measuring cartilage calcium and phosphorus (Fig. 38), but also, by indirect ion-substitution techniques, of identifying the various glycosaminoglycan fractions of cartilage and of determining their relative proportions (Maroudas, 1970). When cartilage is reacted, under controlled conditions, with polyanionic dyes such as Alcian blue, the extent to which different cartilage zones retain the dye also affords a measure of the zonal distribution of the glycosaminoglycans (Scott, 1970). Since Alcian blue possesses a copper moiety, the electron microprobe can be used to detect local concentrations of glycosaminoglycans, simultaneously with measurements of other elements important to cartilage surface structure. The binding of the dye ruthenium red, a property now often used to fix and simultaneously to stain cartilage matrix glycosaminoglycans, offers yet another way of studying matrix structure by electron microprobe analysis. Ruthenium is easily detected by gas or by lithium/silicon x-ray spectrophotometry. The latter principle offers, technically, a means by which the elemental analysis of cartilage surface structure can be undertaken on material that is being viewed by scanning electron microscopy.

In a single pilot experiment of this kind, I easily identified osmium and ruthenium red crystals on the surface of cartilage being scanned in a Stereoscan 4 instrument. In a similar pilot experiment, I failed to identify the copper of Alcian blue at articular cartilaginous surfaces, perhaps because the binding capacity of the limited amount of glycosaminoglycan in the 3 to 5 μm. surface lamina of cartilage is too small to allow an accumulation of enough dye to be identifiable by the particular electron microprobe used.

CONFIGURATION OF LOADED SURFACES *in vivo*

Having established that living articular cartilaginous surfaces bear the same undulating features as can be detected *in vitro* by incident light microscopy and by low-power scanning electron microscopy, it was of paramount importance to determine whether bearing surfaces loaded during normal movement, in life, display the same irregularities. Many recent biophysical measurements of cartilage stiffness, deformation, or loading behaviour have been made with excised material that is, to a greater or lesser extent, non-physiological (Wright, Longfield, and Dowson, 1969; Kempson, Muir, Swanson, and Freeman, 1970; Radin, Paul, and Pollock, 1970). It remained possible that loading would eliminate the undulations, restoring the surfaces to an essentially smooth condition, *i.e.* a smooth state as defined by the techniques used in this low-resolution survey. If this were so, current lubrication theory would be profoundly influenced. Although it seemed possible, at first, to devise a fibre optic probe that could be passed into the loaded joint of small mammals, allowing the living

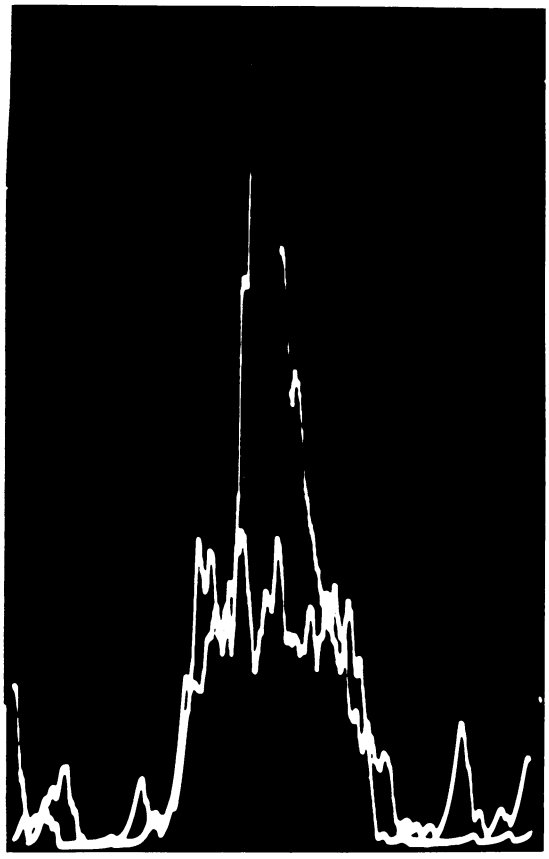

FIG. 38 *Electron microprobe analysis of calcium in turkey epiphyseal cartilage. Curves indicate high peak of calcium trace, flatter peaks of silver trace.* (From Gardner and Hall, 1969).

loaded surface to be studied, and although arthroscopy offered a means of approaching this problem in man, it soon became clear that an alternative preliminary technique could prove more informative.

Something is known of the extent of rat-knee articular surfaces, and of the loads to which they are subjected (Simon, 1970, 1971). Rats were therefore chosen for the present study. The cartilage surfaces of anaesthetized animals were exposed. To the flattest contour of the femur was fitted a small plastic cup, with a glass upper surface, bearing a side-arm that could be connected to a pressure source and a second side-arm where a transducer could be used to measure applied pressures (Fig. 39). The glass cup was filled with a medium, such as polyvinyl alcohol, having synovial fluid-like characteristics. The joint surface was illuminated by coaxial light and viewed by means of a Leitz incident light microscope. However, so small was the femoral plateau, and consequently, so narrow the cup, that when it became desirable to use oblique in place of coaxial illumination no suitable

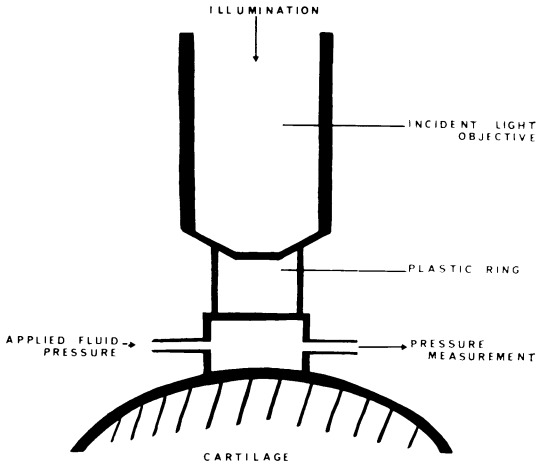

FIG. 39 *Arrangement of glass/metal cup for loading articular surfaces, viewing surface through cup by reflected light*

fibre optic could be inserted into the cup margin. Rather than replace the experimental animal species by another with larger articular surfaces to which a larger cup could be applied, a simpler approach was selected. It was decided to apply loading pressures by means of a glass slide.

A further series of experiments therefore followed:

(1) It was found that contact between the glass and the cartilage surface established a meniscus through which the surface could be viewed by means of a Zeiss operating microscope (Fig. 40; col. pl.).

(2) It was found possible to delineate the zone of contact by allowing Evans Blue or Pellikan ink (colloidal carbon) to encircle the island of contact.

(3) It was demonstrated that, at critical angles of contact, clusters of highly reflective scintillations could be seen in the centre of the loaded zones (Fig. 41: col. pl.).

(4) A motor drive fitted to a Nikon camera was used to photograph these reflection patterns at the limits of resolution of the operating microscope (Fig. 42, opposite).

It was concluded from these operations *in vivo* that the foci of high reflectivity seen on loaded areas corresponded to zones in which tertiary cartilage undulations persisted under load. The evidence supported the suspicion that loaded surfaces, in the living animal, retain the non-smooth surface seen in the unloaded state.

In the course of the experiments there was an opportunity immediately after the load was removed to survey the influence of sustained pressure on the morphology of the whole loaded femoral surface. It

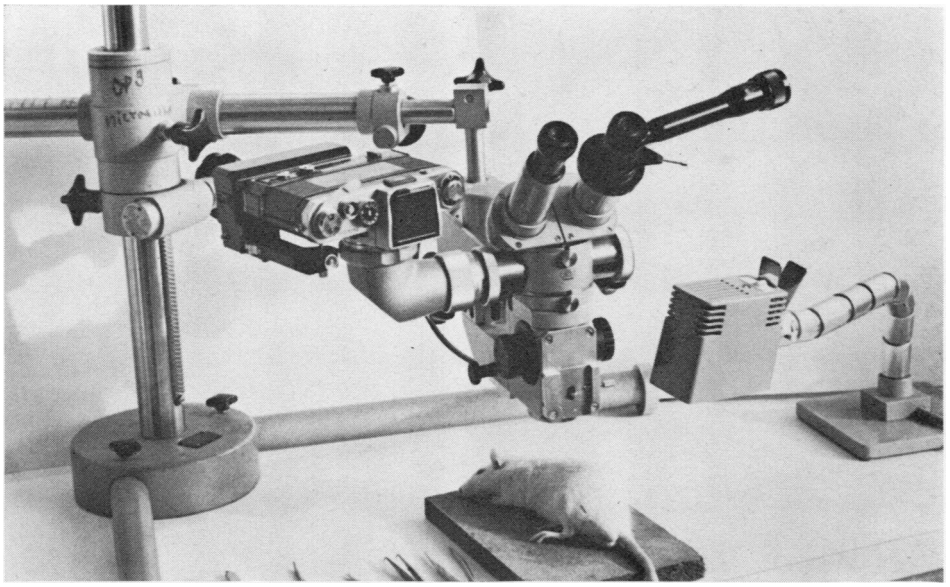

FIG. 42 *Zeiss operating microscope with Nikon camera fitted with motor drive adapted to study loaded cartilage surfaces*

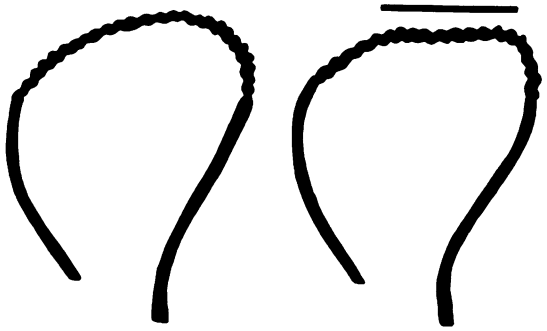

FIG. 43 *Configuration of cartilage surface under prolonged load. Normal femoral condylar surface (left). Loaded femoral condylar surface (right). Contour of cartilage is flattened but tertiary undulations are, to a varying extent, preserved*

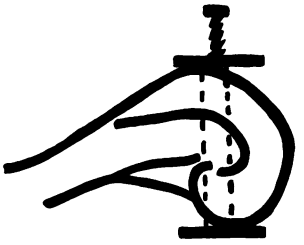

FIG. 44 *Arrangement of screw clamp for loading living knee joint across femoral surfaces*

was noted that the appearances of individual undulations were unchanged by comparison with the surface structure seen before loading (Fig. 43). The primary cartilage contours had changed, however, and the altered contours persisted for some minutes after removal of the load.

A further series of experiments was then performed to elucidate the 3-dimensional structure of the living loaded cartilage surface. Metal screw slips, carefully cleaned by sonication, were placed *in vivo* over the condylar and opposing anterior patellar surfaces of the femur, the joint having been carefully opened without touching the cartilage, and the blood supply to the joint being preserved until the last moment before the clamp was tightened (Fig. 44). Very shortly after the clamp was secured, thus loading the condylar and marginal patellar surfaces, the femur was divided and the whole preparation plunged into liquid nitrogen. The frozen material was then freeze-dried at –25°C. for 24 hrs, the clamp was removed, and the joint was trimmed under the dissecting microscope to give a specimen that would readily enter the scanning electron microscope. After having been coated with a gold/palladium alloy, the trimmed material was scanned at magnifications of from 20× to 5000× at 20 kV.

The results obtained by this technique substantially confirmed the earlier experiments of Walker and others (1969) *in vitro*. The loaded freeze-dried surfaces (Figs 45, 46, overleaf) were never smooth. The irregularities seen did not conform with those present on the metal surface of the clamp used to apply the load: two clamps were examined by scanning electron microscopy after careful cleaning and the metallic irregularities were defined (Fig. 47, overleaf).

No exact measure of the pressure applied to the cartilage surface was obtained in these first studies and it remained possible that excess pressure could

FIG. 45 *Margin of patellar surface of rat femur from joint loaded by application of steel clamp during life and subsequently snap-frozen, freeze-dried, coated with gold-palladium alloy, and viewed by scanning electron microscopy.* ×190

FIG. 46 *Surface of nearby zone of rat femoral cartilage scanned after loading during life and freeze-drying.* ×1,130

have imprinted the papillary irregularities of the underlying bony end-plate on the superficial overlying cartilage. A further study was therefore made. The condylar surfaces of the femur were loaded by pressing them *in vivo* against the upper complementary surface of the tibia. The two bones, held in opposition by lateral springs, were supported in vertical alignment by a splinting device. After pressure had been maintained for 5 mins. the bones were divided above and below the knee; the preparation was then plunged into liquid nitrogen and freeze-dried, and the loaded surfaces were examined with the scanning electron microscope (Fig. 48, opposite). The results showed that there was a flattening of the contours of the joint viewed at low power, but substantial retention of the tertiary undulations.

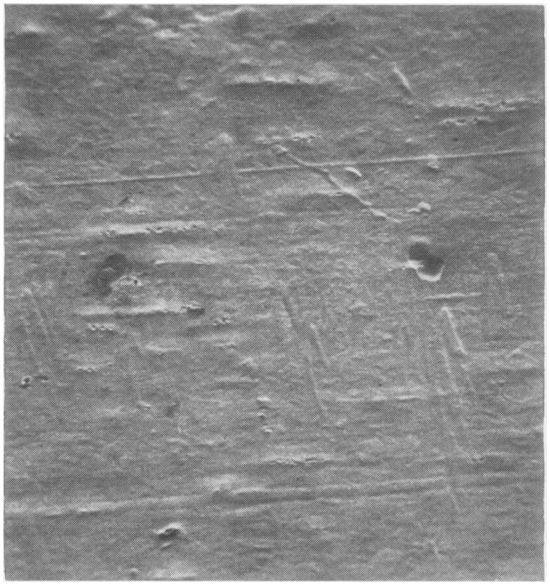

FIG. 47 *Surface of metal clamp, cleaned by sonication, before application to cartilage as shown in Fig.* 45. ×612

Conclusions

Until 1968–9, the surfaces of mammalian articular hyaline cartilage were very generally thought to be 'strikingly smooth'. The commercial availability of the scanning electron microscope then encouraged British and Japanese, and, more recently, American and German investigators, to use this new form of microscope to survey joint surface structure. It quickly became clear that the term 'smooth' could be applied to hyaline cartilage in only the most superficial sense: at every order of magnification, with all forms of microscopy, irregularities could be detected ranging in diameter from coarse secondary structures visible with a hand lens down to collagen fibre bundles detectable only by high resolution scanning electron microscopy or by the transmission electron microscopy of 2-stage carbon surface replicas.

In this Oration, the technological evolution of modern light, acoustic, and electron microscopes has been briefly traced, and an account has been given of

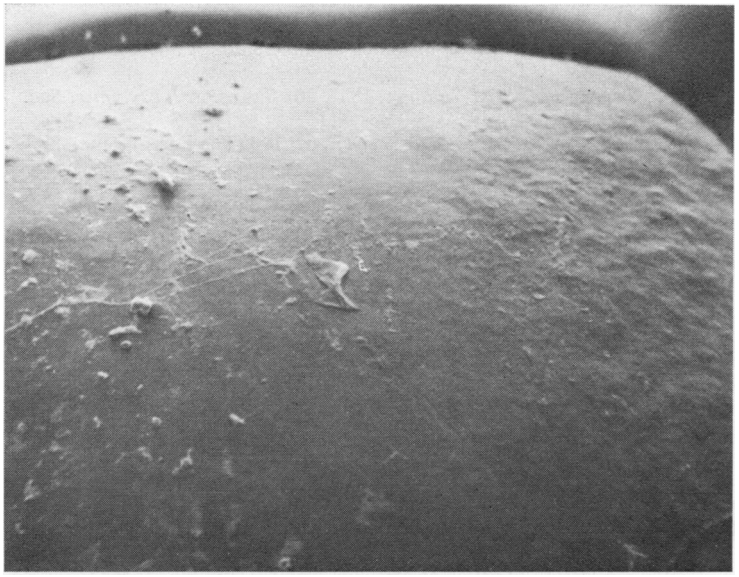

FIG. 48 *Scanning electron micrograph of central part of rat femoral condyle after loading during life by technique described in text. Note flattening of condylar contour (centre area) but persistence of tertiary cartilage undulations.* ×230

the numerous preparative techniques used in this laboratory for the investigation of articular cartilaginous surfaces by many of these microscopic techniques.

Cartilage surface structure in the living animal is not necessarily identical with the microscopic anatomy of an ischaemic joint or with excised cartilage. All 'fixatives' are liable to introduce artefact. The use of ruthenium red and of cetylpyrridinium chloride as additives to conserve glycosaminoglycans is advocated, but studies of the intact living joint are preferred. Unfortunately, microscopy *in vivo* is not compatible with the use of the high resolution scanning and transmission electron microscopes. There is a possibility that the acoustic microscope may overcome this problem. Meanwhile, valuable information can be gained by incident light microscopy in the normal and interference modes.

Hyaline articular cartilage surfaces surveyed during life at low magnification, prepared as undecalcified cryostat sections, after excision in the freeze-dried state, after glutaraldehyde or osmium fixation (with or without ruthenium red), by transmission phase contrast, incident-light, or interference microscopy, and by scanning electron microscopy, are not smooth. Superimposed on the anatomical contours are coarse irregularities visible with a simple microscope and an orderly undulating structure related to the ellipsoid chondrocytes that lie 2 to 6 μm. beneath the articular surface. Interference microscopy reveals that the undulations are 1·2 to 2·6 μm. in height and that they are of widely varying diameter, height, and contour.

The surface structure is formed by a collagen-rich *skin* resistant to deformation by loads *in vivo* or *in vitro*. The surface skin is permeable to water, electrolytes, small enzyme molecules, and dyes, and is composed of interlacing bundles of collagen that lie parallel with the surface. The collagen-rich surface lamina contains little metachromatic or Alcian blue-staining material. Enzymes such as cathepsin D, that effectively degrade embryonic cartilage in culture, penetrate the surface lamina *in vitro*, leaving the lamina intact but degrading the proteoglycan-rich underlying matrix as far as the bony end-plate. Cartilage surfaces digested in this way appear to be intact by scanning electron microscopy at magnifications up to ×6000. By contrast, more severely destructive enzymes such as papain degrade the whole surface structure: the scanning electron microscope then reveals early gross destruction with loss of collagen, matrix, and eventually, of cells.

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